CHIRAL CONTROL

Cross-Reference to Related Applications

[0001] This application claims priority to United States Provisional Application Serial No. 61/671,655, filed July 13, 2012, 61/671,656, filed July 13, 2012, 61/671,722, filed July 14, 2012, and 61/671,724, filed July 14, 2012, the entirety of each of which is incorporated herein by reference.

Background of the Invention

[0002] Oligonucleotides are useful in therapeutic, diagnostic, research and nanomaterials applications. The use of naturally occurring nucleic acids (e.g., unmodified DNA or RNA) for therapeutics can be limited, for example, because of their instability against extra- and intracellular nucleases and/or their poor cell penetration and distribution. Additionally, *in vitro* studies have shown that properties of antisense oligonucleotides such as binding affinity, sequence specific binding to the complementary RNA (Cosstick and Eckstein, 1985; LaPlanche *et al.,* 1986; Latimer *et al.,* 1989; Hacia *et al.,* 1994; Mesmaeker *et al.,* 1995), and stability to nucleases can be affected by the absolute stereochemical configurations of the phosphorus atoms (Cook, *et al.* US005599797A).. Therefore, there is a need for new and improved oligonucleotide compositions.

Summary of the Invention

[0003] The present invention encompasses the recognition that there exists a need for chirally controlled oligonucleotide compositions and new methods for synthesizing the same. The invention specifically encompasses the identification of the source of certain problems with prior methodologies for preparing chiral oligonucleotides, including problems that prohibit preparation of fully chirally controlled compositions, particularly compositions comprising a plurality of oligonucleotide types.

[0004] In some embodiments, the present invention provides chirally controlled oligonulcleotide compositions.

[0005] In some embodiments, the present invention provides methods of making chirally controlled oligonulcleotides and chirally controlled oligonucleotide compositions.

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[0006] In some embodiments, the present invention provides methods of using chirally controlled oligonulcleotide and chirally controlled oligonucleotide compositions.

[0007] All publications and patent documents cited in this application are incorporated herein by reference in their entirety.

Definitions

[0008] *Aliphatic*: The term "aliphatic" or "aliphatic group", as used herein, means a straight-chain (i.e., unbranched) or branched, substituted or unsubstituted hydrocarbon chain that is completely saturated or that contains one or more units of unsaturation, or a monocyclic hydrocarbon or bicyclic or polycyclic hydrocarbon that is completely saturated or that contains one or more units of unsaturation, but which is not aromatic (also referred to herein as "carbocycle" "cycloaliphatic" or "cycloalkyl"), that has a single point of attachment to the rest of the molecule. In some embodiments, aliphatic groups contain 1-50 aliphatic carbon atoms. Unless otherwise specified, aliphatic groups contain 1-10 aliphatic carbon atoms. In some embodiments, aliphatic groups contain 1-6 aliphatic carbon atoms. In some embodiments, aliphatic groups contain 1-5 aliphatic carbon atoms. In other embodiments, aliphatic groups contain 1-4 aliphatic carbon atoms. In still other embodiments, aliphatic groups contain 1-3 aliphatic carbon atoms, and in yet other embodiments, aliphatic groups contain 1-2 aliphatic carbon atoms. In some embodiments, "cycloaliphatic" (or "carbocycle" or "cycloalkyl") refers to a monocyclic or bicyclic C_3-C_{10} hydrocarbon that is completely saturated or that contains one or more units of unsaturation, but which is not aromatic, that has a single point of attachment to the rest of the molecule. In some embodiments, "cycloaliphatic" (or "carbocycle" or "cycloalkyl") refers to a monocyclic C_3–C_6 hydrocarbon that is completely saturated or that contains one or more units of unsaturation, but which is not aromatic, that has a single point of attachment to the rest of the molecule. Suitable aliphatic groups include, but are not limited to, linear or branched, substituted or unsubstituted alkyl, alkenyl, alkynyl groups and hybrids thereof such as (cycloalkyl)alkyl, (cycloalkenyl)alkyl or (cycloalkyl)alkenyl.

[0009] *Alkylene*: The term "alkylene" refers to a bivalent alkyl group. An "alkylene chain" is a polymethylene group, i.e., $-(CH_2)_n-$, wherein n is a positive integer, preferably from 1 to 6, from 1 to 4, from 1 to 3, from 1 to 2, or from 2 to 3. A substituted alkylene chain is a

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polymethylene group in which one or more methylene hydrogen atoms are replaced with a substituent. Suitable substituents include those described below for a substituted aliphatic group.

[0010] *Alkenylene*: The term "alkenylene" refers to a bivalent alkenyl group. A substituted alkenylene chain is a polymethylene group containing at least one double bond in which one or more hydrogen atoms are replaced with a substituent. Suitable substituents include those described below for a substituted aliphatic group.

[0011] *Animal:* As used herein, the term "animal" refers to any member of the animal kingdom. In some embodiments, "animal" refers to humans, at any stage of development. In some embodiments, "animal" refers to non-human animals, at any stage of development. In certain embodiments, the non-human animal is a mammal (*e.g.*, a rodent, a mouse, a rat, a rabbit, a monkey, a dog, a cat, a sheep, cattle, a primate, and/or a pig). In some embodiments, animals include, but are not limited to, mammals, birds, reptiles, amphibians, fish, and/or worms. In some embodiments, an animal may be a transgenic animal, a genetically-engineered animal, and/or a clone.

[0012] *Approximately:* As used herein, the terms "approximately" or "about" in reference to a number are generally taken to include numbers that fall within a range of 5%, 10%, 15%, or 20% in either direction (greater than or less than) of the number unless otherwise stated or otherwise evident from the context (except where such number would be less than 0% or exceed 100% of a possible value). In some embodiments, use of the term "about" in reference to dosages means ± 5 mg/kg/day.

[0013] *Aryl:* The term "aryl" used alone or as part of a larger moiety as in "aralkyl," "aralkoxy," or "aryloxyalkyl," refers to monocyclic and bicyclic ring systems having a total of five to fourteen ring members, wherein at least one ring in the system is aromatic and wherein each ring in the system contains three to seven ring members. The term "aryl" may be used interchangeably with the term "aryl ring." In certain embodiments of the present invention, "aryl" refers to an aromatic ring system which includes, but not limited to, phenyl, biphenyl, naphthyl, anthracyl and the like, which may bear one or more substituents. Also included within the scope of the term "aryl," as it is used herein, is a group in which an aromatic ring is fused to one or more non–aromatic rings, such as indanyl, phthalimidyl, naphthimidyl, phenanthridinyl, or tetrahydronaphthyl, and the like.

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[0014] *Characteristic portion*: As used herein, the phrase a "characteristic portion" of a protein or polypeptide is one that contains a continuous stretch of amino acids, or a collection of continuous stretches of amino acids, that together are characteristic of a protein or polypeptide. Each such continuous stretch generally will contain at least two amino acids. Furthermore, those of ordinary skill in the art will appreciate that typically at least 5, 10, 15, 20 or more amino acids are required to be characteristic of a protein. In general, a characteristic portion is one that, in addition to the sequence identity specified above, shares at least one functional characteristic with the relevant intact protein.

[0015] *Characteristic sequence*: A "characteristic sequence" is a sequence that is found in all members of a family of polypeptides or nucleic acids, and therefore can be used by those of ordinary skill in the art to define members of the family.

[0016] *Characteristic structural element:* The term "characteristic structural element" refers to a distinctive structural element (e.g., core structure, collection of pendant moieties, sequence element, etc) that is found in all members of a family of polypeptides, small molecules, or nucleic acids, and therefore can be used by those of ordinary skill in the art to define members of the family.

[0017] *Comparable:* The term "comparable" is used herein to describe two (or more) sets of conditions or circumstances that are sufficiently similar to one another to permit comparison of results obtained or phenomena observed. In some embodiments, comparable sets of conditions or circumstances are characterized by a plurality of substantially identical features and one or a small number of varied features. Those of ordinary skill in the art will appreciate that sets of conditions are comparable to one another when characterized by a sufficient number and type of substantially identical features to warrant a reasonable conclusion that differences in results obtained or phenomena observed under the different sets of conditions or circumstances are caused by or indicative of the variation in those features that are varied.

[0018] *Dosing regimen:* As used herein, a "dosing regimen" or "therapeutic regimen" refers to a set of unit doses (typically more than one) that are administered individually to a subject, typically separated by periods of time. In some embodiments, a given therapeutic agent has a recommended dosing regimen, which may involve one or more doses. In some embodiments, a dosing regimen comprises a plurality of doses each of which are separated from one another by a time period of the same length; in some embodiments, a dosing regime

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comprises a plurality of doses and at least two different time periods separating individual doses. In some embodiments, all doses within a dosing regimen are of the same unit dose amount. In some embodiments, different doses within a dosing regimen are of different amounts. In some embodiments, a dosing regimen comprises a first dose in a first dose amount, followed by one or more additional doses in a second dose amount different from the first dose amount. In some embodiments, a dosing regimen comprises a first dose in a first dose amount, followed by one or more additional doses in a second dose amount same as the first dose amount.

[0019] *Equivalent agents:* Those of ordinary skill in the art, reading the present disclosure, will appreciate that the scope of useful agents in the context of the present invention is not limited to those specifically mentioned or exemplified herein. In particular, those skilled in the art will recognize that active agents typically have a structure that consists of a core and attached pendant moieties, and furthermore will appreciate that simple variations of such core and/or pendant moieties may not significantly alter activity of the agent. For example, in some embodiments, substitution of one or more pendant moieties with groups of comparable three-dimensional structure and/or chemical reactivity characteristics may generate a substituted compound or portion equivalent to a parent reference compound or portion. In some embodiments, addition or removal of one or more pendant moieties may generate a substituted compound equivalent to a parent reference compound. In some embodiments, alteration of core structure, for example by addition or removal of a small number of bonds (typically not more than 5, 4, 3, 2, or 1 bonds, and often only a single bond) may generate a substituted compound equivalent to a parent reference compound. In many embodiments, equivalent compounds may be prepared by methods illustrated in general reaction schemes as, for example, described below, or by modifications thereof, using readily available starting materials, reagents and conventional or provided synthesis procedures. In these reactions, it is also possible to make use of variants, which are in themselves known, but are not mentioned here.

[0020] *Equivalent Dosage:* The term "equivalent dosage" is used herein to compare dosages of different pharmaceutically active agents that effect the same biological result. Dosages of two different agents are considered to be "equivalent" to one another in accordance with the present invention if they achieve a comparable level or extent of the biological result. In some embodiments, equivalent dosages of different pharmaceutical agents for use in accordance with the present invention are determined using *in vitro* and/or *in vivo* assays as described herein.

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In some embodiments, one or more lysosomal activating agents for use in accordance with the present invention is utilized at a dose equivalent to a dose of a reference lysosomal activating agent; in some such embodiments, the reference lysosomal activating agent for such purpose is selected from the group consisting of small molecule allosteric activators (e.g., pyrazolpyrimidines), imminosugars (e.g., isofagomine), antioxidants (e.g., n-acetyl-cysteine), and regulators of cellular trafficking (e.g., Rab1a polypeptide).

[0021] *Heteroaliphatic*: The term "heteroaliphatic" refers to an aliphatic group wherein one or more units selected from C, CH, CH_2, or CH_3 are independently replaced by a heteroatom. In some embodiments, a heteroaliphatic group is heteroalkyl. In some embodiments, a heteroaliphatic group is heteroalkenyl.

[0022] *Heteroaryl*: The terms "heteroaryl" and "heteroar–," used alone or as part of a larger moiety, e.g., "heteroaralkyl," or "heteroaralkoxy," refer to groups having 5 to 10 ring atoms, preferably 5, 6, or 9 ring atoms; having 6, 10, or 14 π electrons shared in a cyclic array; and having, in addition to carbon atoms, from one to five heteroatoms. The term "heteroatom" refers to nitrogen, oxygen, or sulfur, and includes any oxidized form of nitrogen or sulfur, and any quaternized form of a basic nitrogen. Heteroaryl groups include, without limitation, thienyl, furanyl, pyrrolyl, imidazolyl, pyrazolyl, triazolyl, tetrazolyl, oxazolyl, isoxazolyl, oxadiazolyl, thiazolyl, isothiazolyl, thiadiazolyl, pyridyl, pyridazinyl, pyrimidinyl, pyrazinyl, indolizinyl, purinyl, naphthyridinyl, and pteridinyl. The terms "heteroaryl" and "heteroar–," as used herein, also include groups in which a heteroaromatic ring is fused to one or more aryl, cycloaliphatic, or heterocyclyl rings, where the radical or point of attachment is on the heteroaromatic ring. Nonlimiting examples include indolyl, isoindolyl, benzothienyl, benzofuranyl, dibenzofuranyl, indazolyl, benzimidazolyl, benzthiazolyl, quinolyl, isoquinolyl, cinnolinyl, phthalazinyl, quinazolinyl, quinoxalinyl, 4*H*–quinolizinyl, carbazolyl, acridinyl, phenazinyl, phenothiazinyl, phenoxazinyl, tetrahydroquinolinyl, tetrahydroisoquinolinyl, and pyrido[2,3–b]–1,4–oxazin–3(4H)–one. A heteroaryl group may be mono– or bicyclic. The term "heteroaryl" may be used interchangeably with the terms "heteroaryl ring," "heteroaryl group," or "heteroaromatic," any of which terms include rings that are optionally substituted. The term "heteroaralkyl" refers to an alkyl group substituted by a heteroaryl, wherein the alkyl and heteroaryl portions independently are optionally substituted.

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[0023] *Heteroatom*: The term "heteroatom" means one or more of oxygen, sulfur, nitrogen, phosphorus, or silicon (including, any oxidized form of nitrogen, sulfur, phosphorus, or silicon; the quaternized form of any basic nitrogen or; a substitutable nitrogen of a heterocyclic ring, for example N (as in 3,4-dihydro-2*H*-pyrrolyl), NH (as in pyrrolidinyl) or NR^+ (as in N-substituted pyrrolidinyl)).

[0024] *Heterocycle*: As used herein, the terms "heterocycle," "heterocyclyl," "heterocyclic radical," and "heterocyclic ring" are used interchangeably and refer to a stable 3– to 7–membered monocyclic or 7–10–membered bicyclic heterocyclic moiety that is either saturated or partially unsaturated, and having, in addition to carbon atoms, one or more, preferably one to four, heteroatoms, as defined above. When used in reference to a ring atom of a heterocycle, the term "nitrogen" includes a substituted nitrogen. As an example, in a saturated or partially unsaturated ring having 0–3 heteroatoms selected from oxygen, sulfur or nitrogen, the nitrogen may be N (as in 3,4–dihydro–2*H*–pyrrolyl), NH (as in pyrrolidinyl), or $^+$NR (as in *N*–substituted pyrrolidinyl).

[0025] A heterocyclic ring can be attached to its pendant group at any heteroatom or carbon atom that results in a stable structure and any of the ring atoms can be optionally substituted. Examples of such saturated or partially unsaturated heterocyclic radicals include, without limitation, tetrahydrofuranyl, tetrahydrothiophenyl pyrrolidinyl, piperidinyl, pyrrolinyl, tetrahydroquinolinyl, tetrahydroisoquinolinyl, decahydroquinolinyl, oxazolidinyl, piperazinyl, dioxanyl, dioxolanyl, diazepinyl, oxazepinyl, thiazepinyl, morpholinyl, and quinuclidinyl. The terms "heterocycle," "heterocyclyl," "heterocyclyl ring," "heterocyclic group," "heterocyclic moiety," and "heterocyclic radical," are used interchangeably herein, and also include groups in which a heterocyclyl ring is fused to one or more aryl, heteroaryl, or cycloaliphatic rings, such as indolinyl, 3*H*–indolyl, chromanyl, phenanthridinyl, or tetrahydroquinolinyl, where the radical or point of attachment is on the heterocyclyl ring. A heterocyclyl group may be mono– or bicyclic. The term "heterocyclylalkyl" refers to an alkyl group substituted by a heterocyclyl, wherein the alkyl and heterocyclyl portions independently are optionally substituted.

[0026] *Intraperitoneal:* The phrases "intraperitoneal administration" and "administered intraperitonealy" as used herein have their art-understood meaning referring to administration of a compound or composition into the peritoneum of a subject.

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[0027] *In vitro*: As used herein, the term "*in vitro*" refers to events that occur in an artificial environment, *e.g.*, in a test tube or reaction vessel, in cell culture, *etc.*, rather than within an organism (*e.g.,* animal, plant, and/or microbe).

[0028] *In vivo:* As used herein, the term "*in vivo*" refers to events that occur within an organism (*e.g.,* animal, plant, and/or microbe).

[0029] *Lower alkyl:* The term "lower alkyl" refers to a C_{1-4} straight or branched alkyl group. Exemplary lower alkyl groups are methyl, ethyl, propyl, isopropyl, butyl, isobutyl, and tert-butyl.

[0030] *Lower haloalkyl:* The term "lower haloalkyl" refers to a C_{1-4} straight or branched alkyl group that is substituted with one or more halogen atoms.

[0031] *Optionally substituted:* As described herein, compounds of the invention may contain "optionally substituted" moieties. In general, the term "substituted," whether preceded by the term "optionally" or not, means that one or more hydrogens of the designated moiety are replaced with a suitable substituent. Unless otherwise indicated, an "optionally substituted" group may have a suitable substituent at each substitutable position of the group, and when more than one position in any given structure may be substituted with more than one substituent selected from a specified group, the substituent may be either the same or different at every position. Combinations of substituents envisioned by this invention are preferably those that result in the formation of stable or chemically feasible compounds. The term "stable," as used herein, refers to compounds that are not substantially altered when subjected to conditions to allow for their production, detection, and, in certain embodiments, their recovery, purification, and use for one or more of the purposes disclosed herein.

[0032] Suitable monovalent substituents on a substitutable carbon atom of an "optionally substituted" group are independently halogen; $-(CH_2)_{0-4}R°$; $-(CH_2)_{0-4}OR°$; $-O(CH_2)_{0-4}R°$, $-O-(CH_2)_{0-4}C(O)OR°$; $-(CH_2)_{0-4}CH(OR°)_2$; $-(CH_2)_{0-4}SR°$; $-(CH_2)_{0-4}Ph$, which may be substituted with R°; $-(CH_2)_{0-4}O(CH_2)_{0-1}Ph$ which may be substituted with R°; –CH=CHPh, which may be substituted with R°; $-(CH_2)_{0-4}O(CH_2)_{0-1}$-pyridyl which may be substituted with R°; $-NO_2$; –CN; $-N_3$; $-(CH_2)_{0-4}N(R°)_2$; $-(CH_2)_{0-4}N(R°)C(O)R°$; $-N(R°)C(S)R°$; $-(CH_2)_{0-4}N(R°)C(O)NR°_2$; $-N(R°)C(S)NR°_2$; $-(CH_2)_{0-4}N(R°)C(O)OR°$; $-N(R°)N(R°)C(O)R°$; $-N(R°)N(R°)C(O)NR°_2$; $-N(R°)N(R°)C(O)OR°$; $-(CH_2)_{0-4}C(O)R°$; $-C(S)R°$; $-(CH_2)_{0-4}C(O)OR°$; $-(CH_2)_{0-4}C(O)SR°$; $-(CH_2)_{0-4}C(O)OSiR°_3$; $-(CH_2)_{0-4}OC(O)R°$; $-OC(O)(CH_2)_{0-4}SR-$, $SC(S)SR°$; $-(CH_2)_{0-4}SC(O)R°$;

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$-(CH_2)_{0-4}C(O)NR^\circ_2$; $-C(S)NR^\circ_2$; $-C(S)SR^\circ$; $-SC(S)SR^\circ$, $-(CH_2)_{0-4}OC(O)NR^\circ_2$; $-C(O)N(OR^\circ)R^\circ$; $-C(O)C(O)R^\circ$; $-C(O)CH_2C(O)R^\circ$; $-C(NOR^\circ)R^\circ$; $-(CH_2)_{0-4}SSR^\circ$; $-(CH_2)_{0-4}S(O)_2R^\circ$; $-(CH_2)_{0-4}S(O)_2OR^\circ$; $-(CH_2)_{0-4}OS(O)_2R^\circ$; $-S(O)_2NR^\circ_2$; $-(CH_2)_{0-4}S(O)R^\circ$; $-N(R^\circ)S(O)_2NR^\circ_2$; $-N(R^\circ)S(O)_2R^\circ$; $-N(OR^\circ)R^\circ$; $-C(NH)NR^\circ_2$; $-P(O)_2R^\circ$; $-P(O)R^\circ_2$; $-OP(O)R^\circ_2$; $-OP(O)(OR^\circ)_2$; $-SiR^\circ_3$; $-(C_{1-4}$ straight or branched alkylene$)O-N(R^\circ)_2$; or $-(C_{1-4}$ straight or branched alkylene$)C(O)O-N(R^\circ)_2$, wherein each R° may be substituted as defined below and is independently hydrogen, C_{1-6} aliphatic, $-CH_2Ph$, $-O(CH_2)_{0-1}Ph$, $-CH_2$-(5-6 membered heteroaryl ring), or a 5–6–membered saturated, partially unsaturated, or aryl ring having 0–4 heteroatoms independently selected from nitrogen, oxygen, or sulfur, or, notwithstanding the definition above, two independent occurrences of R°, taken together with their intervening atom(s), form a 3–12–membered saturated, partially unsaturated, or aryl mono– or bicyclic ring having 0–4 heteroatoms independently selected from nitrogen, oxygen, or sulfur, which may be substituted as defined below.

[0033] Suitable monovalent substituents on R° (or the ring formed by taking two independent occurrences of R° together with their intervening atoms), are independently halogen, $-(CH_2)_{0-2}R^\bullet$, $-(haloR^\bullet)$, $-(CH_2)_{0-2}OH$, $-(CH_2)_{0-2}OR^\bullet$, $-(CH_2)_{0-2}CH(OR^\bullet)_2$; $-O(haloR^\bullet)$, $-CN$, $-N_3$, $-(CH_2)_{0-2}C(O)R^\bullet$, $-(CH_2)_{0-2}C(O)OH$, $-(CH_2)_{0-2}C(O)OR^\bullet$, $-(CH_2)_{0-2}SR^\bullet$, $-(CH_2)_{0-2}SH$, $-(CH_2)_{0-2}NH_2$, $-(CH_2)_{0-2}NHR^\bullet$, $-(CH_2)_{0-2}NR^\bullet_2$, $-NO_2$, $-SiR^\bullet_3$, $-OSiR^\bullet_3$, $-C(O)SR^\bullet$, $-(C_{1-4}$ straight or branched alkylene$)C(O)OR^\bullet$, or $-SSR^\bullet$ wherein each $R^\bullet$ is unsubstituted or where preceded by "halo" is substituted only with one or more halogens, and is independently selected from C_{1-4} aliphatic, $-CH_2Ph$, $-O(CH_2)_{0-1}Ph$, or a 5–6–membered saturated, partially unsaturated, or aryl ring having 0–4 heteroatoms independently selected from nitrogen, oxygen, or sulfur. Suitable divalent substituents on a saturated carbon atom of R° include =O and =S.

[0034] Suitable divalent substituents on a saturated carbon atom of an "optionally substituted" group include the following: =O, =S, $=NNR^*_2$, $=NNHC(O)R^*$, $=NNHC(O)OR^*$, $=NNHS(O)_2R^*$, $=NR^*$, $=NOR^*$, $-O(C(R^*_2))_{2-3}O-$, or $-S(C(R^*_2))_{2-3}S-$, wherein each independent occurrence of R^* is selected from hydrogen, C_{1-6} aliphatic which may be substituted as defined below, or an unsubstituted 5–6–membered saturated, partially unsaturated, or aryl ring having 0–4 heteroatoms independently selected from nitrogen, oxygen, or sulfur. Suitable divalent

substituents that are bound to vicinal substitutable carbons of an "optionally substituted" group include: $-O(CR^*_2)_{2-3}O-$, wherein each independent occurrence of R^* is selected from hydrogen, C_{1-6} aliphatic which may be substituted as defined below, or an unsubstituted 5–6–membered saturated, partially unsaturated, or aryl ring having 0–4 heteroatoms independently selected from nitrogen, oxygen, or sulfur.

[0035] Suitable substituents on the aliphatic group of R^* include halogen, $-R^\bullet$, -(halo$R^\bullet$), -OH, $-OR^\bullet$, -(halo$R^\bullet$), –CN, –C(O)OH, $-C(O)OR^\bullet$, $-NH_2$, $-NHR^\bullet$, $-NR^\bullet_2$, or $-NO_2$, wherein each $R^\bullet$ is unsubstituted or where preceded by "halo" is substituted only with one or more halogens, and is independently C_{1-4} aliphatic, $-CH_2Ph$, $-O(CH_2)_{0-1}Ph$, or a 5–6–membered saturated, partially unsaturated, or aryl ring having 0–4 heteroatoms independently selected from nitrogen, oxygen, or sulfur.

[0036] Suitable substituents on a substitutable nitrogen of an "optionally substituted" group include $-R^\dagger$, $-NR^\dagger_2$, $-C(O)R^\dagger$, $-C(O)OR^\dagger$, $-C(O)C(O)R^\dagger$, $-C(O)CH_2C(O)R^\dagger$, $-S(O)_2R^\dagger$, $-S(O)_2NR^\dagger_2$, $-C(S)NR^\dagger_2$, $-C(NH)NR^\dagger_2$, or $-N(R^\dagger)S(O)_2R^\dagger$; wherein each $R^\dagger$ is independently hydrogen, C_{1-6} aliphatic which may be substituted as defined below, unsubstituted –OPh, or an unsubstituted 5–6–membered saturated, partially unsaturated, or aryl ring having 0–4 heteroatoms independently selected from nitrogen, oxygen, or sulfur, or, notwithstanding the definition above, two independent occurrences of $R^\dagger$, taken together with their intervening atom(s) form an unsubstituted 3–12–membered saturated, partially unsaturated, or aryl mono– or bicyclic ring having 0–4 heteroatoms independently selected from nitrogen, oxygen, or sulfur.

[0037] Suitable substituents on the aliphatic group of $R^\dagger$ are independently halogen, $-R^\bullet$, -(halo$R^\bullet$), –OH, $-OR^\bullet$, -(halo$R^\bullet$), –CN, –C(O)OH, $-C(O)OR^\bullet$, $-NH_2$, $-NHR^\bullet$, $-NR^\bullet_2$, or $-NO_2$, wherein each $R^\bullet$ is unsubstituted or where preceded by "halo" is substituted only with one or more halogens, and is independently C_{1-4} aliphatic, $-CH_2Ph$, $-O(CH_2)_{0-1}Ph$, or a 5–6–membered saturated, partially unsaturated, or aryl ring having 0–4 heteroatoms independently selected from nitrogen, oxygen, or sulfur.

[0038] *Oral:* The phrases "oral administration" and "administered orally" as used herein have their art-understood meaning referring to administration by mouth of a compound or composition.

[0039] *Parenteral:* The phrases "parenteral administration" and "administered parenterally" as used herein have their art-understood meaning referring to modes of

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administration other than enteral and topical administration, usually by injection, and include, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticulare, subcapsular, subarachnoid, intraspinal, and intrasternal injection and infusion.

[0040] *Partially unsaturated:* As used herein, the term "partially unsaturated" refers to a ring moiety that includes at least one double or triple bond. The term "partially unsaturated" is intended to encompass rings having multiple sites of unsaturation, but is not intended to include aryl or heteroaryl moieties, as herein defined.

[0041] *Pharmaceutical composition:* As used herein, the term "pharmaceutical composition" refers to an active agent, formulated together with one or more pharmaceutically acceptable carriers. In some embodiments, active agent is present in unit dose amount appropriate for administration in a therapeutic regimen that shows a statistically significant probability of achieving a predetermined therapeutic effect when administered to a relevant population. In some embodiments, pharmaceutical compositions may be specially formulated for administration in solid or liquid form, including those adapted for the following: oral administration, for example, drenches (aqueous or non-aqueous solutions or suspensions), tablets, *e.g.*, those targeted for buccal, sublingual, and systemic absorption, boluses, powders, granules, pastes for application to the tongue; parenteral administration, for example, by subcutaneous, intramuscular, intravenous or epidural injection as, for example, a sterile solution or suspension, or sustained-release formulation; topical application, for example, as a cream, ointment, or a controlled-release patch or spray applied to the skin, lungs, or oral cavity; intravaginally or intrarectally, for example, as a pessary, cream, or foam; sublingually; ocularly; transdermally; or nasally, pulmonary, and to other mucosal surfaces.

[0042] *Pharmaceutically acceptable:* As used herein, the phrase "pharmaceutically acceptable" refers to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

[0043] *Pharmaceutically acceptable carrier:* As used herein, the term "pharmaceutically acceptable carrier" means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, or solvent encapsulating material, involved in

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carrying or transporting the subject compound from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: sugars, such as lactose, glucose and sucrose; starches, such as corn starch and potato starch; cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients, such as cocoa butter and suppository waxes; oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; glycols, such as propylene glycol; polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; esters, such as ethyl oleate and ethyl laurate; agar; buffering agents, such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol; pH buffered solutions; polyesters, polycarbonates and/or polyanhydrides; and other non-toxic compatible substances employed in pharmaceutical formulations.

[0044] *Pharmaceutically acceptable salt:* The term "pharmaceutically acceptable salt", as used herein, refers to salts of such compounds that are appropriate for use in pharmaceutical contexts, i.e., salts which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of humans and lower animals without undue toxicity, irritation, allergic response and the like, and are commensurate with a reasonable benefit/risk ratio. Pharmaceutically acceptable salts are well known in the art. For example, S. M. Berge, et al. describes pharmaceutically acceptable salts in detail in J. Pharmaceutical Sciences, 66: 1-19 (1977). In some embodiments, pharmaceutically acceptable salt include, but are not limited to, nontoxic acid addition salts, which are salts of an amino group formed with inorganic acids such as hydrochloric acid, hydrobromic acid, phosphoric acid, sulfuric acid and perchloric acid or with organic acids such as acetic acid, maleic acid, tartaric acid, citric acid, succinic acid or malonic acid or by using other methods used in the art such as ion exchange. In some embodiments, pharmaceutically acceptable salts include, but are not limited to, adipate, alginate, ascorbate, aspartate, benzenesulfonate, benzoate, bisulfate, borate, butyrate, camphorate, camphorsulfonate, citrate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, formate, fumarate, glucoheptonate, glycerophosphate, gluconate, hemisulfate, heptanoate, hexanoate, hydroiodide, 2-hydroxy-ethanesulfonate, lactobionate, lactate, laurate, lauryl sulfate,

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malate, maleate, malonate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, nitrate, oleate, oxalate, palmitate, pamoate, pectinate, persulfate, 3-phenylpropionate, phosphate, picrate, pivalate, propionate, stearate, succinate, sulfate, tartrate, thiocyanate, *p*-toluenesulfonate, undecanoate, valerate salts, and the like. Representative alkali or alkaline earth metal salts include sodium, lithium, potassium, calcium, magnesium, and the like. In some embodiments, pharmaceutically acceptable salts include, when appropriate, nontoxic ammonium, quaternary ammonium, and amine cations formed using counterions such as halide, hydroxide, carboxylate, sulfate, phosphate, nitrate, alkyl having from 1 to 6 carbon atoms, sulfonate and aryl sulfonate.

[0045] *Prodrug:* A general, a "prodrug," as that term is used herein and as is understood in the art, is an entity that, when administered to an organism, is metabolized in the body to deliver an active (e.g., therapeutic or diagnostic) agent of interest. Typically, such metabolism involves removal of at least one "prodrug moiety" so that the active agent is formed. Various forms of "prodrugs" are known in the art. For examples of such prodrug moieties, see:

a) *Design of Prodrugs*, edited by H. Bundgaard, (Elsevier, 1985) and *Methods in Enzymology*, 42:309-396, edited by K. Widder, *et al. (Academic* Press, 1985);

b) *Prodrugs and Targeted Delivery,* edited by by J. Rautio (Wiley, 2011);

c) *Prodrugs and Targeted Delivery,* edited by by J. Rautio (Wiley, 2011);

d) A Textbook of Drug Design and Development, edited by Krogsgaard-Larsen;

e) Bundgaard, Chapter 5 "Design and Application of Prodrugs", by H. Bundgaard, p. 113-191 (1991);

f) Bundgaard, *Advanced Drug Delivery Reviews*, 8:1-38 (1992);

g) Bundgaard, *et al., Journal of Pharmaceutical Sciences*, 77:285 (1988); and

h) Kakeya, *et al., Chem. Pharm. Bull.*, 32:692 (1984).

[0046] As with other compounds described herein, prodrugs may be provided in any of a variety of forms, e.g., crystal forms, salt forms etc. In some embodiments, prodrugs are provided as pharmaceutically acceptable salts thereof.

[0047] *Protecting group:* The term "protecting group," as used herein, is well known in the art and includes those described in detail in *Protecting Groups in Organic Synthesis*, T. W. Greene and P. G. M. Wuts, 3rd edition, John Wiley & Sons, 1999, the entirety of which is incorporated herein by reference. Also included are those protecting groups specially adapted for nucleoside and nucleotide chemistry described in *Current Protocols in Nucleic Acid*

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Chemistry, edited by Serge L. Beaucage *et al.* 06/2012, the entirety of Chapter 2 is incorporated herein by reference. Suitable amino–protecting groups include methyl carbamate, ethyl carbamante, 9–fluorenylmethyl carbamate (Fmoc), 9–(2–sulfo)fluorenylmethyl carbamate, 9–(2,7–dibromo)fluoroenylmethyl carbamate, 2,7–di–*t*–butyl–[9–(10,10–dioxo–10,10,10,10–tetrahydrothioxanthyl)]methyl carbamate (DBD–Tmoc), 4–methoxyphenacyl carbamate (Phenoc), 2,2,2–trichloroethyl carbamate (Troc), 2–trimethylsilylethyl carbamate (Teoc), 2–phenylethyl carbamate (hZ), 1–(1–adamantyl)–1–methylethyl carbamate (Adpoc), 1,1–dimethyl–2–haloethyl carbamate, 1,1–dimethyl–2,2–dibromoethyl carbamate (DB–*t*–BOC), 1,1–dimethyl–2,2,2–trichloroethyl carbamate (TCBOC), 1–methyl–1–(4–biphenylyl)ethyl carbamate (Bpoc), 1–(3,5–di–*t*–butylphenyl)–1–methylethyl carbamate (*t*–Bumeoc), 2–(2'– and 4'–pyridyl)ethyl carbamate (Pyoc), 2–(*N,N*–dicyclohexylcarboxamido)ethyl carbamate, *t*–butyl carbamate (BOC), 1–adamantyl carbamate (Adoc), vinyl carbamate (Voc), allyl carbamate (Alloc), 1–isopropylallyl carbamate (Ipaoc), cinnamyl carbamate (Coc), 4–nitrocinnamyl carbamate (Noc), 8–quinolyl carbamate, *N*–hydroxypiperidinyl carbamate, alkyldithio carbamate, benzyl carbamate (Cbz), *p*–methoxybenzyl carbamate (Moz), *p*–nitobenzyl carbamate, *p*–bromobenzyl carbamate, *p*–chlorobenzyl carbamate, 2,4–dichlorobenzyl carbamate, 4–methylsulfinylbenzyl carbamate (Msz), 9–anthrylmethyl carbamate, diphenylmethyl carbamate, 2–methylthioethyl carbamate, 2–methylsulfonylethyl carbamate, 2–(*p*–toluenesulfonyl)ethyl carbamate, [2–(1,3–dithianyl)]methyl carbamate (Dmoc), 4–methylthiophenyl carbamate (Mtpc), 2,4–dimethylthiophenyl carbamate (Bmpc), 2–phosphonioethyl carbamate (Peoc), 2–triphenylphosphonioisopropyl carbamate (Ppoc), 1,1–dimethyl–2–cyanoethyl carbamate, *m*–chloro–*p*–acyloxybenzyl carbamate, *p*–(dihydroxyboryl)benzyl carbamate, 5–benzisoxazolylmethyl carbamate, 2–(trifluoromethyl)–6–chromonylmethyl carbamate (Tcroc), *m*–nitrophenyl carbamate, 3,5–dimethoxybenzyl carbamate, *o*–nitrobenzyl carbamate, 3,4–dimethoxy–6–nitrobenzyl carbamate, phenyl(*o*–nitrophenyl)methyl carbamate, phenothiazinyl–(10)–carbonyl derivative, *N'*–*p*–toluenesulfonylaminocarbonyl derivative, *N'*–phenylaminothiocarbonyl derivative, *t*–amyl carbamate, *S*–benzyl thiocarbamate, *p*–cyanobenzyl carbamate, cyclobutyl carbamate, cyclohexyl carbamate, cyclopentyl carbamate, cyclopropylmethyl carbamate, *p*–decyloxybenzyl carbamate, 2,2–dimethoxycarbonylvinyl carbamate, *o*–(*N,N*–dimethylcarboxamido)benzyl carbamate, 1,1–dimethyl–3–(*N,N*–dimethylcarboxamido)propyl carbamate, 1,1–dimethylpropynyl carbamate, di(2–pyridyl)methyl

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carbamate, 2–furanylmethyl carbamate, 2–iodoethyl carbamate, isoborynl carbamate, isobutyl carbamate, isonicotinyl carbamate, *p*–(*p'*–methoxyphenylazo)benzyl carbamate, 1–methylcyclobutyl carbamate, 1–methylcyclohexyl carbamate, 1–methyl–1–cyclopropylmethyl carbamate, 1–methyl–1–(3,5–dimethoxyphenyl)ethyl carbamate, 1–methyl–1–(*p*–phenylazophenyl)ethyl carbamate, 1–methyl–1–phenylethyl carbamate, 1–methyl–1–(4–pyridyl)ethyl carbamate, phenyl carbamate, *p*–(phenylazo)benzyl carbamate, 2,4,6–tri–*t*–butylphenyl carbamate, 4–(trimethylammonium)benzyl carbamate, 2,4,6–trimethylbenzyl carbamate, formamide, acetamide, chloroacetamide, trichloroacetamide, trifluoroacetamide, phenylacetamide, 3–phenylpropanamide, picolinamide, 3–pyridylcarboxamide, *N*–benzoylphenylalanyl derivative, benzamide, *p*–phenylbenzamide, *o*–nitophenylacetamide, *o*–nitrophenoxyacetamide, acetoacetamide, (*N'*–dithiobenzyloxycarbonylamino)acetamide, 3–(*p*–hydroxyphenyl)propanamide, 3–(*o*–nitrophenyl)propanamide, 2–methyl–2–(*o*–nitrophenoxy)propanamide, 2–methyl–2–(*o*–phenylazophenoxy)propanamide, 4–chlorobutanamide, 3–methyl–3–nitrobutanamide, *o*–nitrocinnamide, *N*–acetylmethionine derivative, *o*–nitrobenzamide, *o*–(benzoyloxymethyl)benzamide, 4,5–diphenyl–3–oxazolin–2–one, *N*–phthalimide, *N*–dithiasuccinimide (Dts), *N*–2,3–diphenylmaleimide, *N*–2,5–dimethylpyrrole, *N*–1,1,4,4–tetramethyldisilylazacyclopentane adduct (STABASE), 5–substituted 1,3–dimethyl–1,3,5–triazacyclohexan–2–one, 5–substituted 1,3–dibenzyl–1,3,5–triazacyclohexan–2–one, 1–substituted 3,5–dinitro–4–pyridone, *N*–methylamine, *N*–allylamine, *N*–[2–(trimethylsilyl)ethoxy]methylamine (SEM), *N*–3–acetoxypropylamine, *N*–(1–isopropyl–4–nitro–2–oxo–3–pyroolin–3–yl)amine, quaternary ammonium salts, *N*–benzylamine, *N*–di(4–methoxyphenyl)methylamine, *N*–5–dibenzosuberylamine, *N*–triphenylmethylamine (Tr), *N*–[(4–methoxyphenyl)diphenylmethyl]amine (MMTr), *N*–9–phenylfluorenylamine (PhF), *N*–2,7–dichloro–9–fluorenylmethyleneamine, *N*–ferrocenylmethylamino (Fcm), *N*–2–picolylamino *N'*–oxide, *N*–1,1–dimethylthiomethyleneamine, *N*–benzylideneamine, *N*–*p*–methoxybenzylideneamine, *N*–diphenylmethyleneamine, *N*–[(2–pyridyl)mesityl]methyleneamine, *N*–(*N',N'*–dimethylaminomethylene)amine, *N,N'*–isopropylidenediamine, *N*–*p*–nitrobenzylideneamine, *N*–salicylideneamine, *N*–5–chlorosalicylideneamine, *N*–(5–chloro–2–hydroxyphenyl)phenylmethyleneamine, *N*–cyclohexylideneamine, *N*–(5,5–dimethyl–3–oxo–1–cyclohexenyl)amine, *N*–borane derivative, *N*–diphenylborinic acid derivative, *N*–[phenyl(pentacarbonylchromium– or

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tungsten)carbonyl]amine, *N*–copper chelate, *N*–zinc chelate, *N*–nitroamine, *N*–nitrosoamine, amine *N*–oxide, diphenylphosphinamide (Dpp), dimethylthiophosphinamide (Mpt), diphenylthiophosphinamide (Ppt), dialkyl phosphoramidates, dibenzyl phosphoramidate, diphenyl phosphoramidate, benzenesulfenamide, *o*–nitrobenzenesulfenamide (Nps), 2,4–dinitrobenzenesulfenamide, pentachlorobenzenesulfenamide, 2–nitro–4–methoxybenzenesulfenamide, triphenylmethylsulfenamide, 3–nitropyridinesulfenamide (Npys), *p*–toluenesulfonamide (Ts), benzenesulfonamide, 2,3,6,–trimethyl–4–methoxybenzenesulfonamide (Mtr), 2,4,6–trimethoxybenzenesulfonamide (Mtb), 2,6–dimethyl–4–methoxybenzenesulfonamide (Pme), 2,3,5,6–tetramethyl–4–methoxybenzenesulfonamide (Mte), 4–methoxybenzenesulfonamide (Mbs), 2,4,6–trimethylbenzenesulfonamide (Mts), 2,6–dimethoxy–4–methylbenzenesulfonamide (iMds), 2,2,5,7,8–pentamethylchroman–6–sulfonamide (Pmc), methanesulfonamide (Ms), β–trimethylsilylethanesulfonamide (SES), 9–anthracenesulfonamide, 4–(4',8'–dimethoxynaphthylmethyl)benzenesulfonamide (DNMBS), benzylsulfonamide, trifluoromethylsulfonamide, and phenacylsulfonamide.

[0048] Suitably protected carboxylic acids further include, but are not limited to, silyl–, alkyl–, alkenyl–, aryl–, and arylalkyl–protected carboxylic acids. Examples of suitable silyl groups include trimethylsilyl, triethylsilyl, t–butyldimethylsilyl, t–butyldiphenylsilyl, triisopropylsilyl, and the like. Examples of suitable alkyl groups include methyl, benzyl, p–methoxybenzyl, 3,4–dimethoxybenzyl, trityl, t–butyl, tetrahydropyran–2–yl. Examples of suitable alkenyl groups include allyl. Examples of suitable aryl groups include optionally substituted phenyl, biphenyl, or naphthyl. Examples of suitable arylalkyl groups include optionally substituted benzyl (*e.g.,* p–methoxybenzyl (MPM), 3,4–dimethoxybenzyl, O–nitrobenzyl, p–nitrobenzyl, p–halobenzyl, 2,6–dichlorobenzyl, p–cyanobenzyl), and 2– and 4–picolyl.

[0049] Suitable hydroxyl protecting groups include methyl, methoxylmethyl (MOM), methylthiomethyl (MTM), *t*–butylthiomethyl, (phenyldimethylsilyl)methoxymethyl (SMOM), benzyloxymethyl (BOM), *p*–methoxybenzyloxymethyl (PMBM), (4–methoxyphenoxy)methyl (*p*–AOM), guaiacolmethyl (GUM), *t*–butoxymethyl, 4–pentenyloxymethyl (POM), siloxymethyl, 2–methoxyethoxymethyl (MEM), 2,2,2–trichloroethoxymethyl, bis(2–chloroethoxy)methyl, 2–(trimethylsilyl)ethoxymethyl (SEMOR), tetrahydropyranyl (THP), 3–bromotetrahydropyranyl, tetrahydrothiopyranyl, 1–methoxycyclohexyl, 4–

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methoxytetrahydropyranyl (MTHP), 4–methoxytetrahydrothiopyranyl, 4–methoxytetrahydrothiopyranyl S,S–dioxide, 1–[(2–chloro–4–methyl)phenyl]–4–methoxypiperidin–4–yl (CTMP), 1,4–dioxan–2–yl, tetrahydrofuranyl, tetrahydrothiofuranyl, 2,3,3a,4,5,6,7,7a–octahydro–7,8,8–trimethyl–4,7–methanobenzofuran–2–yl, 1–ethoxyethyl, 1–(2–chloroethoxy)ethyl, 1–methyl–1–methoxyethyl, 1–methyl–1–benzyloxyethyl, 1–methyl–1–benzyloxy–2–fluoroethyl, 2,2,2–trichloroethyl, 2–trimethylsilylethyl, 2–(phenylselenyl)ethyl, *t*–butyl, allyl, *p*–chlorophenyl, *p*–methoxyphenyl, 2,4–dinitrophenyl, benzyl, *p*–methoxybenzyl, 3,4–dimethoxybenzyl, *o*–nitrobenzyl, *p*–nitrobenzyl, *p*–halobenzyl, 2,6–dichlorobenzyl, *p*–cyanobenzyl, *p*–phenylbenzyl, 2–picolyl, 4–picolyl, 3–methyl–2–picolyl *N*–oxido, diphenylmethyl, *p,p'*–dinitrobenzhydryl, 5–dibenzosuberyl, triphenylmethyl, α–naphthyldiphenylmethyl, *p*–methoxyphenyldiphenylmethyl, di(*p*–methoxyphenyl)phenylmethyl, tri(*p*–methoxyphenyl)methyl, 4–(4'–bromophenacyloxyphenyl)diphenylmethyl, 4,4',4''–tris(4,5–dichlorophthalimidophenyl)methyl, 4,4',4''–tris(levulinoyloxyphenyl)methyl, 4,4',4''–tris(benzoyloxyphenyl)methyl, 3–(imidazol–1–yl)bis(4',4''–dimethoxyphenyl)methyl, 1,1–bis(4–methoxyphenyl)–1'–pyrenylmethyl, 9–anthryl, 9–(9–phenyl)xanthenyl, 9–(9–phenyl–10–oxo)anthryl, 1,3–benzodithiolan–2–yl, benzisothiazolyl S,S–dioxido, trimethylsilyl (TMS), triethylsilyl (TES), triisopropylsilyl (TIPS), dimethylisopropylsilyl (IPDMS), diethylisopropylsilyl (DEIPS), dimethylthexylsilyl, *t*–butyldimethylsilyl (TBDMS), *t*–butyldiphenylsilyl (TBDPS), tribenzylsilyl, tri–*p*–xylylsilyl, triphenylsilyl, diphenylmethylsilyl (DPMS), *t*–butylmethoxyphenylsilyl (TBMPS), formate, benzoylformate, acetate, chloroacetate, dichloroacetate, trichloroacetate, trifluoroacetate, methoxyacetate, triphenylmethoxyacetate, phenoxyacetate, *p*–chlorophenoxyacetate, 3–phenylpropionate, 4–oxopentanoate (levulinate), 4,4–(ethylenedithio)pentanoate (levulinoyldithioacetal), pivaloate, adamantoate, crotonate, 4–methoxycrotonate, benzoate, *p*–phenylbenzoate, 2,4,6–trimethylbenzoate (mesitoate), alkyl methyl carbonate, 9–fluorenylmethyl carbonate (Fmoc), alkyl ethyl carbonate, alkyl 2,2,2–trichloroethyl carbonate (Troc), 2–(trimethylsilyl)ethyl carbonate (TMSEC), 2–(phenylsulfonyl) ethyl carbonate (Psec), 2–(triphenylphosphonio) ethyl carbonate (Peoc), alkyl isobutyl carbonate, alkyl vinyl carbonate alkyl allyl carbonate, alkyl *p*–nitrophenyl carbonate, alkyl benzyl carbonate, alkyl *p*–methoxybenzyl carbonate, alkyl 3,4–dimethoxybenzyl carbonate, alkyl *o*–nitrobenzyl carbonate, alkyl *p*–nitrobenzyl carbonate, alkyl *S*–benzyl thiocarbonate, 4–ethoxy–1–napththyl carbonate, methyl dithiocarbonate, 2–iodobenzoate, 4–azidobutyrate, 4–nitro–4–

methylpentanoate, *o*–(dibromomethyl)benzoate, 2–formylbenzenesulfonate, 2–(methylthiomethoxy)ethyl, 4–(methylthiomethoxy)butyrate, 2–(methylthiomethoxymethyl)benzoate, 2,6–dichloro–4–methylphenoxyacetate, 2,6–dichloro–4–(1,1,3,3–tetramethylbutyl)phenoxyacetate, 2,4–bis(1,1–dimethylpropyl)phenoxyacetate, chlorodiphenylacetate, isobutyrate, monosuccinoate, (*E*)–2–methyl–2–butenoate, *o*–(methoxycarbonyl)benzoate, α–naphthoate, nitrate, alkyl *N,N,N',N'*–tetramethylphosphorodiamidate, alkyl *N*–phenylcarbamate, borate, dimethylphosphinothioyl, alkyl 2,4–dinitrophenylsulfenate, sulfate, methanesulfonate (mesylate), benzylsulfonate, and tosylate (Ts). For protecting 1,2– or 1,3–diols, the protecting groups include methylene acetal, ethylidene acetal, 1–*t*–butylethylidene ketal, 1–phenylethylidene ketal, (4–methoxyphenyl)ethylidene acetal, 2,2,2–trichloroethylidene acetal, acetonide, cyclopentylidene ketal, cyclohexylidene ketal, cycloheptylidene ketal, benzylidene acetal, *p*–methoxybenzylidene acetal, 2,4–dimethoxybenzylidene ketal, 3,4–dimethoxybenzylidene acetal, 2–nitrobenzylidene acetal, methoxymethylene acetal, ethoxymethylene acetal, dimethoxymethylene ortho ester, 1–methoxyethylidene ortho ester, 1–ethoxyethylidine ortho ester, 1,2–dimethoxyethylidene ortho ester, α–methoxybenzylidene ortho ester, 1–(*N,N*–dimethylamino)ethylidene derivative, α–(*N,N'*–dimethylamino)benzylidene derivative, 2–oxacyclopentylidene ortho ester, di–*t*–butylsilylene group (DTBS), 1,3–(1,1,3,3–tetraisopropyldisiloxanylidene) derivative (TIPDS), tetra–*t*–butoxydisiloxane–1,3–diylidene derivative (TBDS), cyclic carbonates, cyclic boronates, ethyl boronate, and phenyl boronate.

[0050] In some embodiments, a hydroxyl protecting group is acetyl, t-butyl, t-butoxymethyl, methoxymethyl, tetrahydropyranyl, 1 -ethoxyethyl, 1 -(2-chloroethoxy)ethyl, 2-trimethylsilylethyl, p-chlorophenyl, 2,4-dinitrophenyl, benzyl, benzoyl, p-phenylbenzoyl, 2,6-dichlorobenzyl, diphenylmethyl, p-nitrobenzyl, triphenylmethyl (trityl), 4,4'-dimethoxytrityl, trimethylsilyl, triethylsilyl, t-butyldimethylsilyl, t-butyldiphenylsilyl, triphenylsilyl, triisopropylsilyl, benzoylformate, chloroacetyl, trichloroacetyl, trifiuoroacetyl, pivaloyl, 9-fluorenylmethyl carbonate, mesylate, tosylate, triflate, trityl, monomethoxytrityl (MMTr), 4,4'-dimethoxytrityl, (DMTr) and 4,4',4"-trimethoxytrityl (TMTr), 2-cyanoethyl (CE or Cne), 2-(trimethylsilyl)ethyl (TSE), 2-(2-nitrophenyl)ethyl, 2-(4-cyanophenyl)ethyl 2-(4-nitrophenyl)ethyl (NPE), 2-(4-nitrophenylsulfonyl)ethyl, 3,5-dichlorophenyl, 2,4-dimethylphenyl, 2-nitrophenyl, 4-nitrophenyl, 2,4,6-trimethylphenyl, 2-(2-nitrophenyl)ethyl,

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butylthiocarbonyl, 4,4',4"-tris(benzoyloxy)trityl, diphenylcarbamoyl, levulinyl, 2-(dibromomethyl)benzoyl (Dbmb), 2-(isopropylthiomethoxymethyl)benzoyl (Ptmt), 9-phenylxanthen-9-yl (pixyl) or 9-(p-methoxyphenyl)xanthine-9-yl (MOX). In some embodiments, each of the hydroxyl protecting groups is, independently selected from acetyl, benzyl, t- butyldimethylsilyl, t-butyldiphenylsilyl and 4,4'-dimethoxytrityl. In some embodiments, the hydroxyl protecting group is selected from the group consisting of trityl, monomethoxytrityl and 4,4'-dimethoxytrityl group.

[0051] In some embodiments, a phosphorous protecting group is a group attached to the internucleotide phosphorous linkage throughout oligonucleotide synthesis. In some embodiments, the phosphorous protecting group is attached to the sulfur atom of the internucleotide phosphorothioate linkage. In some embodiments, the phosphorous protecting group is attached to the oxygen atom of the internucleotide phosphorothioate linkage. In some embodiments, the phosphorous protecting group is attached to the oxygen atom of the internucleotide phosphate linkage. In some embodiments the phosphorous protecting group is 2-cyanoethyl (CE or Cne), 2-trimethylsilylethyl, 2-nitroethyl, 2-sulfonylethyl, methyl, benzyl, *o*-nitrobenzyl, 2-(*p*-nitrophenyl)ethyl (NPE or Npe), 2-phenylethyl, 3-(*N*-*tert*-butylcarboxamido)-1-propyl, 4-oxopentyl, 4-methylthio-l-butyl, 2-cyano-1,1-dimethylethyl, 4-*N*-methylaminobutyl, 3-(2-pyridyl)-1-propyl, 2-[*N*-methyl-*N*-(2-pyridyl)]aminoethyl, 2-(*N*-formyl,*N*-methyl)aminoethyl, 4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl.

[0052] *Protein:* As used herein, the term "protein" refers to a polypeptide (*i.e.*, a string of at least two amino acids linked to one another by peptide bonds). In some embodiments, proteins include only naturally-occurring amino acids. In some embodiments, proteins include one or more non-naturally-occurring amino acids (e.g., moieties that form one or more peptide bonds with adjacent amino acids). In some embodiments, one or more residues in a protein chain contain a non-amino-acid moiety (e.g., a glycan, etc). In some embodiments, a protein includes more than one polypeptide chain, for example linked by one or more disulfide bonds or associated by other means. In some embodiments, proteins contain L-amino acids, D-amino acids, or both; in some embodiments, proteins contain one or more amino acid modifications or analogs known in the art. Useful modifications include, *e.g.*, terminal acetylation, amidation, methylation, *etc.* The term "peptide" is generally used to refer to a polypeptide having a length of less than about 100 amino acids, less than about 50 amino acids, less than 20 amino acids, or

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less than 10 amino acids. In some embodiments, proteins are antibodies, antibody fragments, biologically active portions thereof, and/or characteristic portions thereof.

[0053] *Sample*: As used herein, the term "sample" refers to a biological sample obtained or derived from a source of interest, as described herein. In some embodiments, a source of interest comprises an organism, such as an animal or human. In some embodiments, a biological sample comprises biological tissue or fluid. In some embodiments, a biological sample is or comprises bone marrow; blood; blood cells; ascites; tissue or fine needle biopsy samples; cell-containing body fluids; free floating nucleic acids; sputum; saliva; urine; cerebrospinal fluid, peritoneal fluid; pleural fluid; feces; lymph; gynecological fluids; skin swabs; vaginal swabs; oral swabs; nasal swabs; washings or lavages such as a ductal lavages or broncheoalveolar lavages; aspirates; scrapings; bone marrow specimens; tissue biopsy specimens; surgical specimens; feces, other body fluids, secretions, and/or excretions; and/or cells therefrom, *etc.* In some embodiments, a biological sample is or comprises cells obtained from an individual. In some embodiments, a sample is a "primary sample" obtained directly from a source of interest by any appropriate means. For example, in some embodiments, a primary biological sample is obtained by methods selected from the group consisting of biopsy (*e.g.*, fine needle aspiration or tissue biopsy), surgery, collection of body fluid (*e.g.,* blood, lymph, feces *etc.*), *etc.* In some embodiments, as will be clear from context, the term "sample" refers to a preparation that is obtained by processing (e.g., by removing one or more components of and/or by adding one or more agents to) a primary sample. For example, filtering using a semi-permeable membrane. Such a "processed sample" may comprise, for example nucleic acids or proteins extracted from a sample or obtained by subjecting a primary sample to techniques such as amplification or reverse transcription of mRNA, isolation and/or purification of certain components, etc.

[0054] *Stereochemically isomeric forms:* The phrase "stereochemically isomeric forms," as used herein, refers to different compounds made up of the same atoms bonded by the same sequence of bonds but having different three-dimensional structures which are not interchangeable. In some embodiments of the invention, provided chemical compositions may be or include pure preparations of individual stereochemically isomeric forms of a compound; in some embodiments, provided chemical compositions may be or include mixtures of two or more stereochemically isomeric forms of the compound. In certain embodiments, such mixtures contain equal amounts of different stereochemically isomeric forms; in certain embodiments,

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such mixtures contain different amounts of at least two different stereochemically isomeric forms. In some embodiments, a chemical composition may contain all diastereomers and/or enantiomers of the compound. In some embodiments, a chemical composition may contain less than all diastereomers and/or enantiomers of a compound. In some embodiments, if a particular enantiomer of a compound of the present invention is desired, it may be prepared, for example, by asymmetric synthesis, or by derivation with a chiral auxiliary, where the resulting diastereomeric mixture is separated and the auxiliary group cleaved to provide the pure desired enantiomers. Alternatively, where the molecule contains a basic functional group, such as amino, diastereomeric salts are formed with an appropriate optically-active acid, and resolved, for example, by fractional crystallization.

[0055] *Subject:* As used herein, the term "subject" or "test subject" refers to any organism to which a provided compound or composition is administered in accordance with the present invention *e.g.*, for experimental, diagnostic, prophylactic, and/or therapeutic purposes. Typical subjects include animals (*e.g.*, mammals such as mice, rats, rabbits, non-human primates, and humans; insects; worms; *etc.*) and plants. In some embodiments, a subject may be suffering from, and/or susceptible to a disease, disorder, and/or condition.

[0056] *Substantially:* As used herein, the term "substantially" refers to the qualitative condition of exhibiting total or near-total extent or degree of a characteristic or property of interest. One of ordinary skill in the biological arts will understand that biological and chemical phenomena rarely, if ever, go to completion and/or proceed to completeness or achieve or avoid an absolute result. The term "substantially" is therefore used herein to capture the potential lack of completeness inherent in many biological and/or chemical phenomena.

[0057] *Suffering from*: An individual who is "suffering from" a disease, disorder, and/or condition has been diagnosed with and/or displays one or more symptoms of a disease, disorder, and/or condition.

[0058] *Susceptible to*: An individual who is "susceptible to" a disease, disorder, and/or condition is one who has a higher risk of developing the disease, disorder, and/or condition than does a member of the general public. In some embodiments, an individual who is susceptible to a disease, disorder and/or condition may not have been diagnosed with the disease, disorder, and/or condition. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition may exhibit symptoms of the disease, disorder, and/or condition. In some

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embodiments, an individual who is susceptible to a disease, disorder, and/or condition may not exhibit symptoms of the disease, disorder, and/or condition. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition will develop the disease, disorder, and/or condition. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition will not develop the disease, disorder, and/or condition.

[0059] *Systemic:* The phrases "systemic administration," "administered systemically," "peripheral administration," and "administered peripherally" as used herein have their art-understood meaning referring to administration of a compound or composition such that it enters the recipient's system.

[0060] *Tautomeric forms:* The phrase "tautomeric forms," as used herein, is used to describe different isomeric forms of organic compounds that are capable of facile interconversion. Tautomers may be characterized by the formal migration of a hydrogen atom or proton, accompanied by a switch of a single bond and adjacent double bond. In some embodiments, tautomers may result from prototropic tautomerism (*i.e.,* the relocation of a proton). In some embodiments, tautomers may result from valence tautomerism (*i.e.,* the rapid reorganization of bonding electrons). All such tautomeric forms are intended to be included within the scope of the present invention. In some embodiments, tautomeric forms of a compound exist in mobile equilibrium with each other, so that attempts to prepare the separate substances results in the formation of a mixture. In some embodiments, tautomeric forms of a compound are separable and isolatable compounds. In some embodiments of the invention, chemical compositions may be provided that are or include pure preparations of a single tautomeric form of a compound. In some embodiments of the invention, chemical compositions may be provided as mixtures of two or more tautomeric forms of a compound. In certain embodiments, such mixtures contain equal amounts of different tautomeric forms; in certain embodiments, such mixtures contain different amounts of at least two different tautomeric forms of a compound. In some embodiments of the invention, chemical compositions may contain all tautomeric forms of a compound. In some embodiments of the invention, chemical compositions may contain less than all tautomeric forms of a compound. In some embodiments of the invention, chemical compositions may contain one or more tautomeric forms of a compound in amounts that vary over time as a result of interconversion. In some embodiments of the invention, the tautomerism is keto-enol tautomerism. One of skill in the chemical arts would

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recognize that a keto-enol tautomer can be "trapped" (i.e., chemically modified such that it remains in the "enol" form) using any suitable reagent known in the chemical arts in to provide an enol derivative that may subsequently be isolated using one or more suitable techniques known in the art. Unless otherwise indicated, the present invention encompasses all tautomeric forms of relevant compounds, whether in pure form or in admixture with one another.

[0061] *Therapeutic agent:* As used herein, the phrase "therapeutic agent" refers to any agent that, when administered to a subject, has a therapeutic effect and/or elicits a desired biological and/or pharmacological effect. In some embodiments, a therapeutic agent is any substance that can be used to alleviate, ameliorate, relieve, inhibit, prevent, delay onset of, reduce severity of, and/or reduce incidence of one or more symptoms or features of a disease, disorder, and/or condition.

[0062] *Therapeutically effective amount:* As used herein, the term "therapeutically effective amount" means an amount of a substance (*e.g.*, a therapeutic agent, composition, and/or formulation) that elicits a desired biological response when administered as part of a therapeutic regimen. In some embodiments, a therapeutically effective amount of a substance is an amount that is sufficient, when administered to a subject suffering from or susceptible to a disease, disorder, and/or condition, to treat, diagnose, prevent, and/or delay the onset of the disease, disorder, and/or condition. As will be appreciated by those of ordinary skill in this art, the effective amount of a substance may vary depending on such factors as the desired biological endpoint, the substance to be delivered, the target cell or tissue, *etc.* For example, the effective amount of compound in a formulation to treat a disease, disorder, and/or condition is the amount that alleviates, ameliorates, relieves, inhibits, prevents, delays onset of, reduces severity of and/or reduces incidence of one or more symptoms or features of the disease, disorder, and/or condition. In some embodiments, a therapeutically effective amount is administered in a single dose; in some embodiments, multiple unit doses are required to deliver a therapeutically effective amount.

[0063] *Treat:* As used herein, the term "treat," "treatment," or "treating" refers to any method used to partially or completely alleviate, ameliorate, relieve, inhibit, prevent, delay onset of, reduce severity of, and/or reduce incidence of one or more symptoms or features of a disease, disorder, and/or condition. Treatment may be administered to a subject who does not exhibit signs of a disease, disorder, and/or condition. In some embodiments, treatment may be

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administered to a subject who exhibits only early signs of the disease, disorder, and/or condition, for example for the purpose of decreasing the risk of developing pathology associated with the disease, disorder, and/or condition.

[0064] *Unsaturated:* The term "unsaturated," as used herein, means that a moiety has one or more units of unsaturation.

[0065] *Unit dose*: The expression "unit dose" as used herein refers to an amount administered as a single dose and/or in a physically discrete unit of a pharmaceutical composition. In many embodiments, a unit dose contains a predetermined quantity of an active agent. In some embodiments, a unit dose contains an entire single dose of the agent. In some embodiments, more than one unit dose is administered to achieve a total single dose. In some embodiments, administration of multiple unit doses is required, or expected to be required, in order to achieve an intended effect. A unit dose may be, for example, a volume of liquid (e.g., an acceptable carrier) containing a predetermined quantity of one or more therapeutic agents, a predetermined amount of one or more therapeutic agents in solid form, a sustained release formulation or drug delivery device containing a predetermined amount of one or more therapeutic agents, etc. It will be appreciated that a unit dose may be present in a formulation that includes any of a variety of components in addition to the therapeutic agent(s). For example, acceptable carriers (e.g., pharmaceutically acceptable carriers), diluents, stabilizers, buffers, preservatives, etc., may be included as described infra. It will be appreciated by those skilled in the art, in many embodiments, a total appropriate daily dosage of a particular therapeutic agent may comprise a portion, or a plurality, of unit doses, and may be decided, for example, by the attending physician within the scope of sound medical judgment. In some embodiments, the specific effective dose level for any particular subject or organism may depend upon a variety of factors including the disorder being treated and the severity of the disorder; activity of specific active compound employed; specific composition employed; age, body weight, general health, sex and diet of the subject; time of administration, and rate of excretion of the specific active compound employed; duration of the treatment; drugs and/or additional therapies used in combination or coincidental with specific compound(s) employed, and like factors well known in the medical arts.

[0066] *Wild-type:* As used herein, the term "wild-type" has its art-understood meaning that refers to an entity having a structure and/or activity as found in nature in a "normal" (as

contrasted with mutant, diseased, altered, etc) state or context. Those of ordinary skill in the art will appreciate that wild type genes and polypeptides often exist in multiple different forms (e.g., alleles).

[0067] *Nucleic acid*: The term "nucleic acid" includes any nucleotides, analogs thereof, and polymers thereof. The term "polynucleotide" as used herein refer to a polymeric form of nucleotides of any length, either ribonucleotides (RNA) or deoxyribonucleotides (DNA). These terms refer to the primary structure of the molecules and, thus, include double- and single-stranded DNA, and double- and single-stranded RNA. These terms include, as equivalents, analogs of either RNA or DNA made from nucleotide analogs and modified polynucleotides such as, though not limited to, methylated, protected and/or capped nucleotides or polynucleotides. The terms encompass poly- or oligo-ribonucleotides (RNA) and poly- or oligo-deoxyribonucleotides (DNA); RNA or DNA derived from N-glycosides or C-glycosides of nucleobases and/or modified nucleobases; nucleic acids derived from sugars and/or modified sugars; and nucleic acids derived from phosphate bridges and/or modified phosphorus-atom bridges (also referred to herein as "internucleotide linkages"). The term encompasses nucleic acids containing any combinations of nucleobases, modified nucleobases, sugars, modified sugars, phosphate bridges or modified phosphorus atom bridges. Examples include, and are not limited to, nucleic acids containing ribose moieties, the nucleic acids containing deoxy-ribose moieties, nucleic acids containing both ribose and deoxyribose moieties, nucleic acids containing ribose and modified ribose moieties. The prefix poly- refers to a nucleic acid containing 2 to about 10,000 nucleotide monomer units and wherein the prefix oligo- refers to a nucleic acid containing 2 to about 200 nucleotide monomer units.

[0068] *Nucleotide:* The term "nucleotide" as used herein refers to a monomeric unit of a polynucleotide that consists of a heterocyclic base, a sugar, and one or more phosphate groups or phosphorus-containing internucleotidic linkages. The naturally occurring bases, (guanine, (G), adenine, (A), cytosine, (C), thymine, (T), and uracil (U)) are derivatives of purine or pyrimidine, though it should be understood that naturally and non-naturally occurring base analogs are also included. The naturally occurring sugar is the pentose (five-carbon sugar) deoxyribose (which forms DNA) or ribose (which forms RNA), though it should be understood that naturally and non-naturally occurring sugar analogs are also included. Nucleotides are linked via internucleotidic linkages to form nucleic acids, or polynucleotides. Many internucleotidic

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linkages are known in the art (such as, though not limited to, phosphate, phosphorothioates, boranophosphates and the like). Artificial nucleic acids include PNAs (peptide nucleic acids), phosphotriesters, phosphorothionates, *H*-phosphonates, phosphoramidates, boranophosphates, methylphosphonates, phosphonoacetates, thiophosphonoacetates and other variants of the phosphate backbone of native nucleic acids, such as those described herein.

[0069] *Nucleoside*: The term "nucleoside" refers to a moiety wherein a nucleobase or a modified nucleobase is covalently bound to a sugar or modified sugar.

[0070] *Sugar:* The term "sugar" refers to a monosaccharide in closed and/or open form. Sugars include, but are not limited to, ribose, deoxyribose, pentofuranose, pentopyranose, and hexopyranose moieties. As used herein, the term also encompasses structural analogs used in lieu of conventional sugar molecules, such as glycol, polymer of which forms the backbone of the nucleic acid analog, glycol nucleic acid ("GNA").

[0071] *Modified sugar*: The term "modified sugar" refers to a moiety that can replace a sugar. The modified sugar mimics the spatial arrangement, electronic properties, or some other physicochemical property of a sugar.

[0072] *Nucleobase*: The term "nucleobase" refers to the parts of nucleic acids that are involved in the hydrogen-bonding that binds one nucleic acid strand to another complementary strand in a sequence specific manner. The most common naturally-occurring nucleobases are adenine (A), guanine (G), uracil (U), cytosine (C), and thymine (T). In some embodiments, the naturally-occurring nucleobases are modified adenine, guanine, uracil, cytosine, or thymine. In some embodiments, the naturally-occurring nucleobases are methylated adenine, guanine, uracil, cytosine, or thymine. In some embodiments, a nucleobase is a "modified nucleobase," e.g., a nucleobase other than adenine (A), guanine (G), uracil (U), cytosine (C), and thymine (T). In some embodiments, the modified nucleobases are methylated adenine, guanine, uracil, cytosine, or thymine. In some embodiments, the modified nucleobase mimics the spatial arrangement, electronic properties, or some other physicochemical property of the nucleobase and retains the property of hydrogen-bonding that binds one nucleic acid strand to another in a sequence specific manner. In some embodiments, a modified nucleobase can pair with all of the five naturally occurring bases (uracil, thymine, adenine, cytosine, or guanine) without substantially affecting the melting behavior, recognition by intracellular enzymes or activity of the oligonucleotide duplex.

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[0073] *Chiral ligand*: The term "chiral ligand" or "chiral auxiliary" refers to a moiety that is chiral and can be incorporated into a reaction so that the reaction can be carried out with certain stereoselectivity.

[0074] *Condensing reagent*: In a condensation reaction, the term "condensing reagent" refers to a reagent that activates a less reactive site and renders it more susceptible to attack by another reagent. In some embodiments, such another reagent is a nucleophile.

[0075] *Blocking group*: The term "blocking group" refers to a group that masks the reactivity of a functional group. The functional group can be subsequently unmasked by removal of the blocking group. In some embodiments, a blocking group is a protecting group.

[0076] *Moiety*: The term "moiety" refers to a specific segment or functional group of a molecule. Chemical moieties are often recognized chemical entities embedded in or appended to a molecule.

[0077] *Solid support*: The term "solid support" refers to any support which enables synthesis of nucleic acids. In some embodiments, the term refers to a glass or a polymer, that is insoluble in the media employed in the reaction steps performed to synthesize nucleic acids, and is derivatized to comprise reactive groups. In some embodiments, the solid support is Highly Cross-linked Polystyrene (HCP) or Controlled Pore Glass (CPG). In some embodiments, the solid support is Controlled Pore Glass (CPG). In some embodiments, the solid support is hybrid support of Controlled Pore Glass (CPG) and Highly Cross-linked Polystyrene (HCP).

[0078] *Linking moiety*: The term "linking moiety" refers to any moiety optionally positioned between the terminal nucleoside and the solid support or between the terminal nucleoside and another nucleoside, nucleotide, or nucleic acid.

[0079] *DNA molecule*: A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in its either single stranded form or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences can be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the non-transcribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA).

[0080] *Coding sequence*: A DNA "coding sequence" or "coding region" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide *in vivo* when placed under the control of appropriate expression control sequences. The boundaries of the coding sequence (the "open reading frame" or "ORF") are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and synthetic DNA sequences. A polyadenylation signal and transcription termination sequence is, usually, be located 3' to the coding sequence. The term "non-coding sequence" or "non-coding region" refers to regions of a polynucleotide sequence that are not translated into amino acids (e.g. 5′ and 3′ un-translated regions).

[0081] *Reading frame*: The term "reading frame" refers to one of the six possible reading frames, three in each direction, of the double stranded DNA molecule. The reading frame that is used determines which codons are used to encode amino acids within the coding sequence of a DNA molecule.

[0082] *Antisense*: As used herein, an "antisense" nucleic acid molecule comprises a nucleotide sequence which is complementary to a "sense" nucleic acid encoding a protein, e.g., complementary to the coding strand of a double-stranded cDNA molecule, complementary to an mRNA sequence or complementary to the coding strand of a gene. Accordingly, an antisense nucleic acid molecule can associate via hydrogen bonds to a sense nucleic acid molecule.

[0083] *Wobble position*: As used herein, a "wobble position" refers to the third position of a codon. Mutations in a DNA molecule within the wobble position of a codon, in some embodiments, result in silent or conservative mutations at the amino acid level. For example, there are four codons that encode Glycine, i.e., GGU, GGC, GGA and GGG, thus mutation of any wobble position nucleotide, to any other nucleotide selected from A, U , C and G, does not result in a change at the amino acid level of the encoded protein and, therefore, is a silent substitution.

[0084] *Silent substitution*: a "silent substitution" or "silent mutation" is one in which a nucleotide within a codon is modified, but does not result in a change in the amino acid residue encoded by the codon. Examples include mutations in the third position of a codon, as well in

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the first position of certain codons such as in the codon "CGG" which, when mutated to AGG, still encodes Arg.

[0085] *Gene*: The terms "gene," "recombinant gene" and "gene construct" as used herein, refer to a DNA molecule, or portion of a DNA molecule, that encodes a protein or a portion thereof. The DNA molecule can contain an open reading frame encoding the protein (as exon sequences) and can further include intron sequences. The term "intron" as used herein, refers to a DNA sequence present in a given gene which is not translated into protein and is found in some, but not all cases, between exons. It can be desirable for the gene to be operably linked to, (or it can comprise), one or more promoters, enhancers, repressors and/or other regulatory sequences to modulate the activity or expression of the gene, as is well known in the art.

[0086] *Complementary DNA*: As used herein, a "complementary DNA" or "cDNA" includes recombinant polynucleotides synthesized by reverse transcription of mRNA and from which intervening sequences (introns) have been removed.

[0087] *Homology*: "Homology" or "identity" or "similarity" refers to sequence similarity between two nucleic acid molecules. Homology and identity can each be determined by comparing a position in each sequence which can be aligned for purposes of comparison. When an equivalent position in the compared sequences is occupied by the same base, then the molecules are identical at that position; when the equivalent site occupied by the same or a similar nucleic acid residue (e.g., similar in steric and/or electronic nature), then the molecules can be referred to as homologous (similar) at that position. Expression as a percentage of homology/similarity or identity refers to a function of the number of identical or similar nucleic acids at positions shared by the compared sequences. A sequence which is "unrelated" or "non-homologous" shares less than 40% identity, less than 35% identity, less than 30% identity, or less than 25% identity with a sequence described herein. In comparing two sequences, the absence of residues (amino acids or nucleic acids) or presence of extra residues also decreases the identity and homology/similarity.

[0088] In some embodiments, the term "homology" describes a mathematically based comparison of sequence similarities which is used to identify genes with similar functions or motifs. The nucleic acid sequences described herein can be used as a "query sequence" to perform a search against public databases, for example, to identify other family members, related

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sequences or homologs. In some embodiments, such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. (1990) J. Mol. Biol. 215:403-10. In some embodiments, BLAST nucleotide searches can be performed with the NBLAST program, score=100, wordlength=12 to obtain nucleotide sequences homologous to nucleic acid molecules of the invention. In some embodiments, to obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) Nucleic Acids Res. 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and BLAST) can be used (See www.ncbi.nlm.nih.gov).

[0089] *Identity*: As used herein, "identity" means the percentage of identical nucleotide residues at corresponding positions in two or more sequences when the sequences are aligned to maximize sequence matching, i.e., taking into account gaps and insertions. Identity can be readily calculated by known methods, including but not limited to those described in (Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., SIAM J. Applied Math., 48: 1073 (1988). Methods to determine identity are designed to give the largest match between the sequences tested. Moreover, methods to determine identity are codified in publicly available computer programs. Computer program methods to determine identity between two sequences include, but are not limited to, the GCG program package (Devereux, J., et al., Nucleic Acids Research 12(1): 387 (1984)), BLASTP, BLASTN, and FASTA (Altschul, S. F. et al., J. Molec. Biol. 215: 403-410 (1990) and Altschul et al. Nuc. Acids Res. 25: 3389-3402 (1997)). The BLAST X program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, Md. 20894; Altschul, S., et al., J. Mol. Biol. 215: 403-410 (1990). The well-known Smith Waterman algorithm can also be used to determine identity.

[0090] *Heterologous*: A "heterologous" region of a DNA sequence is an identifiable segment of DNA within a larger DNA sequence that is not found in association with the larger

sequence in nature. Thus, when the heterologous region encodes a mammalian gene, the gene can usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. Another example of a heterologous coding sequence is a sequence where the coding sequence itself is not found in nature (e.g., a cDNA where the genomic coding sequence contains introns or synthetic sequences having codons or motifs different than the unmodified gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

[0091] *Transition mutation:* The term "transition mutations" refers to base changes in a DNA sequence in which a pyrimidine (cytidine (C) or thymidine (T) is replaced by another pyrimidine, or a purine (adenosine (A) or guanosine (G) is replaced by another purine.

[0092] *Transversion mutation*: The term "transversion mutations" refers to base changes in a DNA sequence in which a pyrimidine (cytidine (C) or thymidine (T) is replaced by a purine (adenosine (A) or guanosine (G), or a purine is replaced by a pyrimidine.

[0093] *Oligonucleotide:* the term "oligonucleotide" refers to a polymer or oligomer of nucleotide monomers, containing any combination of nucleobases, modified nucleobases, sugars, modified sugars, phosphate bridges, or modified phosphorus atom bridges (also referred to herein as "internucleotidic linkage", defined further herein).

[0094] Oligonucleotides can be single-stranded or double-stranded. As used herein, the term "oligonucleotide strand" encompasses a single-stranded oligonucleotide. A single-stranded oligonucleotide can have double-stranded regions and a double-stranded oligonucleotide can have single-stranded regions. Exemplary oligonucleotides include, but are not limited to structural genes, genes including control and termination regions, self-replicating systems such as viral or plasmid DNA, single-stranded and double-stranded siRNAs and other RNA interference reagents (RNAi agents or iRNA agents), shRNA, antisense oligonucleotides, ribozymes, microRNAs, microRNA mimics, supermirs, aptamers, antimirs, antagomirs, Ul adaptors, triplex-forming oligonucleotides, G-quadruplex oligonucleotides, RNA activators, immuno-stimulatory oligonucleotides, and decoy oligonucleotides.

[0095] Double-stranded and single-stranded oligonucleotides that are effective in inducing RNA interference are also referred to as siRNA, RNAi agent, or iRNA agent, herein. In some embodiments, these RNA interference inducing oligonucleotides associate with a cytoplasmic multi-protein complex known as RNAi-induced silencing complex (RISC). In many

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embodiments, single-stranded and double-stranded RNAi agents are sufficiently long that they can be cleaved by an endogenous molecule, e.g., by Dicer, to produce smaller oligonucleotides that can enter the RISC machinery and participate in RISC mediated cleavage of a target sequence, e.g. a target mRNA.

[0096] Oligonucleotides of the present invention can be of various lengths. In particular embodiments, oligonucleotides can range from about 2 to about 200 nucleotides in length. In various related embodiments, oligonucleotides, single-stranded, double-stranded, and triple-stranded, can range in length from about 4 to about 10 nucleotides, from about 10 to about 50 nucleotides, from about 20 to about 50 nucleotides, from about 15 to about 30 nucleotides, from about 20 to about 30 nucleotides in length. In some embodiments, the oligonucleotide is from about 9 to about 39 nucleotides in length. In some embodiments, the oligonucleotide is at least 4 nucleotides in length. In some embodiments, the oligonucleotide is at least 5 nucleotides in length. In some embodiments, the oligonucleotide is at least 6 nucleotides in length. In some embodiments, the oligonucleotide is at least 7 nucleotides in length. In some embodiments, the oligonucleotide is at least 8 nucleotides in length. In some embodiments, the oligonucleotide is at least 9 nucleotides in length. In some embodiments, the oligonucleotide is at least 10 nucleotides in length. In some embodiments, the oligonucleotide is at least 11 nucleotides in length. In some embodiments, the oligonucleotide is at least 12 nucleotides in length. In some embodiments, the oligonucleotide is at least 15 nucleotides in length. In some embodiments, the oligonucleotide is at least 20 nucleotides in length. In some embodiments, the oligonucleotide is at least 25 nucleotides in length. In some embodiments, the oligonucleotide is at least 30 nucleotides in length. In some embodiments, the oligonucleotide is a duplex of complementary strands of at least 18 nucleotides in length. In some embodiments, the oligonucleotide is a duplex of complementary strands of at least 21 nucleotides in length.

[0097] *Internucleotidic linkage:* As used herein, the phrase "internucleotidic linkage" refers generally to the phosphorus-containing linkage between nucleotide units of an oligonucleotide, and is interchangeable with "inter-sugar linkage" and "phosphorus atom bridge," as used above and herein. In some embodiments, an internucleotidic linkage is a phosphodiester linkage, as found in naturally occurring DNA and RNA molecules. In some embodiments, an internucleotidic linkage is a "modified internucleotidic linkage" wherein each oxygen atom of the phosphodiester linkage is optionally and independently replaced by an

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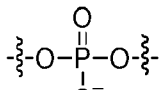
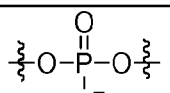
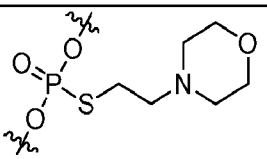
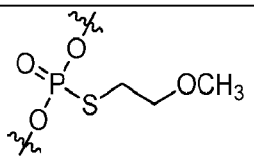
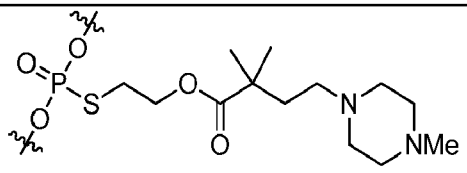
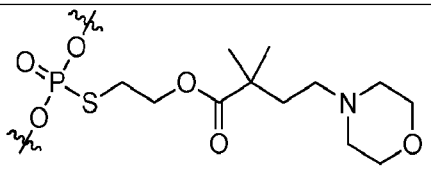
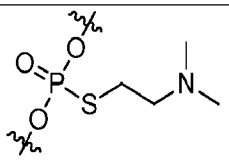
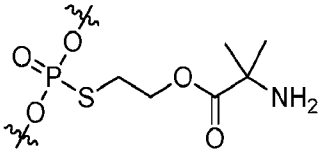
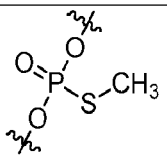
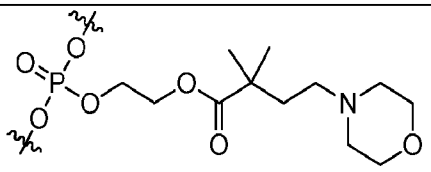
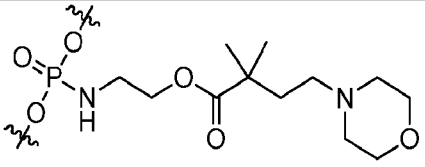
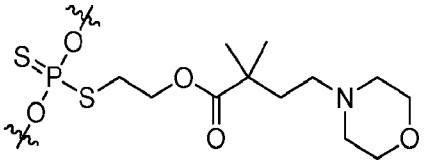
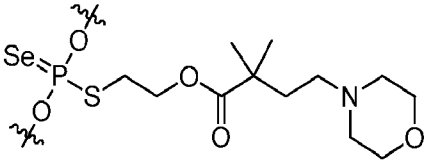
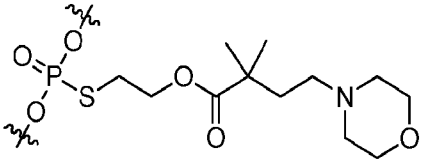
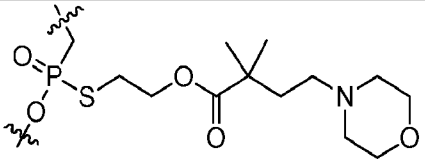
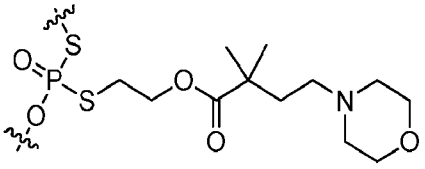
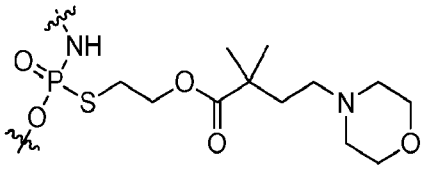
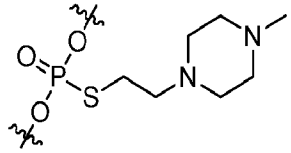
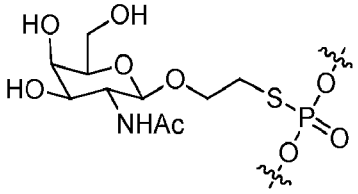
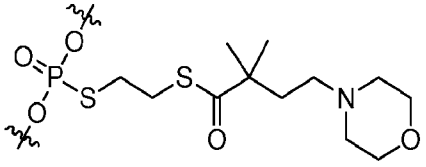
organic or inorganic moiety. In some embodiments, such an organic or inorganic moiety is selected from but not limited to =S, =Se, =NR', –SR', –SeR', –$N(R')_2$, $B(R')_3$, –S–, –Se–, and –N(R')–, wherein each R' is independently as defined and described below. In some embodiments, an internucleotidic linkage is a phosphotriester linkage, phosphorothioate diester linkage (), or modified phosphorothioate triester linkage. It is understood by a person of ordinary skill in the art that the internucleotidic linkage may exist as an anion or cation at a given pH due to the existence of acid or base moieties in the linkage.

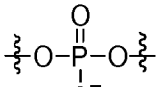
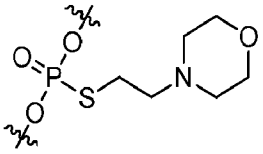
[0098] Unless otherwise specified, when used with an oligonucleotide sequence, each of s, s1, s2, s3, s4, s5, s6 and s7 independently represents the following modified internucleotidic linkage as illustrated in Table 1, below.

[0099] Table 1. Exemplary Modified Internucleotidic Linkage.

Symbol	Modified Internucleotidic Linkage
s	 phosphorothioate ( )
s1	
s2	
s3	
s4	

s5	
s6	
s7	
s8	
s9	
s10	
s11	
s12	
s13	
s14	

s15	
s16	
s17	
s18	

[00100] For instance, (*R*p, *S*p)–ATsCs1GA has 1) a phosphorothioate internucleotidic linkage () between T and C; and 2) a phosphorothioate triester internucleotidic linkage having the structure of  between C and G. Unless otherwise specified, the *R*p/*S*p designations preceding an oligonucleotide sequence describe the configurations of chiral linkage phosphorus atoms in the internucleotidic linkages sequentially from 5' to 3' of the oligonucleotide sequence. For instance, in (*R*p, *S*p)–ATsCs1GA, the phosphorus in the "s" linkage between T and C has *R*p configuration and the phosphorus in "s1" linkage between C and G has *S*p configuration. In some embodiments, "All-(*R*p)" or "All-(*S*p)" is used to indicate that all chiral linkage phosphorus atoms in oligonucleotide have the same *R*p or *S*p configuration, respectively. For instance, All-(*R*p)-GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC indicates that all the chiral linkage phosphorus atoms in the oligonucleotide have *R*p configuration; All-(*S*p)-

GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC indicates that all the chiral linkage phosphorus atoms in the oligonucleotide have *S*p configuration.

[00101] *Oligonucleotide type:* As used herein, the phrase "oligonucleotide type" is used to define an oligonucleotide that has a particular base sequence, pattern of backbone linkages (i.e., pattern of internucleotidic linkage types, for example, phosphate, phosphorothioate, etc), pattern of backbone chiral centers (i.e. pattern of linkage phosphorus stereochemistry (*R*p/*S*p)), and pattern of backbone phosphorus modifications (e.g., pattern of "-XLR^1" groups in formula **I**). Oligonucleotides of a common designated "type" are structurally identical to one another.

[00102] One of skill in the art will appreciate that synthetic methods of the present invention provide for a degree of control during the synthesis of an oligonucleotide strand such that each nucleotide unit of the oligonucleotide strand can be designed and/or selected in advance to have a particular stereochemistry at the linkage phosphorus and/or a particular modification at the linkage phosphorus, and/or a particular base, and/or a particular sugar. In some embodiments, an oligonucleotide strand is designed and/or selected in advance to have a particular combination of stereocenters at the linkage phosphorus. In some embodiments, an oligonucleotide strand is designed and/or determined to have a particular combination of modifications at the linkage phosphorus. In some embodiments, an oligonucleotide strand is designed and/or selected to have a particular combination of bases. In some embodiments, an oligonucleotide strand is designed and/or selected to have a particular combination of one or more of the above structural characteristics. The present invention provides compositions comprising or consisting of a plurality of oligonucleotide molecules (e.g., chirally controlled oligonucleotide compositions). In some embodiments, all such molecules are of the same type (i.e., are structurally identical to one another). In many embodiments, however, provided compositions comprise a plurality of oligonucleotides of different types, typically in pre-determined relative amounts.

[00103] *Chiral control:* As used herein, "chiral control" refers to an ability to control the stereochemical designation of every chiral linkage phosphorus within an oligonucleotide strand. The phrase "chirally controlled oligonucleotide" refers to an oligonucleotide which exists in a single diastereomeric form with respect to the chiral linkage phosphorus.

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[00104] *Chirally controlled oligonucleotide composition:* As used herein, the phrase "chirally controlled oligonucleotide composition" refers to an oligonucleotide composition that contains predetermined levels of individual oligonucleotide types. For instance, in some embodiments a chirally controlled oligonucleotide composition comprises one oligonucleotide type. In some embodiments, a chirally controlled oligonucleotide composition comprises more than one oligonucleotide type. In some embodiments, a chirally controlled oligonucleotide composition comprises a mixture of multiple oligonucleotide types. Exemplary chirally controlled oligonucleotide compositions are described further herein.

[00105] *Chirally pure:* as used herein, the phrase "chirally pure" is used to describe a chirally controlled oligonucleotide composition in which all of the oligonucleotides exist in a single diastereomeric form with respect to the linkage phosphorus.

[00106] *Chirally uniform:* as used herein, the phrase "chirally uniform" is used to describe an oligonucleotide molecule or type in which all nucleotide units have the same stereochemistry at the linkage phosphorus. For instance, an oligonucleotide whose nucleotide units all have *R*p stereochemistry at the linkage phosphorus is chirally uniform. Likewise, an oligonucleotide whose nucleotide units all have *S*p stereochemistry at the linkage phosphorus is chirally uniform.

[00107] *Predetermined:* By predetermined is meant deliberately selected, for example as opposed to randomly occurring or achieved. Those of ordinary skill in the art, reading the present specification, will appreciate that the present invention provides new and surprising technologies that permit selection of particular oligonucleotide types for preparation and/or inclusion in provided compositions, and further permits controlled preparation of precisely the selected particular types, optionally in selected particular relative amounts, so that provided compositions are prepared. Such provided compositions are "predetermined" as described herein. Compositions that may contain certain individual oligonucleotide types because they happen to have been generated through a process that cannot be controlled to intentionally generate the particular oligonucleotide types is not a "predetermined" composition. In some embodiments, a predetermined composition is one that can be intentionally reproduced (e.g., through repetition of a controlled process).

[00108] *Linkage phosphorus:* as defined herein, the phrase "linkage phosphorus" is used to indicate that the particular phosphorus atom being referred to is the phosphorus atom present in the internucleotidic linkage, which phosphorus atom corresponds to the phosphorus atom of a

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phosphodiester of an internucleotidic linkage as occurs in naturally occurring DNA and RNA. In some embodiments, a linkage phosphorus atom is in a modified internucleotidic linkage, wherein each oxygen atom of a phosphodiester linkage is optionally and independently replaced by an organic or inorganic moiety. In some embodiments, a linkage phosphorus atom is P* of formula **I**. In some embodiments, a linkage phosphorus atom is chiral. In some embodiments, a chiral linkage phosphorus atom is P* of formula **I**.

[00109] *P-modification:* as used herein, the term "P-modification" refers to any modification at the linkage phosphorus other than a stereochemical modification. In some embodiments, a P-modification comprises addition, substitution, or removal of a pendant moiety covalently attached to a linkage phosphorus. In some embodiments, the "P-modification" is –X–L–R^1 wherein each of X, L and R^1 is independently as defined and described herein and below.

[00110] *Blockmer:* the term "blockmer," as used herein, refers to an oligonucleotide strand whose pattern of structural features characterizing each individual nucleotide unit is characterized by the presence of at least two consecutive nucleotide units sharing a common structural feature at the internucleotidic phosphorus linkage. By common structural feature is meant common stereochemistry at the linkage phosphorus or a common modification at the linkage phosphorus. In some embodiments, the at least two consecutive nucleotide units sharing a common structure feature at the internucleotidic phosphours linkage are referred to as a "block".

[00111] In some embodiments, a blockmer is a "stereoblockmer," e.g., at least two consecutive nucleotide units have the same stereochemistry at the linkage phosphorus. Such at lest two consecutive nucleotide units form a "stereoblock." For instance, (*S*p, *S*p)-ATsCs1GA is a stereoblockmer because at least two consecutive nucleotide units, the Ts and the Cs1, have the same stereochemistry at the linkage phosphorus (both *S*p). In the same oligonucleotide (*S*p, *S*p)-ATsCs1GA, TsCs1 forms a block, and it is a stereoblock.

[00112] In some embodiments, a blockmer is a "P-modification blockmer," e.g., at least two consecutive nucleotide units have the same modification at the linkage phosphorus. Such at lest two consecutive nucleotide units form a "P-modification block". For instance, (*R*p, *S*p)-ATsCsGA is a P-modification blockmer because at least two consecutive nucleotide units, the Ts and the Cs, have the same P-modification (i.e., both are a phosphorothioate diester). In the same oligonucleotide of (*R*p, *S*p)-ATsCsGA, TsCs forms a block, and it is a P-modification block.

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[00113] In some embodiments, a blockmer is a "linkage blockmer," e.g., at least two consecutive nucleotide units have identical stereochemistry and identical modifications at the linkage phosphorus. At least two consecutive nucleotide units form a "linkage block". For instance, (*R*p, *R*p)-ATsCsGA is a linkage blockmer because at least two consecutive nucleotide units, the Ts and the Cs, have the same stereochemistry (both *R*p) and P-modification (both phosphorothioate). In the same oligonucleotide of (*R*p, *R*p)-ATsCsGA, TsCs forms a block, and it is a linkage block.

[00114] In some embodiments, a blockmer comprises one or more blocks independently selected from a stereoblock, a P-modification block and a linkage block. In some embodiments, a blockmer is a stereoblockmer with respect to one block, and/or a P-modification blockmer with respect to another block, and/or a linkage blockmer with respect to yet another block. For instance, (*R*p, *R*p, *R*p, *R*p, *R*p, *S*p, *S*p, *S*p)–AAsTsCsGsAs1Ts1Cs1Gs1ATCG is a stereoblockmer with respect to the stereoblock AsTsCsGsAs1 (all *R*p at linkage phosphorus) or Ts1Cs1Gs1 (all *S*p at linkage phosphorus), a P-modification blockmer with respect to the P-modification block AsTsCsGs (all s linkage) or As1Ts1Cs1Gs1 (all s1 linkage), or a linkage blockmer with respect to the linkage block AsTsCsGs (all *R*p at linkage phosphorus and all s linkage) or Ts1Cs1Gs1 (all *S*p at linkage phosphorus and all s1 linkage).

[00115] *Altmer:* the term "altmer," as used herein, refers to an oligonucleotide strand whose pattern of structural features characterizing each individual nucleotide unit is characterized in that no two consecutive nucleotide units of the oligonucleotide strand share a particular structural feature at the internucleotidic phosphorus linkage. In some embodiments, an altmer is designed such that it comprises a repeating pattern. In some embodiments, an altmer is designed such that it does not comprise a repeating pattern.

[00116] In some embodiments, an altmer is a "stereoaltmer," e.g., no two consecutive nucleotide units have the same stereochemistry at the linkage phosphorus. For instance, (*R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p)-GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC.

[00117] In some embodiments, an altmer is a "P-modification altmer" e.g., no two consecutive nucleotide units have the same modification at the linkage phosphorus. For instance, All-(*S*p)-CAs1GsT, in which each linkage phosphorus has a different P-modification than the others.

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[00118] In some embodiments, an altmer is a "linkage altmer," e.g., no two consecutive nucleotide units have identical stereochemistry or identical modifications at the linkage phosphorus. For instance, (*R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p, *S*p *R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p)-GsCs1CsTs1CsAs1GsTs1CsTs1GsCs1TsTs2CsGs3CsAs4CsC.

[00119] *Unimer:* the term "unimer," as used herein, refers to an oligonucleotide strand whose pattern of structural features characterizing each individual nucleotide unit is such that all nucleotide units within the strand share at least one common structural feature at the internucleotidic phosphorus linkage. By common structural feature is meant common stereochemistry at the linkage phosphorus or a common modification at the linkage phosphorus.

[00120] In some embodiments, a unimer is a "stereounimer," e.g., all nucleotide units have the same stereochemistry at the linkage phosphorus. For instance, All-(*S*p)-CsAs1GsT, in which all the linkages have *S*p phosphorus.

[00121] In some embodiments, a unimer is a "P-modification unimer", e.g., all nucleotide units have the same modification at the linkage phosphorus. For instance, (*R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p, *S*p *R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p)-GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC, in which all the internucleotidic linkages are phosphorothioate diester.

[00122] In some embodiments, a unimer is a "linkage unimer," e.g., all nucleotide units have the same stereochemistry and the same modifications at the linkage phosphorus. For instance, All-(*S*p)-GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC, in which all the internucleotidic linkages are phosphorothioate diester having *S*p linkage phosphorus.

[00123] *Gapmer*: as used herein, the term "gapmer" refers to an oligonucleotide strand characterized in that at least one internucleotidic phosphorus linkage of the oligonucleotide strand is a phosphate diester linkage, for example such as those found in naturally occurring DNA or RNA. In some embodiments, more than one internucleotidic phosphorus linkage of the oligonucleotide strand is a phosphate diester linkage such as those found in naturally occurring DNA or RNA. For instance, All-(*S*p)-CAs1GsT, in which the internucleotidic linkage between C and A is a phosphate diester linkage.

[00124] *Skipmer*: as used herein, the term "skipmer" refers to a type of gapmer in which every other internucleotidic phosphorus linkage of the oligonucleotide strand is a phosphate diester linkage, for example such as those found in naturally occurring DNA or RNA, and every

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other internucleotidic phosphorus linkage of the oligonucleotide strand is a modified internucleotidic linkage. For instance, All-(*S*p)-AsTCs1GAs2TCs3G.

[00125] For purposes of this invention, the chemical elements are identified in accordance with the Periodic Table of the Elements, CAS version, *Handbook of Chemistry and Physics*, 67th Ed., 1986-87, inside cover.

[00126] The methods and structures described herein relating to compounds and compositions of the invention also apply to the pharmaceutically acceptable acid or base addition salts and all stereoisomeric forms of these compounds and compositions.

Brief Description of the Drawing

[00127] *Figure 1.* Chirally controlled oligonucleotide has significantly different retention time on HPLC compared to the stereorandom oligonucleotide. A: crude chirally controlled oligonucleotide (Oligonucleotide 101); C: the corresponding stereorandom oligonucleotide (Oligonucleotide 118).

[00128] *Figure 2.* HPLC of chirally controlled oligonucleotides and stereorandom oligonucleotide. A: Oligonucleotide 101 (all-*R*p); B: Oligonucleotide 102 (all-*S*p); and C: Oligonucleotide 118 (stereorandom).

[00129] *Figure 3.* Tm of chirally controlled oligonucleotides and stereorandom oligonucleotide.

[00130] *Figure 4.* Representative Data: Melting Curve Analysis of the target and endogenous control pairs yield single amplicons.

[00131] *Figure 5.* Representative data and IC_{50} curves for compounds.

[00132] *Figure 6.* HPLC of crude (*R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC] ((RRS)$_6$-R, stereoblockmer and P-modification unimer (s-unimer)).

[00133] *Figure 7.* HPLC of purified (*R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC] ((RRS)$_6$-R, stereoblockmer and P-modification unimer (s-unimer)).

[00134] *Figure 8.* LCMS of (*R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC] ((RRS)$_6$-R, stereoblockmer and P-modification unimer (s-unimer)).

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[00135] *Figure 9.* HPLC of crude (*S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC] (S-(RRS)$_6$, stereoblockmer and P-modification unimer (s-unimer)).

[00136] *Figure 10.* HPLC of purified (*S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC] (S-(RRS)$_6$, stereoblockmer and P-modification unimer (s-unimer)).

[00137] *Figure 11.* LCMS of (Sp, Rp, Rp, Sp, Rp, Rp, Sp, Rp, Rp, Sp, Rp, Rp, Sp, Rp, Rp, Sp, Rp, Rp, Sp)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC] (S-(RRS)$_6$, stereoblockmer and P-modification unimer (s-unimer)).

[00138] *Figure 12.* HPLC of crude (*R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p) d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC] (RS-(RRS)$_5$-RR, stereoblockmer and P-modification unimer (s-unimer)).

[00139] *Figure 13.* HPLC of purified (*R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p) d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC] (RS-(RRS)$_5$-RR, stereoblockmer and P-modification unimer (s-unimer)).

[00140] *Figure 14.* LCMS of (*R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC] (RS-(RRS)$_5$-RR, stereoblockmer and P-modification unimer (s-unimer)).

[00141] *Figure 15.* HPLC of crude (*R*p, *R*p, *R*p, *S*p, *S*p, *S*p, *S*p, *S*p, *R*p, *R*p, *R*p)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G] (3R-5S-3R, stereoblockmer and P-modification unimer (s1-unimer)).

[00142] *Figure 16.* HPLC of purified (*R*p, *R*p, *R*p, *S*p, *S*p, *S*p, *S*p, *S*p, *R*p, *R*p, *R*p)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G] (3R-5S-3R, stereoblockmer and P-modification unimer (s1-unimer)).

[00143] *Figure 17.* LCMS of (*R*p, *R*p, *R*p, *S*p, *S*p, *S*p, *S*p, *S*p, *R*p, *R*p, *R*p)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G] (3R-5S-3R, stereoblockmer and P-modification unimer (s1-unimer)).

[00144] *Figure 18.* HPLC of crude All-(*R*p)-d[Cs3As3Gs3T] (P-modification unimer (s3-unimer), stereounimer and linkage unimer).

[00145] *Figure 19.* LCMS of All-(*R*p)-d[Cs3As3Gs3T] (P-modification unimer (s3-unimer), stereounimer and linkage unimer).

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[00146] *Figure 20.* HPLC of crude All-(*R*p)-d[Cs2As2Gs2T] (P-modification unimer (s2-unimer), stereounimer and linkage unimer).

[00147] *Figure 21.* LCMS of All-(*R*p)-d[Cs2As2Gs2T] (P-modification unimer (s2-unimer), stereounimer and linkage unimer).

[00148] *Figure 22.* HPLC of crude All-(*S*p)-d[Cs1AGs1T] (gapmer, stereoaltmer, P-modification altmer and linkage altmer).

[00149] *Figure 23.* LCMS of All-(*S*p)-d[Cs1AGs1T] (gapmer stereoaltmer, P-modification altmer and linkage altmer).

[00150] *Figure 24.* Crude All-(*R*p)-d[TsCs1AsT] (stereounimer, P-modification altmer and linkage altmer).

[00151] *Figure 25.* LCMS of All-(*R*p)-d[TsCs1AsT] (stereounimer, P-modification altmer and linkage altmer).

[00152] *Figure 26.* Exemplary oligonucleotides described in WO2012/030683 and contemplated for synthesis using methods of the present invention.

[00153] *Figure 27.* Exemplary oligonucleotides described in WO2012/030683 and contemplated for synthesis using methods of the present invention.

[00154] *Figure 28.* Exemplary oligonucleotides described in WO2012/030683 and contemplated for synthesis using methods of the present invention.

[00155] *Figure 29.* Exemplary oligonucleotides described in WO2012/030683 and contemplated for synthesis using methods of the present invention.

[00156] *Figure 30.* Exemplary oligonucleotides described in WO2012/030683 and contemplated for synthesis using methods of the present invention.

[00157] *Figure 31.* Exemplary linkers described in WO2012/030683 for use in methods of the present invention.

[00158] *Figure 32.* Exemplary linkers described in WO2012/030683 for use in methods of the present invention.

[00159] *Figure 33.* Exemplary linkers described in WO2012/030683 for use in methods of the present invention.

[00160] *Figure 34.* Exemplary linkers described in WO2012/030683 for use in methods of the present invention.

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[00161] *Figure 35.* RP-HPLC of crude DMT on oligonucleotide: ONT-75 (Panel A); ONT-80 (Panel B); ONT-77 (Panel C); ONT-81 (Panel D); ONT-87 (Panel E); ONT-88 (Panel F); ONT-89 (Panel G); ONT-82 (Panel H); ONT-84 (Panel I); ONT-85 (Panel J); ONT-86 (Panel K).

[00162] *Figure 36.* RP-HPLC of purified DMT off oligonucleotide: ONT-75 (Panel A); ONT-80 (Panel B); ONT-77 (Panel C); ONT-81 (Panel D); ONT-87 (Panel E); ONT-88 (Panel F); ONT-89 (Panel G); ONT-82 (Panel H); ONT-84 (Panel I); ONT-85 (Panel J); ONT-86 (Panel K).

[00163] *Figure 37.* Overlay of RP-HPLC traces of purified DMT off oligonucleotide: ONT-75, ONT-77, ONT-80, ONT-81, ONT-87, ONT-88, ONT-89, and ONT-41 (Panel A); expanded view of overlay of ONT-75, ONT-77, ONT-80, ONT-81, ONT-87, ONT-88, ONT-89, and ONT-41 (Panel B).

[00164] *Figure 38.* Overlay of RP-HPLC traces of purified DMT off oligonucleotide: ONT-82, ONT-84, ONT-85, ONT-86, and ONT-83 (Panel A); expanded view of overlay of ONT-82, ONT-84, ONT-85, ONT-86, and ONT-83 (Panel B).

[00165] *Figure 39.* Tm overlay of chirally controlled oligonucleotides ONT-81, ONT-41, ONT-75, ONT-77, and ONT-80.

[00166] *Figure 40.* Graphical representation of timecourse of serum human apolipoprotein B protein levels relative to PBS after 5 mg/kg stereoisomer or mipomersen IP dosing in huApoB mice for ONT-41, ONT-75, ONT-80, ONT-77, and ONT-81. A downward arrow indicates dosing days.

[00167] *Figure 41.* Graphical representation of timecourse of serum human apolipoprotein B protein levels relative to PBS after 5 mg/kg stereoisomer or mipomersen IP dosing in huApoB mice for mipomersen, "full R" mipomersen, "full S" mipomersen, "RSR" mipomersen, and "SRS" mipomersen. A downward arrow indicates dosing days.

[00168] *Figure 42.* Graphical representation of timecourse of serum human apolipoprotein B protein levels relative to PBS after 10 mg/kg stereoisomer or mipomersen IP dosing in huApoB mice for mipomersen, "full R" mipomersen, "full S" mipomersen, "RSR" mipomersen, and "SRS" mipomersen. A downward arrow indicates dosing days.

[00169] *Figure 43.* Graphical representation of timecourse of serum human apolipoprotein B protein levels relative to PBS after 5 mg/kg stereoisomer or mipomersen IP

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dosing in huApoB mice for mipomersen, ONT-87, ONT-88, and ONT-89. A downward arrow indicates dosing days.

[00170] *Figure 44.* Graphical representation of timecourse of serum human apolipoprotein B protein levels relative to PBS after 10 mg/kg stereoisomer or mipomersen IP dosing in huApoB mice for ONT-87, ONT-88, and ONT-89. A downward arrow indicates dosing days.

[00171] *Figure 45.* Graphical representation of % PCSK-9 mRNA remaining after Hep3B treatment with siRNA duplex.

[00172] *Figure 46.* Graphical representation of % PCSK-9 mRNA remaining after Hep3B treatment with siRNA duplex curve fit.

[00173]

[00174] *Figure 47.* Graphical representation of % PCSK-9 mRNA remaining after HeLa treatment with siRNA duplex.

[00175] *Figure 48.* Graphical representation of % PCSK-9 mRNA remaining after HeLa treatment with siRNA duplex curve fit.

[00176] *Figure 49.* Graphical representation of % PCSK-9 mRNA remaining after HeLa treatment with siRNA duplex containing 3 Phophorothiate stereo-centers.

[00177] *Figure 50.* Graphical representation of % PCSK-9 mRNA remaining after HeLa treatment with siRNA duplex containing 3 Phophorothiate stereo-centers curve fit.

[00178] *Figure 51.* Overlay of RP-HPLC traces of purified DMT off oligonucleotide: ONT-108, ONT-109, and ONT-114.

[00179] *Figure 52.* Overlay of RP-HPLC traces of purified DMT off oligonucleotide: ONT-106, ONT-107, and ONT-114.

[00180] *Figure 53.* Graphical representation of timecourse of serum human apolipoprotein B protein levels relative to PBS after 10 mg/kg stereoisomer or mipomersen IP dosing in huApoB mice. A downward arrow indicates dosing days.

[00181] *Figure 54.* Graphical representation of timecourse of serum human apolipoprotein B protein levels relative to PBS after multiple IP doses of 5 mg/kg stereoisomer or mipomersen in huApoB mice (n = 3-4). A downward arrow indicates dosing days.

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[00182] *Figure 55.* Day 17 serum human apolipoprotein B protein levels relative to PBS after 10 mg/kg stereoisomer (ONT-87, ONT-88 or ONT-89) or mipomersen IP dosing in huApoB mice.

[00183] *Figure 56.* Day 24 Serum Human Apolipoprotein B Protein Levels Relative to PBS After 10 mg/kg Stereoisomer (ONT-87, ONT-88 or ONT-89) or Mipomersen IP Dosing in huApoB Mice.

[00184] *Figure 57.* Serum Human Apolipoprotein B Protein Levels Relative to PBS After 10 mg/kg Stereoisomer (ONT-41, ONT-87, ONT-88 or ONT-89) Dosing in huApoB Mice.

[00185] *Figure 58.* Serum Human Apolipoprotein B Protein Levels Relative to PBS After 10 mg/kg Stereoisomer (ONT-87, ONT-88 or ONT-89) Dosing in huApoB Mice.

[00186] *Figure 59.* Plot of IEX-HPLC quantification analysis of svPDE digestion study for oligonucleotides ONT-75, ONT-77, ONT-80, ONT-81, ONT-87, ONT-88, ONT-89 and ONT-41.

[00187] *Figure 60.* IEX-HPLC of enzymatic digestion study using nP1 for oligonucleotide ONT-75 (All (*R*p))-Gs5mCs5mCsTs5mCsAsGsTs5mCsTsGs5mCsTsTs5mCsGs 5mCsAs5mCs5mC.

[00188] *Figure 61.* IEX-HPLC of enzymatic digestion study using nP1 for oligonucleotide ONT-77 (*R*p, *R*p, *R*p, *R*p, *R*p, *S*p, *S*p, *S*p, *S*p, *S*p, *S*p, *S*p, *S*p, *S*p, *S*p, *R*p, *R*p, *R*p, *R*p)- Gs5mCs5mCsTs5mCsAsGsTs5mCsTsGs5mCsTsTs5mCsGs5mCsAs5mCs5mC (5R-10S-4R).

[00189] *Figure 62.* IEX-HPLC of enzymatic digestion study using nP1 for oligonucleotide ONT-80 (All (*S*p))-Gs5mCs5mCsTs5mCsAsGsTs5mCsTsGs5mCsTsTs5mCs Gs5mCsAs 5mCs5mC.

[00190] *Figure 63.* IEX-HPLC of enzymatic digestion study using nP1 for oligonucleotide ONT-81 (*S*p, *S*p, *S*p, *S*p, *S*p, *R*p, *R*p, *R*p, *R*p, *R*p, *R*p, *R*p, *R*p, *R*p, *S*p, *S*p, *S*p, *S*p)-Gs5mCs5mCsTs5mCsAsGsTs5mCsTsGs5mCsTsTs5mCsGs5mCsAs5mCs5mC (5S-10R-4S).

[00191] *Figure 64.* IEX-HPLC of enzymatic digestion study using nP1 for oligonucleotide ONT-87 (*R*p, *R*p, *R*p, *R*p, *R*p, *S*p, *S*p, *R*p, *S*p, *S*p, *R*p, *S*p, *S*p, *R*p, *R*p, *R*p, *R*p, *R*p, *R*p)-Gs5mCs5mCsTs5mCsAsGsTs5mCsTsGs5mCsTsTs5mCsGs5mCsAs5mCs5mC (5R-(SSR)$_3$ -5R).

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[00192] *Figure 65.* IEX-HPLC of enzymatic digestion study using nP1 for oligonucleotide ONT-88 (*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*)-Gs5mCs5mCsTs5mCsAsGsTs5mCsTsGs5mCsTsTs5mCsGs5mCsAs 5mCs5mC (5S-(RRS)$_3$-5S).

[00193] *Figure 66.* IEX-HPLC of enzymatic digestion study using nP1 for oligonucleotide ONT-89 (*Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*)-Gs5mCs5mCsTs5mCsAsGsTs5mCsTsGs5mCsTsTs5mCsGs5mCsAs5mCs5mC ((SR)$_9$S).

[00194] *Figure 67.* IEX-HPLC of enzymatic digestion study using nP1 for oligonucleotide ONT-41 (*diastereomixture*)-Gs5mCs5mCsTs5mCsAsGsTs5mCsTsGs 5mCsTsTs5mCsGs5mCsAs5mCs5mC.

[00195] *Figure 68.* Comparison of stability of chirally pure oligonucleotides ONT-75 and ONT-77 with the stereorandom "parental" oligonucleotide ONT-41 (Mipomersen) in preincubated rat whole liver homogenate.

[00196] *Figure 69.* UPLC profile in producing oligonucleotide derivative using the monomer of **13b**.

[00197] *Figure 70.* UPLC profile in producing oligonucleotide derivative using the monomer of **27**.

[00198] *Figure 71.* Mouse Apolipoprotein B/GAPDH mRNA Levels Relative to Mock and Untreated Controls after Transfection of Primary Mouse Hepatocytes with Stereoisomer (ONT-82, ONT-83, ONT-84, ONT-85 or ONT-86).

[00199] *Figure 72.* Mouse Apolipoprotein B/GAPDH mRNA Levels Relative to Mock and Untreated Controls after Transfection of Primary Mouse Hepatocytes with Stereoisomer (ONT-83, ONT-84, ONT-85 or ONT-86).

Detailed Description of Certain Embodiments

[00200] Synthetic oligonucleotides provide useful molecular tools in a wide variety of applications. For example, oligonucleotides are useful in therapeutic, diagnostic, research, and new nanomaterials applications. The use of naturally occurring nucleic acids (e.g., unmodified DNA or RNA) is limited, for example, by their susceptibility to endo- and exo-nucleases. As such, various synthetic counterparts have been developed to circumvent these shortcomings. These include synthetic oligonucleotides that contain backbone modifications, which render

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these molecules less susceptible to degradation. From a structural point of view, such modifications to internucleotide phosphate linkages introduce chirality. It has become clear that certain properties of oligonucleotides may be affected by the configurations of the phosphorus atoms that form the backbone of the oligonucleotides. For example, *in vitro* studies have shown that the properties of antisense nucleotides such as binding affinity, sequence specific binding to the complementary RNA, stability to nucleases are affected by, inter alia, chirality of the backbone (e.g., the configurations of the phosphorus atoms). Thus, the present invention encompasses the recognition that there is a need for chirally controlled oligonucleotides which comprise phosphorus atom-modified nucleic acids, as well as related compositions and methods. In some embodiments, the present invention provides chirally controlled oligonucleotides that are structurally optimized to exhibit certain desirable characteristics, such as, e.g., increased stability and improved efficacy for *in vitro* and/or *in vivo* applications.

[00201] Oligonucleotides in which one or two of the two nonbridging oxygen atoms of the internucleotidic phosphates is replaced by a different type of atom or substituent are known to be useful as therapeutic agents and probes to elucidate enzymatic reaction mechanisms. However, such oligonucleotides often exhibit undesirable properties (e.g., susceptibility to degradation by nucleases, poor cell membrane permeability) that prohibit their use in numerous applications. Thus, various types of chemical modifications have been developed in an attempt to improve their properties and/or impart new functionality.

Modified Oligonucleotide Structures

[00202] As noted above, in light of the usefulness of oligonucleotide compositions in various applications and indications, those skilled in the art have endeavoured to develop modifications of oligonucleotide structures that may have preferred or desirable characteristics or attributes as compared with naturally-occurring oligonucleotide molecules, for example as used in particular applications and indications. Exemplary such modifications are described below.

[00203] WO2010/141471 (herein "Traversa I") teaches the modification of different types of nucleic acid constructs modified to have a reduced net polyanionic charge. WO2010/039543 (herein "Travera II") teaches compositions and methods for making neutral polynucleotides (NNs) with reduced polyanionic charge. WO2008/008476 (herein, "Traversa III") describes the synthesis of SATE (Imbach-type) phosphate prodrugs. Traversa I, II, and III do not teach

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chirally controlled oligonucleotides, compositions thereof, and methods of making and using the same, as described by the present invention.

[00204] WO2010/072831 (herein "Girindus et al.") also teaches the modification of oligonucleotides. In particular, Girindus et al. teaches the use of sulfurization reagents to generate phosphorothioate triesters as prodrugs. Girindus et al. does not teach chirally controlled oligonucleotides, compositions thereof, and methods of making and using the same, as described by the present invention.

[00205] Similarly, WO2004/085454 (herein "Avecia I") teaches the preparation of phosphorothioate oligonucleotides through, e.g., transient silylation of poly-*H*-phosphonate diesters. WO2001/027126 (herein "Avecia II") teaches processes for the solid phase synthesis of phosphotriester oligonucleotides by coupling *H*-phosphonate monomers to a solid supported 5'-hydroxyl oligonucleotide and further sulfurization of the resulting *H*-phosphonte diester into a phosphorothioate triester. The disclosure of WO2001/064702 (herein "Avecia III") is similar to Avecia II and further describes solid-phase synthesis on different solid supports. Avecia I, II, and III do not teach chirally controlled oligonucleotides, compositions thereof, and methods of making and using the same, as described by the present invention.

[00206] WO1997/006183 (herein "Chiron") teaches oligonucleotides with cationic internucleotide linkages comprising asymmetric phosphorus, such as stereopure amidates. Chiron teaches stereopure oligonucleotides obtained via crystallization of a mixture of diastereomers or via resolution using, e.g., column chromatography. Chiron does not teach chirally controlled oligonucleotides, compositions thereof, and methods of making and using the same, as described by the present invention.

[00207] WO2009/146123 (herein "Spring Bank I") teaches compositions and methods for treating viral infections using substituted phosphate oligonucleotides and phosphorothioate triesters. WO2007/070598 (herein "Spring Bank II") teaches phosphotriester prodrugs as antiviral nucleic acids and teaches the synthesis of phosphorothioate prodrugs. Spring Bank I and II do not teach chirally controlled oligonucleotides, compositions thereof, and methods of making and using the same, as described by the present invention.

[00208] EP0779893 (herein "Hybridon") teaches lipophilic prodrugs for the increased cellular uptake of antisense oligonucleotides and observes that *R*p and *S*p phosphorothioates and phosphorothioate triester dimers can have different enzymatic stability properties. Hybridon

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does not teach chirally controlled oligonucleotides, compositions thereof, and methods of making and using the same, as described by the present invention.

[00209] WO1997/047637 (herein "Imbach I") teaches generally the Imbach "SATE" (S-acyl thioethyl) prodrug oligonucleotide compositions and methods. Imbach I describes, for example, bioreversible phosphotriester prodrugs and the preparation of certain prodrug oligonucleotides using post-synthestic alkylation or prodrug-group-containing phosphoramidites. US 6,124,445 (herein "Imbach II") teaches modified antisense and chimeric prodrug oligonucleotides. Imbach I and II do not teach chirally controlled oligonucleotides, compositions thereof, and methods of making and using the same, as described by the present invention.

[00210] WO2006/065751 (herein "Beaucage") teaches CpG oligonucleotide phosphorothioate prodrugs that comprise thermolabile substituents (which substituents are introduced via a phosphoramidite monomer), and applications thereof. Beaucage does not teach chirally controlled oligonucleotides, compositions thereof, and methods of making and using the same, as described by the present invention.

[00211] Takeshi Wada et al. developed novel methods for the stereo-controlled synthesis of P-chiral nucleic acids using amidite chiral auxiliaries (JP4348077, WO2005/014609, WO2005/092909, and WO2010/064146, cumulatively referred to herein as "Wada I"). In particular, WO2010/064146 (referred to herein as "Wada II") discloses methods for synthesizing phosphorus atom-modified nucleic acids wherein the stereochemical configuration at phosphorus is controlled. However, the methods of Wada II are limited in that they do not provide for individual P-modification of each chiral linkage phosphorus in a controlled and designed manner. That is, the methods for P-modified linkages of Wada II provide for the generation of a condensed intermediate poly *H*-phosphonate oligonucleotide strand that, once built to a desired length, is mass modified at the linkage phosphorus to provide, e.g., a desired phosphorothioate diester, phosphoramidate or boranophosphate or other such phosphorus atom-modified nucleic acids (referred to as Route B in the document – Scheme 6, page 36). Furthermore, the *H*-phosphonate oligonucleotide strands of Wada II are of shorter lengths (e.g., dimer trimer, or tetramer). Combined with the fact that there is no capping step in route B, which generally presents low crude purity as a result of the accumulation of "n-1"-type byproducts, the Wada II route contains limitations in regards of the synthesis of longer oligonucleotides. While Wada II contemplates generally that a particular oligonucleotide could be envisaged to contain different

modifications at each linkage phosphorus, Wada II does not describe or suggest methods for controlled iterative installation of such modifications, as are described herein. To the extent that Wada II depicts a synthetic cycle that does not require an *H*-phosphonate intermediate oligonucleotide to be completely assembled prior to modification at the linkage phosphorus (therein referred to as Route A, page 35, Scheme 5, "Synthesis of a nucleic acid comprising a chiral X-phosphonate moiety of Formula 1 via Route A"), this general disclosure does not teach certain key steps that are required to install certain P-modifications, as provided by the present invention, and especially not with any degree of efficiency and versatility such that this cycle would be useful in the synthesis of chirally controlled P-modified oligonucleotides, and especially oligonucleotides of longer lengths.

[00212] At least one such inefficiency of Wada II is noted by Wada et al. in WO2012/039448 (herein "Wada III"). Wada III teaches novel chiral auxiliaries for use in Wada II methods to produce *H*-phosphonate oligonucleotides that, once built, can be subsequently modified to provide, *inter alia*, phosphorothioates and the like. Wada et al. observe in Wada III that the four types of chiral auxiliaries disclosed in Wada II formed strong bonds with phosphorus at the linkage phosphorus and thus did not allow for efficient removal. Wada III notes that removal of the Wada II chiral auxiliaries required harsh conditions, which conditions were prone to compromising the integrity of the product oligonucleotide. Wada III observes that this is especially problematic when synthesizing long chain oligonucleotides for at least the reason that as the degradation reaction(s) proceed, additional byproducts are generated that can further react with and degrade the product oligonucleotide. Wada III therefore provides chiral auxiliaries that can be more efficiently cleaved from the oligonucleotide under mild acidic conditions by way of an S_N1 mechanism releasing the *H*-phosphonate internucleotide linkage (route B), or under relatively mild basic conditions, by a β-elimation pathway.

[00213] One of skill in the chemical and synthetic arts will immediately appreciate the complexities associated with generating chirally controlled oligonucleotides such as those provided by the present invention. For instance, in order to synthesize and isolate a chirally controlled oligonucleotide, conditions for each monomer addition must be designed such that (1) the chemistry is compatible with every portion of the growing oligonucleotide; (2) the byproducts generated during each monomer addition do not compromise the structural and stereochemical integrity of the growing oligonucleotide; and (3) the crude final product

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composition is a composition which allows for isolation of the desired chirally controlled oligonucleotide product.

[00214] Oligonucleotide phosphorothioates have shown therapeutic potential (Stein *et al.,* Science (1993), 261:1004-12; Agrawal *et al.,* Antisence Res. and Dev. (1992), 2:261-66; Bayever *et al.,* Antisense Res. and Dev. (1993), 3:383-390). Oligonucleotide phosphorothioates prepared without regard to the stereochemistry of the phosphorothioate exist as a mixture of 2^n diastereomers, where n is the number of internucleotide phosphorothioates linkages. The chemical and biological properties of these diastereomeric phosphorothioates can be distinct. For example, Wada *et al* (Nucleic Acids Symposium Series No. 51 p. 119-120; doi:10.1093/nass/nrm060) found that stereodefined-(*R*p)-(Ups)9U/(Ap)9A duplex showed a higher Tm value than that of natural-(Up)9U/(Ap)9A and stereodefined-(*S*p)-(Ups)9U did not form a duplex. In another example, in a study by Tang *et al.,* (Nucleosides Nucleotides (1995), 14:985-990) stereopure *R*p-oligodeoxyribonucleoside phosphorothioates were found to possess lower stability to nucleases endogenous to human serum that the parent oligodeoxyribonucleoside phosphorothioates with undefined phosphorus chirality.

Chirally Controlled Oligonucleotides and Chirally Controlled Oligonucleotide Compositions

[00215] The present invention provides chirally controlled oligonucleotides, and chirally controlled oligonucleotide compositions which are of high crude purity and of high diastereomeric purity. In some embodiments, the present invention provides chirally controlled oligonucleotides, and chirally controlled oligonucleotide compositions which are of high crude purity. In some embodiments, the present invention provides chirally controlled oligonucleotides, and chirally controlled oligonucleotide compositions which are of high diastereomeric purity.

[00216] In some embodiments, the present invention provides chirally controlled compositions comprising a plurality of oligonucleotides of at least one type, wherein each type is defined by: 1) base sequence; 2) pattern of backbone linkages; 3) pattern of backbone chiral centers; and 4) pattern of backbone P-modifications.

[00217] In some embodiments, the present invention provides chirally controlled compositions comprising a plurality of oligonucleotides of the same type, wherein each type is defined by: 1) base sequence; 2) pattern of backbone linkages; 3) pattern of backbone chiral

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centers; and 4) pattern of backbone P-modifications. In some embodiments, the present invention provides chirally controlled compositions comprising a plurality of oligonucleotides of two or more types, wherein each type is defined by: 1) base sequence; 2) pattern of backbone linkages; 3) pattern of backbone chiral centers; and 4) pattern of backbone P-modifications.

[00218] In some embodiments, the present invention provides oligonucleotides comprising one or more diastereomerically pure internucleotidic linkages with respect to the chiral linkage phosphorus. In some embodiments, the present invention provides oligonucleotides comprising one or more diastereomerically pure internucleotidic linkages having the structure of formula **I**. In some embodiments, the present invention provides oligonucleotides comprising one or more diastereomerically pure internucleotidic linkages with respect to the chiral linkage phosphorus, and one or more phosphate diester linkages. In some embodiments, the present invention provides oligonucleotides comprising one or more diastereomerically pure internucleotidic linkages having the structure of formula **I**, and one or more phosphate diester linkages. In some embodiments, the present invention provides oligonucleotides comprising one or more diastereomerically pure internucleotidic linkages having the structure of formula **I-c**, and one or more phosphate diester linkages. In some embodiments, such oligonucleotides are prepared by using stereoselective oligonucleotide synthesis, as described in this application, to form pre-designed diastereomerically pure internucleotidic linkages with respect to the chiral linkage phosphorus. For instance, in one exemplary oligonucleotide of (*R*p/*S*p, *R*p/*S*p, *R*p/*S*p, *R*p, *R*p, *S*p, *S*p, *S*p, *S*p, *S*p *S*p, *S*p, *S*p, *S*p, *R*p, *R*p, *R*p, *R*p, *R*p)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGs1Cs1As1CsC], the first three internucleotidic linkages are constructed using traditional oligonucleotide synthesis method, and the diastereomerically pure internucleotidic linkages are constructed with stereochemical control as described in this application. Exemplary internucleotidic linkages, including those having structures of formula **I**, are further described below. In some embodiments, such oligonucleotides comprise a sequence further described in the application, including but not limited to those described in Tables 2 and 4, and Appendices A, B and C.

[00219] In some embodiments, a provided oligonucleotide comprises a combination of stereopure and stereorandom internucleotidic linkages with respect to chirality at the linkage phosphorus. For instance, in some embodiments it is desirable to have a block of one or more stereodefined internucleotidic linkages within an oligonucleotide that is otherwise stereorandom

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with respect to chirality at the linkage phosphorus. In some embodiments, it is desirable to have a block of one or more internucleotidic linkages that are stereorandom within an oligonucleotide that is otherwise stereodefined with respect to chirality at the linkage phosphorus.

[00220] In some embodiments, at least one nucleotide unit of a provided oligonucleotide is installed using stereoselective oligonucleotide synthesis, as described in this application, to form a pre-designed diastereomerically pure internucleotidic linkage with respect to the chiral linkage phosphorus. In some embodiments, at least two nucleotide units of a provided oligonucleotide are installed using stereoselective oligonucleotide synthesis, as described in this application, to form at least two pre-designed diastereomerically pure internucleotidic linkages with respect to the chiral linkage phosphorus. In some embodiments, at least three nucleotide units of a provided oligonucleotide are installed using stereoselective oligonucleotide synthesis, as described in this application, to form at least three pre-designed diastereomerically pure internucleotidic linkages with respect to the chiral linkage phosphorus. In some embodiments, the at least one, two, or three pre-designed diastereomerically pure internucleotidic linkages are adjacent to one another. In some embodiments, the at least one, two, or three pre-designed diastereomerically pure internucleotidic linkages are not adjacent to one another.

[00221] In some embodiments, at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, or 50% of nucleotide units of a provided oligonucleotide are installed using stereoselective oligonucleotide synthesis, as described in this application, to form a pre-designed diastereomerically pure internucleotidic linkage with respect to the chiral linkage phosphorus. As described herein, in some embodiments the at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, or 50% of nucleotide units occur in one or more blocks to provide a blockmer. In some embodiments, the at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, or 50% of nucleotide units occur in a an alternating pattern to provide an altmer. One of skill in the relevant arts will recognize that any desirable pattern can be achieved using methods of the present invention and are contemplated herein.

[00222] In some embodiments, the present invention provides a chirally controlled oligonucleotide, wherein at least two of the individual internucleotidic linkages within the oligonucleotide have different stereochemistry and/or different P-modifications relative to one another. In certain embodiments, the present invention provides a chirally controlled oligonucleotide, wherein at least two individual internucleotidic linkages within the

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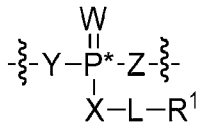
oligonucleotide have different P-modifications relative to one another. In certain embodiments, the present invention provides a chirally controlled oligonucleotide, wherein at least two of the individual internucleotidic linkages within the oligonucleotide have different P-modifications relative to one another, and wherein the chirally controlled oligonucleotide comprises at least one phosphate diester internucleotidic linkage. In certain embodiments, the present invention provides a chirally controlled oligonucleotide, wherein at least two of the individual internucleotidic linkages within the oligonucleotide have different P-modifications relative to one another, and wherein the chirally controlled oligonucleotide comprises at least one phosphate diester internucleotidic linkage and at least one phosphorothioate diester internucleotidic linkage. In certain embodiments, the present invention provides a chirally controlled oligonucleotide, wherein at least two of the individual internucleotidic linkages within the oligonucleotide have different P-modifications relative to one another, and wherein the chirally controlled oligonucleotide comprises at least one phosphorothioate triester internucleotidic linkage. In certain embodiments, the present invention provides a chirally controlled oligonucleotide, wherein at least two of the individual internucleotidic linkages within the oligonucleotide have different P-modifications relative to one another, and wherein the chirally controlled oligonucleotide comprises at least one phosphate diester internucleotidic linkage and at least one phosphorothioate triester internucleotidic linkage.

[00223] In certain embodiments, the present invention provides a chirally controlled oligonucleotide comprising one or more modified internucleotidic linkages independently having the structure of formula **I**:



(I)

wherein each variable is as defined and described below. In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising one or more modified internucleotidic linkages of formula **I**, and wherein individual internucleotidic linkages of formula **I** within the oligonucleotide have different P-modifications relative to one another. In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising one or more modified internucleotidic linkages of formula **I**, and wherein individual internucleotidic linkages of formula **I** within the oligonucleotide have different –X–L–R^1 relative

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to one another. In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising one or more modified internucleotidic linkages of formula **I**, and wherein individual internucleotidic linkages of formula **I** within the oligonucleotide have different X relative to one another. In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising one or more modified internucleotidic linkages of formula **I**, and wherein individual internucleotidic linkages of formula **I** within the oligonucleotide have different –L–R^1 relative to one another.

[00224] In some embodiments, the present invention provides a chirally controlled oligonucleotide, wherein at least two of the individual internucleotidic linkages within the oligonucleotide have different stereochemistry and/or different P-modifications relative to one another. In some embodiments, the present invention provides a chirally controlled oligonucleotide, wherein at least two of the individual internucleotidic linkages within the oligonucleotide have different stereochemistry relative to one another, and wherein at least a portion of the structure of the chirally controlled oligonucleotide is characterized by a repeating pattern of alternating stereochemisty.

[00225] In some embodiments, the present invention provides a chirally controlled oligonucleotide, wherein at least two of the individual internucleotidic linkages within the oligonucleotide have different P-modifications relative to one another, in that they have different X atoms in their –XLR^1 moieties, and/or in that they have different L groups in their –XLR^1 moieties, and/or that they have different R^1 atoms in their –XLR^1 moieties.

[00226] In some embodiments, the present invention provides a chirally controlled oligonucleotide, wherein at least two of the individual internucleotidic linkages within the oligonucleotide have different stereochemistry and/or different P-modifications relative to one another and the oligonucleotide has a structure represented by the following formula:

$$[S^Bn1R^Bn2S^Bn3R^Bn4...S^BnxR^Bny]$$

wherein:

each R^B independently represents a block of nucleotide units having the R configuration at the linkage phosphorus;

each S^B independently represents a block of nucleotide units having the S configuration at the linkage phosphorus;

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each of n1-ny is zero or an integer, with the requirement that at least one odd n and at least one even n must be non-zero so that the oligonucleotide includes at least two individual internucleotidic linkages with different stereochemistry relative to one another; and

wherein the sum of n1-ny is between 2 and 200, and in some embodiments is between a lower limit selected from the group consisting of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more and an upper limit selected from the group consisting of 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, and 200, the upper limit being larger than the lower limit.

[00227] In some such embodiments, each n has the same value; in some embodiments, each even n has the same value as each other even n; in some embodiments, each odd n has the same value each other odd n; in some embodiments, at least two even ns have different values from one another; in some embodiments, at least two odd ns have different values from one another.

[00228] In some embodiments, at least two adjacent ns are equal to one another, so that a provided oligonucleotide includes adjacent blocks of S stereochemistry linkages and R stereochemistry linkages of equal lengths. In some embodiments, provided oligonucleotides include repeating blocks of S and R stereochemistry linkages of equal lengths. In some embodiments, provided oligonucleotides include repeating blocks of S and R stereochemistry linkages, where at least two such blocks are of different lengths from one another; in some such embodiments each S stereochemistry block is of the same length, and is of a different length from each R stereochemistry length, which may optionally be of the same length as one another.

[00229] In some embodiments, at least two skip-adjacent ns are equal to one another, so that a provided oligonucleotide includes at least two blocks of linkages of a first steroechemistry that are equal in length to one another and are separated by a block of linkages of the other stereochemistry, which separating block may be of the same length or a different length from the blocks of first steroechemistry.

[00230] In some embodiments, ns associated with linkage blocks at the ends of a provided oligonucleotide are of the same length. In some embodiments, provided oligonucleotides have terminal blocks of the same linkage stereochemistry. In some such embodiments, the terminal blocks are separated from one another by a middle block of the other linkage stereochemistry.

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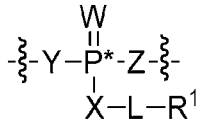
[00231] In some embodiments, a provided oligonucleotide of formula [S^Bn1R^Bn2S^Bn3R^Bn4...S^BnxR^Bny] is a stereoblockmer. In some embodiments, a provided oligonucleotide of formula [S^Bn1R^Bn2S^Bn3R^Bn4...S^BnxR^Bny] is a stereoskipmer. In some embodiments, a provided oligonucleotide of formula [S^Bn1R^Bn2S^Bn3R^Bn4...S^BnxR^Bny] is a stereoaltmer. In some embodiments, a provided oligonucleotide of formula [S^Bn1R^Bn2S^Bn3R^Bn4...S^BnxR^Bny] is a gapmer.

[00232] In some embodiments, a provided oligonucleotide of formula [S^Bn1R^Bn2S^Bn3R^Bn4...S^BnxR^Bny] is of any of the above described patterns and further comprises patterns of P-modifications. For instance, in some embodiments, a provided oligonucleotide of formula [S^Bn1R^Bn2S^Bn3R^Bn4...S^BnxR^Bny] and is a stereoskipmer and P-modification skipmer. In some embodiments, a provided oligonucleotide of formula [S^Bn1R^Bn2S^Bn3R^Bn4...S^BnxR^Bny] and is a stereoblockmer and P-modification altmer. In some embodiments, a provided oligonucleotide of formula [S^Bn1R^Bn2S^Bn3R^Bn4...S^BnxR^Bny] and is a stereoaltmer and P-modification blockmer.

[00233] In some embodiments, a provided oligonucleotide of formula [S^Bn1R^Bn2S^Bn3R^Bn4...S^BnxR^Bny] is a chirally controlled oligonucleotide comprising one or more modified internuceotidic linkages independently having the structure of formula I:



(I)

wherein:

P* is an asymmetric phosphorus atom and is either *R*p or *S*p;

W is O, S or Se;

each of X, Y and Z is independently –O–, –S–, –N(–L–R^1)–, or L;

L is a covalent bond or an optionally substituted, linear or branched C_1–C_{10} alkylene, wherein one or more methylene units of L are optionally and independently replaced by an optionally substituted C_1–C_6 alkylene, C_1–C_6 alkenylene, —C≡C—, –C(R′)$_2$–, –Cy–, –O–, –S–, –S–S–, –N(R′)–, –C(O)–, –C(S)–, –C(NR′)–, –C(O)N(R′)–, –N(R′)C(O)N(R′)-, –N(R′)C(O)–, –

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N(R′)C(O)O–, –OC(O)N(R′)-, –S(O)–, –$S(O)_2$–, –$S(O)_2$N(R′)–, –N(R′)$S(O)_2$–, –SC(O)–, –C(O)S–, –OC(O)–, or –C(O)O–;

R^1 is halogen, R, or an optionally substituted C_1–C_{50} aliphatic wherein one or more methylene units are optionally and independently replaced by an optionally substituted C_1–C_6 alkylene, C_1–C_6 alkenylene, —C≡C—, –C(R′)$_2$–, –Cy–, –O–, –S–, –S–S–, –N(R′)–, –C(O)–, –C(S)–, –C(NR′)–, –C(O)N(R′)–, –N(R′)C(O)N(R′)-, –N(R′)C(O)–, –N(R′)C(O)O–, –OC(O)N(R′)-, –S(O)–, –$S(O)_2$–, –$S(O)_2$N(R′)–, –N(R′)$S(O)_2$–, –SC(O)–, –C(O)S–, –OC(O)–, or –C(O)O–;

each R′ is independently –R, -C(O)R, -CO_2R, or –SO_2R, or:

two R′ on the same nitrogen are taken together with their intervening atoms to form an optionally substituted heterocyclic or heteroaryl ring, or

two R′ on the same carbon are taken together with their intervening atoms to form an optionally substituted aryl, carbocyclic, heterocyclic, or heteroaryl ring;

–Cy– is an optionally substituted bivalent ring selected from phenylene, carbocyclylene, arylene, heteroarylene, or heterocyclylene;

each R is independently hydrogen, or an optionally substituted group selected from C_1–C_6 aliphatic, phenyl, carbocyclyl, aryl, heteroaryl, or heterocyclyl; and

each $-\!\!\lesssim\!\!-$ independently represents a connection to a nucleoside.

[00234] In some embodiments, a chirally controlled oligonucleotide comprises one or more modified internucleotidic phosphorus linkages. In some embodiments, a chirally controlled oligonucleotide comprises, e.g., a phosphorothioate or a phosphorothioate triester linkage. In some embodiments, a chirally controlled oligonucleotide comprises a phosphorothioate triester linkage. In some embodiments, a chirally controlled oligonucleotide comprises at least two phosphorothioate triester linkages. In some embodiments, a chirally controlled oligonucleotide comprises at least three phosphorothioate triester linkages. In some embodiments, a chirally controlled oligonucleotide comprises at least four phosphorothioate triester linkages. In some embodiments, a chirally controlled oligonucleotide comprises at least five phosphorothioate triester linkages. Exemplary such modified internucleotidic phosphorus linkages are described further herein.

[00235] In some embodiments, a chirally controlled oligonucleotide comprises different internucleotidic phosphorus linkages. In some embodiments, a chirally controlled

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oligonucleotide comprises at least one phosphate diester internucleotidic linkage and at least one modified internucleotidic linkage. In some embodiments, a chirally controlled oligonucleotide comprises at least one phosphate diester internucleotidic linkage and at least one phosphorothioate triester linkage. In some embodiments, a chirally controlled oligonucleotide comprises at least one phosphate diester internucleotidic linkage and at least two phosphorothioate triester linkages. In some embodiments, a chirally controlled oligonucleotide comprises at least one phosphate diester internucleotidic linkage and at least three phosphorothioate triester linkages. In some embodiments, a chirally controlled oligonucleotide comprises at least one phosphate diester internucleotidic linkage and at least four phosphorothioate triester linkages. In some embodiments, a chirally controlled oligonucleotide comprises at least one phosphate diester internucleotidic linkage and at least five phosphorothioate triester linkages. Exemplary such modified internucleotidic phosphorus linkages are described further herein.

[00236] In some embodiments, a phosphorothioate triester linkage comprises a chiral auxiliary, which, for example, is used to control the stereoselectivity of a reaction. In some embodiments, a phosphorothioate triester linkage does not comprise a chiral auxiliary. In some embodiments, a phosphorothioate triester linkage is intentionally maintained until and/or during the administration to a subject.

[00237] In some embodiments, a chirally controlled oligonucleotide is linked to a solid support. In some embodiments, a chirally controlled oligonucleotide is cleaved from a solid support.

[00238] In some embodiments, a chirally controlled oligonucleotide comprises at least one phosphate diester internucleotidic linkage and at least two consecutive modified internucleotidic linkages. In some embodiments, a chirally controlled oligonucleotide comprises at least one phosphate diester internucleotidic linkage and at least two consecutive phosphorothioate triester internucleotidic linkages.

[00239] In some embodiments, a chirally controlled oligonucleotide is a blockmer. In some embodiments, a chirally controlled oligonucleotide is a stereoblockmer. In some embodiments, a chirally controlled oligonucleotide is a P-modification blockmer. In some embodiments, a chirally controlled oligonucleotide is a linkage blockmer.

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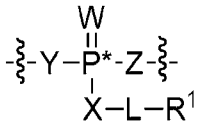
[00240] In some embodiments, a chirally controlled oligonucleotide is an altmer. In some embodiments, a chirally controlled oligonucleotide is a stereoaltmer. In some embodiments, a chirally controlled oligonucleotide is a P-modification altmer. In some embodiments, a chirally controlled oligonucleotide is a linkage altmer.

[00241] In some embodiments, a chirally controlled oligonucleotide is a unimer. In some embodiments, a chirally controlled oligonucleotide is a stereounimer. In some embodiments, a chirally controlled oligonucleotide is a P-modification unimer. In some embodiments, a chirally controlled oligonucleotide is a linkage unimer.

[00242] In some embodiments, a chirally controlled oligonucleotide is a gapmer.

[00243] In some embodiments, a chirally controlled oligonucleotide is a skipmer.

[00244] In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising one or more modified internucleotidic linkages independently having the structure of formula **I**:



(I)

wherein:

P* is an asymmetric phosphorus atom and is either *R*p or *S*p;

W is O, S or Se;

each of X, Y and Z is independently –O–, –S–, –N(–L–R^1)–, or L;

L is a covalent bond or an optionally substituted, linear or branched C_1–C_{10} alkylene, wherein one or more methylene units of L are optionally and independently replaced by an optionally substituted C_1–C_6 alkylene, C_1–C_6 alkenylene, —C≡C—, –C(R′)$_2$–, –Cy–, –O–, –S–, –S–S–, –N(R′)–, –C(O)–, –C(S)–, –C(NR′)–, –C(O)N(R′)–, –N(R′)C(O)N(R′)-, –N(R′)C(O)–, –N(R′)C(O)O–, –OC(O)N(R′)-, –S(O)–, –S(O)$_2$–, –S(O)$_2$N(R′)–, –N(R′)S(O)$_2$–, –SC(O)–, –C(O)S–, –OC(O)–, or –C(O)O–;

R^1 is halogen, R, or an optionally substituted C_1–C_{50} aliphatic wherein one or more methylene units are optionally and independently replaced by an optionally substituted C_1–C_6 alkylene, C_1–C_6 alkenylene, —C≡C—, –C(R′)$_2$–, –Cy–, –O–, –S–, –S–S–, –N(R′)–, –C(O)–, –C(S)–, –

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C(NR′)–, –C(O)N(R′)–, –N(R′)C(O)N(R′)-, –N(R′)C(O)–, –N(R′)C(O)O–, –OC(O)N(R′)-, –S(O)–, $–S(O)_2–$, $–S(O)_2N(R′)–$, $–N(R′)S(O)_2–$, –SC(O)–, –C(O)S–, –OC(O)–, or –C(O)O–;

each R′ is independently –R, -C(O)R, $-CO_2R$, or $–SO_2R$, or:

two R′ on the same nitrogen are taken together with their intervening atoms to form an optionally substituted heterocyclic or heteroaryl ring, or

two R′ on the same carbon are taken together with their intervening atoms to form an optionally substituted aryl, carbocyclic, heterocyclic, or heteroaryl ring;

–Cy– is an optionally substituted bivalent ring selected from phenylene, carbocyclylene, arylene, heteroarylene, or heterocyclylene;

each R is independently hydrogen, or an optionally substituted group selected from C_1–C_6 aliphatic, phenyl, carbocyclyl, aryl, heteroaryl, or heterocyclyl; and

each $-\!\!\lessgtr\!\!-$ independently represents a connection to a nucleoside.

[00245] As defined generally above and herein, P* is an asymmetric phosphorus atom and is either *R*p or *S*p. In some embodiments, P* is *R*p. In other embodiments, P* is *S*p. In some embodiments, an oligonucleotide comprises one or more internucleotidic linkages of formula **I** wherein each P* is independently *R*p or *S*p. In some embodiments, an oligonucleotide comprises one or more internucleotidic linkages of formula **I** wherein each P* is *R*p. In some embodiments, an oligonucleotide comprises one or more internucleotidic linkages of formula **I** wherein each P* is *S*p. In some embodiments, an oligonucleotide comprises at least one internucleotidic linkage of formula **I** wherein P* is *R*p. In some embodiments, an oligonucleotide comprises at least one internucleotidic linkage of formula **I** wherein P* is *S*p. In some embodiments, an oligonucleotide comprises at least one internucleotidic linkage of formula **I** wherein P* is *R*p, and at least one internucleotidic linkage of formula **I** wherein P* is *S*p.

[00246] As defined generally above and herein, W is O, S, or Se. In some embodiments, W is O. In some embodiments, W is S. In some embodiments, W is Se. In some embodiments, an oligonucleotide comprises at least one internucleotidic linkage of formula **I** wherein W is O. In some embodiments, an oligonucleotide comprises at least one internucleotidic linkage of formula **I** wherein W is S. In some embodiments, an oligonucleotide comprises at least one internucleotidic linkage of formula **I** wherein W is Se.

[00247] As defined generally above and herein, each R is independently hydrogen, or an optionally substituted group selected from C_1–C_6 aliphatic, phenyl, carbocyclyl, aryl, heteroaryl, or heterocyclyl.

[00248] In some embodiments, R is hydrogen. In some embodiments, R is an optionally substituted group selected from C_1–C_6 aliphatic, phenyl, carbocyclyl, aryl, heteroaryl, or heterocyclyl.

[00249] In some embodiments, R is an optionally substituted C_1–C_6 aliphatic. In some embodiments, R is an optionally substituted C_1–C_6 alkyl. In some embodiments, R is optionally substituted, linear or branched hexyl. In some embodiments, R is optionally substituted, linear or branched pentyl. In some embodiments, R is optionally substituted, linear or branched butyl. In some embodiments, R is optionally substituted, linear or branched propyl. In some embodiments, R is optionally substituted ethyl. In some embodiments, R is optionally substituted methyl.

[00250] In some embodiments, R is optionally substituted phenyl. In some embodiments, R is substituted phenyl. In some embodiments, R is phenyl.

[00251] In some embodiments, R is optionally substituted carbocyclyl. In some embodiments, R is optionally substituted C_3-C_{10} carbocyclyl. In some embodiments, R is optionally substituted monocyclic carbocyclyl. In some embodiments, R is optionally substituted cycloheptyl. In some embodiments, R is optionally substituted cyclohexyl. In some embodiments, R is optionally substituted cyclopentyl. In some embodiments, R is optionally substituted cyclobutyl. In some embodiments, R is an optionally substituted cyclopropyl. In some embodiments, R is optionally substituted bicyclic carbocyclyl.

[00252] In some embodiments, R is an optionally substituted aryl. In some embodiments, R is an optionally substituted bicyclic aryl ring.

[00253] In some embodiments, R is an optionally substituted heteroaryl. In some embodiments, R is an optionally substituted 5-6 membered monocyclic heteroaryl ring having 1-3 heteroatoms independently selected from nitrogen, sulfur, or oxygen. In some embodiments, R is a substituted 5-6 membered monocyclic heteroaryl ring having 1-3 heteroatoms independently selected from nitrogen, oxygen, or sulfur. In some embodiments, R is an unsubstituted 5-6 membered monocyclic heteroaryl ring having 1-3 heteroatoms independently selected from nitrogen, sulfur, or oxygen.

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[00254] In some embodiments, R is an optionally substituted 5 membered monocyclic heteroaryl ring having 1-3 heteroatoms independently selected from nitrogen, oxygen or sulfur. In some embodiments, R is an optionally substituted 6 membered monocyclic heteroaryl ring having 1-3 heteroatoms independently selected from nitrogen, oxygen, or sulfur.

[00255] In some embodiments, R is an optionally substituted 5-membered monocyclic heteroaryl ring having 1 heteroatom selected from nitrogen, oxygen, or sulfur. In some embodiments, R is selected from pyrrolyl, furanyl, or thienyl.

[00256] In some embodiments, R is an optionally substituted 5-membered heteroaryl ring having 2 heteroatoms independently selected from nitrogen, oxygen, or sulfur. In certain embodiments, R is an optionally substituted 5-membered heteroaryl ring having 1 nitrogen atom, and an additional heteroatom selected from sulfur or oxygen. Exemplary R groups include optionally substituted pyrazolyl, imidazolyl, thiazolyl, isothiazolyl, oxazolyl or isoxazolyl.

[00257] In some embodiments, R is a 6-membered heteroaryl ring having 1–3 nitrogen atoms. In other embodiments, R is an optionally substituted 6-membered heteroaryl ring having 1–2 nitrogen atoms. In some embodiments, R is an optionally substituted 6-membered heteroaryl ring having 2 nitrogen atoms. In certain embodiments, R is an optionally substituted 6-membered heteroaryl ring having 1 nitrogen. Exemplary R groups include optionally substituted pyridinyl, pyrimidinyl, pyrazinyl, pyridazinyl, triazinyl, or tetrazinyl.

[00258] In certain embodiments, R is an optionally substituted 8–10 membered bicyclic heteroaryl ring having 1–4 heteroatoms independently selected from nitrogen, oxygen, or sulfur. In some embodiments, R is an optionally substituted 5,6–fused heteroaryl ring having 1–4 heteroatoms independently selected from nitrogen, oxygen, or sulfur. In other embodiments, R is an optionally substituted 5,6–fused heteroaryl ring having 1–2 heteroatoms independently selected from nitrogen, oxygen, or sulfur. In certain embodiments, R is an optionally substituted 5,6–fused heteroaryl ring having 1 heteroatom independently selected from nitrogen, oxygen, or sulfur. In some embodiments, R is an optionally substituted indolyl. In some embodiments, R is an optionally substituted azabicyclo[3.2.1]octanyl. In certain embodiments, R is an optionally substituted 5,6–fused heteroaryl ring having 2 heteroatoms independently selected from nitrogen, oxygen, or sulfur. In some embodiments, R is an optionally substituted azaindolyl. In some embodiments, R is an optionally substituted benzimidazolyl. In some embodiments, R is an optionally substituted benzothiazolyl. In some embodiments, R is an optionally substituted

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benzoxazolyl. In some embodiments, R is an optionally substituted indazolyl. In certain embodiments, R is an optionally substituted 5,6–fused heteroaryl ring having 3 heteroatoms independently selected from nitrogen, oxygen, or sulfur.

[00259] In certain embodiments, R is an optionally substituted 6,6–fused heteroaryl ring having 1–4 heteroatoms independently selected from nitrogen, oxygen, or sulfur. In some embodiments, R is an optionally substituted 6,6–fused heteroaryl ring having 1–2 heteroatoms independently selected from nitrogen, oxygen, or sulfur. In other embodiments, R is an optionally substituted 6,6–fused heteroaryl ring having 1 heteroatom independently selected from nitrogen, oxygen, or sulfur. In some embodiments, R is an optionally substituted quinolinyl. In some embodiments, R is an optionally substituted isoquinolinyl. According to one aspect, R is an optionally substituted 6,6–fused heteroaryl ring having 2 heteroatoms independently selected from nitrogen, oxygen, or sulfur. In some embodiments, R is a quinazoline or a quinoxaline.

[00260] In some embodiments, R is an optionally substituted heterocyclyl. In some embodiments, R is an optionally substituted 3-7 membered saturated or partially unsaturated heterocyclic ring having 1-2 heteroatoms independently selected from nitrogen, oxygen, or sulfur. In some embodiments, R is a substituted 3-7 membered saturated or partially unsaturated heterocyclic ring having 1-2 heteroatoms independently selected from nitrogen, oxygen, or sulfur. In some embodiments, R is an unsubstituted 3-7 membered saturated or partially unsaturated heterocyclic ring having 1-2 heteroatoms independently selected from nitrogen, oxygen, or sulfur.

[00261] In some embodiments, R is an optionally substituted heterocyclyl. In some embodiments, R is an optionally substituted 6 membered saturated or partially unsaturated heterocyclic ring having 1-2 heteroatoms independently selected from nitrogen, oxygen, or sulfur. In some embodiments, R is an optionally substituted 6 membered partially unsaturated heterocyclic ring having 2 heteroatoms independently selected from nitrogen, oxygen, or sulfur. In some embodiments, R is an optionally substituted 6 membered partially unsaturated heterocyclic ring having 2 oxygen atom.

[00262] In certain embodiments, R is a 3-7 membered saturated or partially unsaturated heterocyclic ring having 1-2 heteroatoms independently selected from nitrogen, oxygen, or sulfur. In certain embodiments, R is oxiranyl, oxetanyl, tetrahydrofuranyl, tetrahydropyranyl,

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oxepaneyl, aziridineyl, azetidineyl, pyrrolidinyl, piperidinyl, azepanyl, thiiranyl, thietanyl, tetrahydrothiophenyl, tetrahydrothiopyranyl, thiepanyl, dioxolanyl, oxathiolanyl, oxazolidinyl, imidazolidinyl, thiazolidinyl, dithiolanyl, dioxanyl, morpholinyl, oxathianyl, piperazinyl, thiomorpholinyl, dithianyl, dioxepanyl, oxazepanyl, oxathiepanyl, dithiepanyl, diazepanyl, dihydrofuranonyl, tetrahydropyranonyl, oxepanonyl, pyrolidinonyl, piperidinonyl, azepanonyl, dihydrothiophenonyl, tetrahydrothiopyranonyl, thiepanonyl, oxazolidinonyl, oxazinanonyl, oxazepanonyl, dioxolanonyl, dioxanonyl, dioxepanonyl, oxathiolinonyl, oxathianonyl, oxathiepanonyl, thiazolidinonyl, thiazinanonyl, thiazepanonyl, imidazolidinonyl, tetrahydropyrimidinonyl, diazepanonyl, imidazolidinedionyl, oxazolidinedionyl, thiazolidinedionyl, dioxolanedionyl, oxathiolanedionyl, piperazinedionyl, morpholinedionyl, thiomorpholinedionyl, tetrahydropyranyl, tetrahydrofuranyl, morpholinyl, thiomorpholinyl, piperidinyl, piperazinyl, pyrrolidinyl, tetrahydrothiophenyl, or tetrahydrothiopyranyl. In some embodiments, R is an optionally substituted 5 membered saturated or partially unsaturated heterocyclic ring having 1-2 heteroatoms independently selected from nitrogen, oxygen, or sulfur.

[00263] In certain embodiments, R is an optionally substituted 5–6 membered partially unsaturated monocyclic ring having 1–2 heteroatoms independently selected from nitrogen, oxygen, or sulfur. In certain embodiments, R is an optionally substituted tetrahydropyridinyl, dihydrothiazolyl, dihydrooxazolyl, or oxazolinyl group.

[00264] In some embodiments, R is an optionally substituted 8-10 membered bicyclic saturated or partially unsaturated heterocyclic ring having 1-4 heteroatoms independently selected from nitrogen, oxygen, or sulfur. In some embodiments, R is an optionally substituted indolinyl. In some embodiments, R is an optionally substituted isoindolinyl. In some embodiments, R is an optionally substituted 1, 2, 3, 4-tetrahydroquinoline. In some embodiments, R is an optionally substituted 1, 2, 3, 4-tetrahydroisoquinoline.

[00265] As defined generally above and herein, each R′ is independently –R, -C(O)R, $-CO_2R$, or $-SO_2R$, or:

two R′ on the same nitrogen are taken together with their intervening atoms to form an optionally substituted heterocyclic or heteroaryl ring, or

two R′ on the same carbon are taken together with their intervening atoms to form an optionally substituted aryl, carbocyclic, heterocyclic, or heteroaryl ring.

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[00266] In some embodiments, R' is –R, -C(O)R, -CO_2R, or –SO_2R, wherein R is as defined above and described herein.

[00267] In some embodiments, R' is –R, wherein R is as defined and described above and herein. In some embodiments, R' is hydrogen.

[00268] In some embodiments, R' is –C(O)R, wherein R is as defined above and described herein. In some embodiments, R' is –CO_2R, wherein R is as defined above and described herein. In some embodiments, R' is –SO_2R, wherein R is as defined above and described herein.

[00269] In some embodiments, two R′ on the same nitrogen are taken together with their intervening atoms to form an optionally substituted heterocyclic or heteroaryl ring. In some embodiments, two R′ on the same carbon are taken together with their intervening atoms to form an optionally substituted aryl, carbocyclic, heterocyclic, or heteroaryl ring.

[00270] As defined generally above and herein, –Cy– is an optionally substituted bivalent ring selected from phenylene, carbocyclylene, arylene, heteroarylene, or heterocyclylene.

[00271] In some embodiments, –Cy– is optionally substituted phenylene. In some embodiments, –Cy– is optionally substituted carbocyclylene. In some embodiments, –Cy– is optionally substituted arylene. In some embodiments, –Cy– is optionally substituted heteroarylene. In some embodiments, –Cy– is optionally substituted heterocyclylene.

[00272] As defined generally above and herein, each of X, Y and Z is independently –O–, –S–, –N(–L–R^1)–, or L, wherein each of L and R^1 is independently as defined above and described below.

[00273] In some embodiments, X is –O–. In some embodiments, X is –S–. In some embodiments, X is –O– or –S–. In some embodiments, an oligonucleotide comprises at least one internucleotidic linkage of formula **I** wherein X is –O–. In some embodiments, an oligonucleotide comprises at least one internucleotidic linkage of formula **I** wherein X is –S–. In some embodiments, an oligonucleotide comprises at least one internucleotidic linkage of formula **I** wherein X is –O–, and at least one internucleotidic linkage of formula **I** wherein X is –S–. In some embodiments, an oligonucleotide comprises at least one internucleotidic linkage of formula **I** wherein X is –O–, and at least one internucleotidic linkage of formula **I** wherein X is –S–, and at least one internucleotidic linkage of formula **I** wherein L is an optionally substituted, linear or branched C_1–C_{10} alkylene, wherein one or more methylene units of L are optionally and

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independently replaced by an optionally substituted C_1–C_6 alkylene, C_1–C_6 alkenylene, —C≡C—, –C(R′)$_2$–, –Cy–, –O–, –S–, –S–S–, –N(R′)–, –C(O)–, –C(S)–, –C(NR′)–, –C(O)N(R′)–, –N(R′)C(O)N(R′)-, –N(R′)C(O)–, –N(R′)C(O)O–, –OC(O)N(R′)-, –S(O)–, –S(O)$_2$–, –S(O)$_2$N(R′)–, –N(R′)S(O)$_2$–, –SC(O)–, –C(O)S–, –OC(O)–, or –C(O)O–.

[00274] In some embodiments, X is –N(–L–R^1)–. In some embodiments, X is –N(R^1)–. In some embodiments, X is –N(R')–. In some embodiments, X is –N(R)–. In some embodiments, X is –NH–.

[00275] In some embodiments, X is L. In some embodiments, X is a covalent bond. In some embodiments, X is or an optionally substituted, linear or branched C_1–C_{10} alkylene, wherein one or more methylene units of L are optionally and independently replaced by an optionally substituted C_1–C_6 alkylene, C_1–C_6 alkenylene, —C≡C—, –C(R′)$_2$–, –Cy–, –O–, –S–, –S–S–, –N(R′)–, –C(O)–, –C(S)–, –C(NR′)–, –C(O)N(R′)–, –N(R′)C(O)N(R′)-, –N(R′)C(O)–, –N(R′)C(O)O–, –OC(O)N(R′)-, –S(O)–, –S(O)$_2$–, –S(O)$_2$N(R′)–, –N(R′)S(O)$_2$–, –SC(O)–, –C(O)S–, –OC(O)–, or –C(O)O–. In some embodiments, X is an optionally substituted C_1–C_{10} alkylene or C_1–C_{10} alkenylene. In some embodiments, X is methylene.

[00276] In some embodiments, Y is –O–. In some embodiments, Y is –S–.

[00277] In some embodiments, Y is –N(–L–R^1)–. In some embodiments, Y is –N(R^1)–. In some embodiments, Y is –N(R')–. In some embodiments, Y is –N(R)–. In some embodiments, Y is –NH–.

[00278] In some embodiments, Y is L. In some embodiments, Y is a covalent bond. In some embodiments, Y is or an optionally substituted, linear or branched C_1–C_{10} alkylene, wherein one or more methylene units of L are optionally and independently replaced by an optionally substituted C_1–C_6 alkylene, C_1–C_6 alkenylene, —C≡C—, –C(R′)$_2$–, –Cy–, –O–, –S–, –S–S–, –N(R′)–, –C(O)–, –C(S)–, –C(NR′)–, –C(O)N(R′)–, –N(R′)C(O)N(R′)-, –N(R′)C(O)–, –N(R′)C(O)O–, –OC(O)N(R′)-, –S(O)–, –S(O)$_2$–, –S(O)$_2$N(R′)–, –N(R′)S(O)$_2$–, –SC(O)–, –C(O)S–, –OC(O)–, or –C(O)O–. In some embodiments, Y is an optionally substituted C_1–C_{10} alkylene or C_1–C_{10} alkenylene. In some embodiments, Y is methylene.

[00279] In some embodiments, Z is –O–. In some embodiments, Z is –S–.

[00280] In some embodiments, Z is –N(–L–R^1)–. In some embodiments, Z is –N(R^1)–. In some embodiments, Z is –N(R')–. In some embodiments, Z is –N(R)–. In some embodiments, Z is –NH–.

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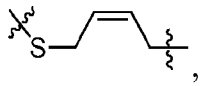
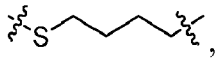
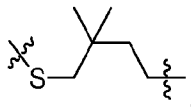
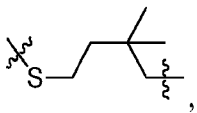
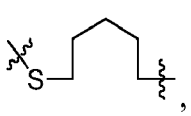
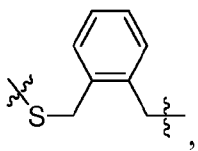
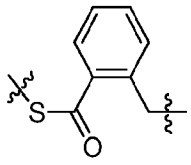
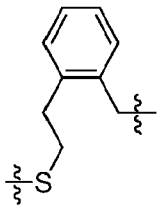
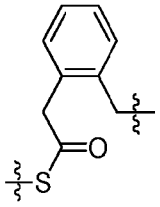
[00281] In some embodiments, Z is L. In some embodiments, Z is a covalent bond. In some embodiments, Z is or an optionally substituted, linear or branched C_1–C_{10} alkylene, wherein one or more methylene units of L are optionally and independently replaced by an optionally substituted C_1–C_6 alkylene, C_1–C_6 alkenylene, —C≡C—, –C(R′)$_2$–, –Cy–, –O–, –S–, –S–S–, –N(R′)–, –C(O)–, –C(S)–, –C(NR′)–, –C(O)N(R′)–, –N(R′)C(O)N(R′)-, –N(R′)C(O)–, –N(R′)C(O)O–, –OC(O)N(R′)-, –S(O)–, –S(O)$_2$–, –S(O)$_2$N(R′)–, –N(R′)S(O)$_2$–, –SC(O)–, –C(O)S–, –OC(O)–, or –C(O)O–. In some embodiments, Z is an optionally substituted C_1–C_{10} alkylene or C_1–C_{10} alkenylene. In some embodiments, Z is methylene.

[00282] As defined generally above and herein, L is a covalent bond or an optionally substituted, linear or branched C_1–C_{10} alkylene, wherein one or more methylene units of L are optionally and independently replaced by an optionally substituted C_1–C_6 alkylene, C_1–C_6 alkenylene, —C≡C—, –C(R′)$_2$–, –Cy–, –O–, –S–, –S–S–, –N(R′)–, –C(O)–, –C(S)–, –C(NR′)–, –C(O)N(R′)–, –N(R′)C(O)N(R′)-, –N(R′)C(O)–, –N(R′)C(O)O–, –OC(O)N(R′)-, –S(O)–, –S(O)$_2$–, –S(O)$_2$N(R′)–, –N(R′)S(O)$_2$–, –SC(O)–, –C(O)S–, –OC(O)–, or –C(O)O–.

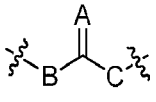
[00283] In some embodiments, L is a covalent bond. In some embodiments, L is an optionally substituted, linear or branched C_1–C_{10} alkylene, wherein one or more methylene units of L are optionally and independently replaced by an optionally substituted C_1–C_6 alkylene, C_1–C_6 alkenylene, —C≡C—, –C(R′)$_2$–, –Cy–, –O–, –S–, –S–S–, –N(R′)–, –C(O)–, –C(S)–, –C(NR′)–, –C(O)N(R′)–, –N(R′)C(O)N(R′)-, –N(R′)C(O)–, –N(R′)C(O)O–, –OC(O)N(R′)-, –S(O)–, –S(O)$_2$–, –S(O)$_2$N(R′)–, –N(R′)S(O)$_2$–, –SC(O)–, –C(O)S–, –OC(O)–, or –C(O)O–.

[00284] In some embodiments, L has the structure of –L^1–V–, wherein:

L^1 is an optionally substituted group selected from , , , , , , , ,



, C_1–C_6 alkylene, C_1-C_6 alkenylene, carbocyclylene, arylene, C_1–C_6 heteroalkylene, heterocyclylene, and heteroarylene;

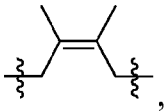
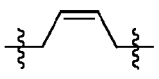
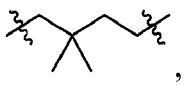
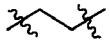
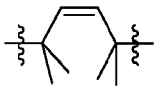
V is selected from –O–, –S–, –NR'–, $C(R')_2$, –S–S–, –B–S–S–C–, 

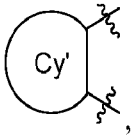
, or an optionally substituted group selected from C_1–C_6 alkylene, arylene, C_1–C_6 heteroalkylene, heterocyclylene, and heteroarylene;

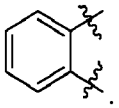
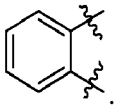
A is =O, =S, =NR', or $=C(R')_2$;

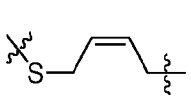
each of B and C is independently –O–, –S–, –NR'–, $–C(R')_2–$, or an optionally substituted group selected from C_1–C_6 alkylene, carbocyclylene, arylene, heterocyclylene, or heteroarylene; and

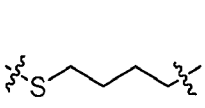
each R' is independently as defined above and described herein.

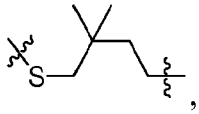
[00285] In some embodiments, L^1 is , , , , , , or .

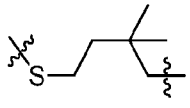
[00286] In some embodiments, L^1 is 

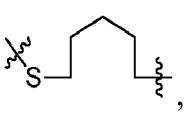
, wherein Ring Cy' is an optionally substituted arylene, carbocyclylene, heteroarylene, or heterocyclylene. In some embodiments, L^1 is optionally substituted . In some embodiments, L^1 is .

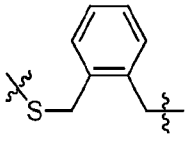
[00287] In some embodiments, L^1 is connected to X. In some embodiments, L^1 is an optionally substituted group selected from 

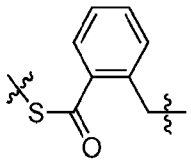
, 

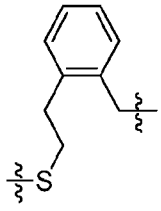
, 

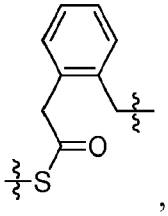
, 

, 

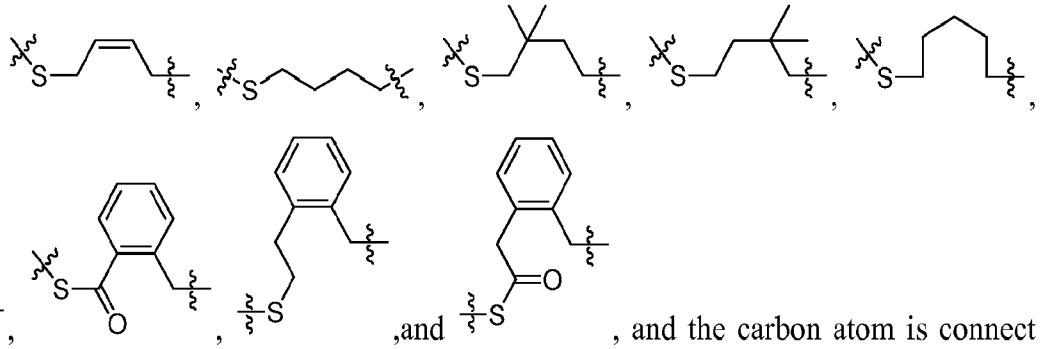
, 

, 

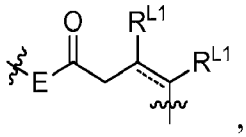
, 

,and 

, and the sulfur atom is connect to V. In some embodiments, L^1 is an optionally substituted group

selected from , and the carbon atom is connect to X.

[00288] In some embodiments, L has the structure of:



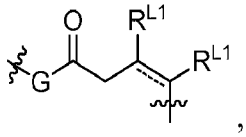
wherein:

E is –O–, –S–, –NR'– or $–C(R')_2–$;

----- is a single or double bond;

the two R^{L1} are taken together with the two carbon atoms to which they are bound to form an optionally substituted aryl, carbocyclic, heteroaryl or heterocyclic ring; and each R' is independently as defined above and described herein.

[00289] In some embodiments, L has the structure of:



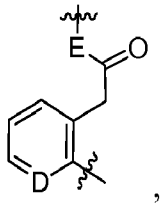
wherein:

G is –O–, –S–, or –NR';

----- is a single or double bond; and

the two R^{L1} are taken together with the two carbon atoms to which they are bound to form an optionally substituted aryl, C_3-C_{10} carbocyclic, heteroaryl or heterocyclic ring.

[00290] In some embodiments, L has the structure of:



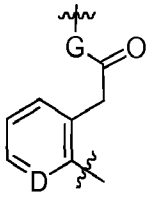
wherein:

E is –O–, –S–, –NR'– or $–C(R')_2–$;

D is =N–, =C(F)–, =C(Cl)–, =C(Br)–, =C(I)–, =C(CN)–, $=C(NO_2)–$, $=C(CO_2–(C_1\text{-}C_6$ aliphatic))–, or $=C(CF_3)–$; and

each R' is independently as defined above and described herein.

[00291] In some embodiments, L has the structure of:



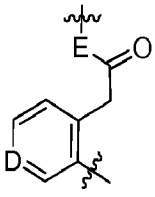
,

wherein:

G is –O–, –S–, or –NR';

D is =N–, =C(F)–, =C(Cl)–, =C(Br)–, =C(I)–, =C(CN)–, $=C(NO_2)–$, $=C(CO_2–(C_1\text{-}C_6$ aliphatic))–, or $=C(CF_3)–$.

[00292] In some embodiments, L has the structure of:



,

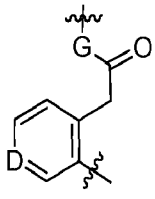
wherein:

E is –O–, –S–, –NR'– or $–C(R')_2–$;

D is =N–, =C(F)–, =C(Cl)–, =C(Br)–, =C(I)–, =C(CN)–, $=C(NO_2)–$, $=C(CO_2–(C_1\text{-}C_6$ aliphatic))–, or $=C(CF_3)–$; and

each R' is independently as defined above and described herein.

[00293] In some embodiments, L has the structure of:



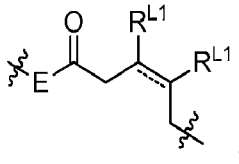
,

wherein:

G is –O–, –S–, or –NR';

D is =N–, =C(F)–, =C(Cl)–, =C(Br)–, =C(I)–, =C(CN)–, $=C(NO_2)–$, $=C(CO_2–(C_1\text{-}C_6$ aliphatic))–, or $=C(CF_3)–$.

[00294] In some embodiments, L has the structure of:



,

wherein:

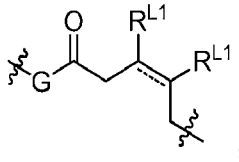
E is –O–, –S–, –NR'– or $–C(R')_2–$;

⁼⁼⁼ is a single or double bond;

the two R^{L1} are taken together with the two carbon atoms to which they are bound to form an optionally substituted aryl, C_3-C_{10} carbocyclic, heteroaryl or heterocyclic ring;

and each R' is independently as defined above and described herein.

[00295] In some embodiments, L has the structure of:



,

wherein:

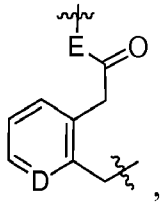
G is –O–, –S–, or –NR';

⁼⁼⁼ is a single or double bond;

the two R^{L1} are taken together with the two carbon atoms to which they are bound to form an optionally substituted aryl, C_3-C_{10} carbocyclic, heteroaryl or heterocyclic ring;

and each R' is independently as defined above and described herein.

[00296] In some embodiments, L has the structure of:



,

wherein:

E is –O–, –S–, –NR'– or $–C(R')_2–$;

D is =N–, =C(F)–, =C(Cl)–, =C(Br)–, =C(I)–, =C(CN)–, $=C(NO_2)–$, $=C(CO_2–(C_1$-C_6 aliphatic))–, or $=C(CF_3)–$; and

each R' is independently as defined above and described herein.

[00297] In some embodiments, L has the structure of:

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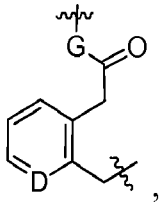


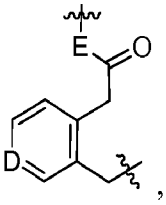
wherein:

G is –O–, –S–, or –NR';

D is =N–, =C(F)–, =C(Cl)–, =C(Br)–, =C(I)–, =C(CN)–, =C(NO_2)–, =C(CO_2–(C_1-C_6 aliphatic))–, or =C(CF_3)–; and

each R' is independently as defined above and described herein.

[00298] In some embodiments, L has the structure of:



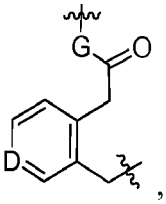
wherein:

E is –O–, –S–, –NR'– or –C(R')$_2$–;

D is =N–, =C(F)–, =C(Cl)–, =C(Br)–, =C(I)–, =C(CN)–, =C(NO_2)–, =C(CO_2–(C_1-C_6 aliphatic))–, or =C(CF_3)–; and

each R' is independently as defined above and described herein.

[00299] In some embodiments, L has the structure of:



wherein:

G is –O–, –S–, or –NR';

D is =N–, =C(F)–, =C(Cl)–, =C(Br)–, =C(I)–, =C(CN)–, =C(NO_2)–, =C(CO_2–(C_1-C_6 aliphatic))–, or =C(CF_3)–; and

each R' is independently as defined above and described herein.

[00300] In some embodiments, L has the structure of:

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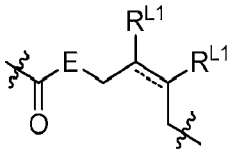


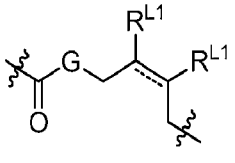
wherein:

E is –O–, –S–, –NR'– or $–C(R')_2–$;

⁼⁼⁼ is a single or double bond;

the two R^{L1} are taken together with the two carbon atoms to which they are bound to form an optionally substituted aryl, C_3-C_{10} carbocyclic, heteroaryl or heterocyclic ring; and each R' is independently as defined above and described herein.

[00301] In some embodiments, L has the structure of:



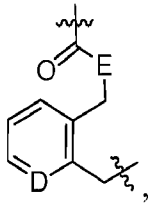
wherein:

G is –O–, –S–, or –NR';

⁼⁼⁼ is a single or double bond;

the two R^{L1} are taken together with the two carbon atoms to which they are bound to form an optionally substituted aryl, C_3-C_{10} carbocyclic, heteroaryl or heterocyclic ring; and each R' is independently as defined above and described herein.

[00302] In some embodiments, L has the structure of:



wherein:

E is –O–, –S–, –NR'– or $–C(R')_2–$;

D is =N–, =C(F)–, =C(Cl)–, =C(Br)–, =C(I)–, =C(CN)–, $=C(NO_2)–$, $=C(CO_2–(C_1$-C_6 aliphatic))–, or $=C(CF_3)–$; and

each R' is independently as defined above and described herein.

[00303] In some embodiments, L has the structure of:

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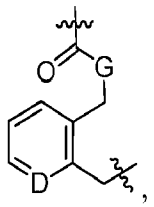


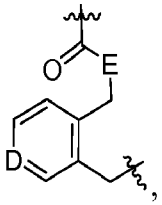
wherein:

G is –O–, –S–, or –NR';

D is =N–, =C(F)–, =C(Cl)–, =C(Br)–, =C(I)–, =C(CN)–, =C(NO_2)–, =C(CO_2–(C_1-C_6 aliphatic))–, or =C(CF_3)–; and

R' is as defined above and described herein.

[00304] In some embodiments, L has the structure of:



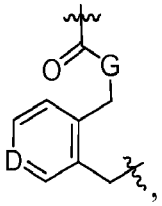
wherein:

E is –O–, –S–, –NR'– or –C(R')$_2$–;

D is =N–, =C(F)–, =C(Cl)–, =C(Br)–, =C(I)–, =C(CN)–, =C(NO_2)–, =C(CO_2–(C_1-C_6 aliphatic))–, or =C(CF_3)–; and

each R' is independently as defined above and described herein.

[00305] In some embodiments, L has the structure of:



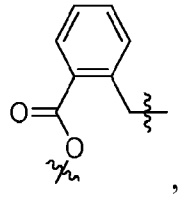
wherein:

G is –O–, –S–, or –NR';

D is =N–, =C(F)–, =C(Cl)–, =C(Br)–, =C(I)–, =C(CN)–, =C(NO_2)–, =C(CO_2–(C_1-C_6 aliphatic))–, or =C(CF_3)–; and

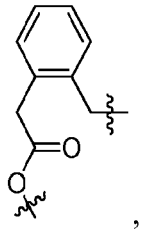
R' is as defined above and described herein.

[00306] In some embodiments, L has the structure of:



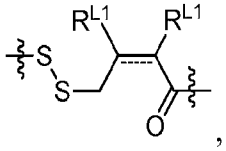
wherein the phenyl ring is optionally substituted. In some embodiments, the phenyl ring is not substituted. In some embodiments, the phenyl ring is substituted.

[00307] In some embodiments, L has the structure of:



wherein the phenyl ring is optionally substituted. In some embodiments, the phenyl ring is not substituted. In some embodiments, the phenyl ring is substituted.

[00308] In some embodiments, L has the structure of:

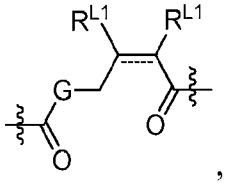


wherein:

==== is a single or double bond; and

the two R^{L1} are taken together with the two carbon atoms to which they are bound to form an optionally substituted aryl, C_3-C_{10} carbocyclic, heteroaryl or heterocyclic ring.

[00309] In some embodiments, L has the structure of:



wherein:

G is –O–, –S–, or –NR';

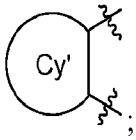
==== is a single or double bond; and

the two R^{L1} are taken together with the two carbon atoms to which they are bound to form an optionally substituted aryl, C_3-C_{10} carbocyclic, heteroaryl or heterocyclic ring.

[00310] As defined generally above and herein, E is –O–, –S–, –NR'– or $–C(R')_2–$, wherein each R' independently as defined above and described herein. In some embodiments, E is –O–, –S–, or –NR'–. In some embodiments, E is –O–, –S–, or –NH–. In some embodiments, E is –O–. In some embodiments, E is –S–. In some embodiments, E is –NH–.

[00311] As defined generally above and herein, G is –O–, –S–, or –NR', wherein each R' independently as defined above and described herein. In some embodiments, G is –O–, –S–, or –NH–. In some embodiments, G is –O–. In some embodiments, G is –S–. In some embodiments, G is –NH–.

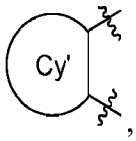
[00312] In some embodiments, L is $–L^3–G–$, wherein:

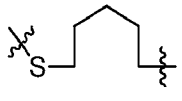
L^3 is an optionally substituted $C_1–C_5$ alkylene or alkenylene, wherein one or more methylene units are optionally and independently replaced by –O–, –S–,–N(R')–, –C(O)–, –C(S)–, –C(NR')–, –S(O)–, $–S(O)_2–$, or 

; and

wherein each of G, R' and Ring Cy' is independently as defined above and described herein.

[00313] In some embodiments, L is $–L^3–S–$, wherein L^3 is as defined above and described herein. In some embodiments, L is $–L^3–O–$, wherein L^3 is as defined above and described herein. In some embodiments, L is $–L^3–N(R')–$, wherein each of L^3 and R' is independently as defined above and described herein. In some embodiments, L is $–L^3–NH–$, wherein each of L^3 and R' is independently as defined above and described herein.

[00314] In some embodiments, L^3 is an optionally substituted C_5 alkylene or alkenylene, wherein one or more methylene units are optionally and independently replaced by –O–, –S–,–N(R')–, –C(O)–, –C(S)–, –C(NR')–, –S(O)–, $–S(O)_2–$, or 

, and each of R' and Ring Cy' is independently as defined above and described herein. In some embodiments, L^3 is an optionally substituted C_5 alkylene. In some embodiments, $–L^3–G–$ is 

.

[00315] In some embodiments, L^3 is an optionally substituted C_4 alkylene or alkenylene, wherein one or more methylene units are optionally and independently replaced by –O–, –S–,–

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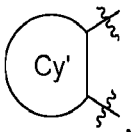
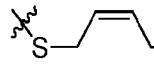
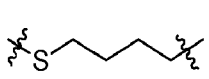
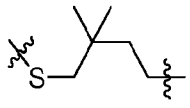
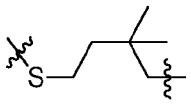
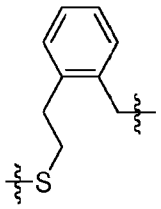
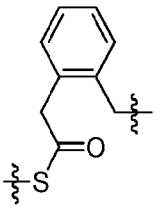
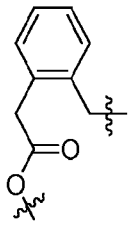
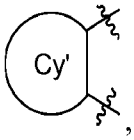
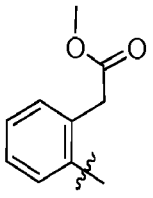
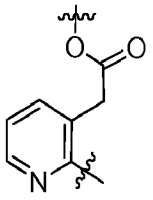
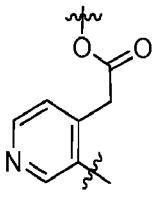
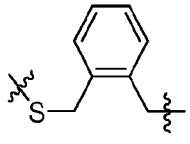
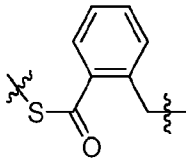
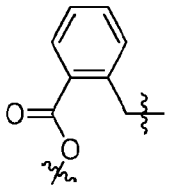
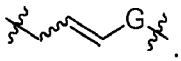
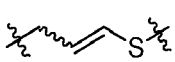
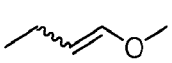
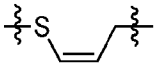
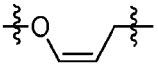
N(R′)–, –C(O)–, –C(S)–, –C(NR′)–, –S(O)–, –S(O)$_2$–, or , and each of R' and Cy' is independently as defined above and described herein.

[00316] In some embodiments, –L^3–G– is , , , , , , or .

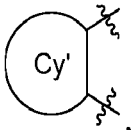
[00317] In some embodiments, L^3 is an optionally substituted C_3 alkylene or alkenylene, wherein one or more methylene units are optionally and independently replaced by –O–, –S–,–

N(R′)–, –C(O)–, –C(S)–, –C(NR′)–, –S(O)–, –S(O)$_2$–, or , and each of R' and Cy' is independently as defined above and described herein.

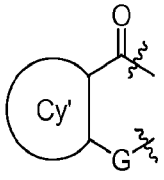
[00318] In some embodiments, –L^3–G– is , , , , , or .

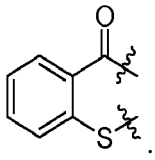
[00319] In some embodiments, L is . In some embodiments, L is  or . In some embodiments, L is  or .

[00320] In some embodiments, L^3 is an optionally substituted C_2 alkylene or alkenylene, wherein one or more methylene units are optionally and independently replaced by –O–, –S–,–

N(R′)–, –C(O)–, –C(S)–, –C(NR′)–, –S(O)–, –S(O)$_2$–, or 

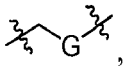
, and each of R' and Cy' is independently as defined above and described herein.

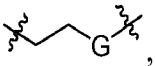
[00321] In some embodiments, –L^3–G– is 

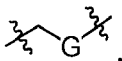
, wherein each of G and Cy' is independently as defined above and described herein. In some embodiments, L is 

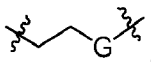
.

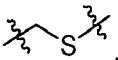
[00322] In some embodiments, L is –L^4–G–, wherein L^4 is an optionally substituted C_1–C_2 alkylene; and G is as defined above and described herein. In some embodiments, L is –L^4–G–, wherein L^4 is an optionally substituted C_1–C_2 alkylene; G is as defined above and described herein; and G is connected to R^1. In some embodiments, L is –L^4–G–, wherein L^4 is an optionally substituted methylene; G is as defined above and described herein; and G is connected to R^1. In some embodiments, L is –L^4–G–, wherein L^4 is methylene; G is as defined above and described herein; and G is connected to R^1. In some embodiments, L is –L^4–G–, wherein L^4 is an optionally substituted –$(CH_2)_2$–; G is as defined above and described herein; and G is connected to R^1. In some embodiments, L is –L^4–G–, wherein L^4 is –$(CH_2)_2$–; G is as defined above and described herein; and G is connected to R^1.

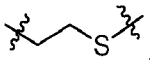
[00323] In some embodiments, L is 

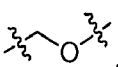
, or 

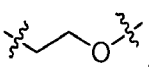
, wherein G is as defined above and described herein, and G is connected to R^1. In some embodiments, L is 

, wherein G is as defined above and described herein, and G is connected to R^1. In some embodiments, L is 

, wherein G is as defined above and described herein, and G is connected to R^1. In some embodiments, L is 

, or 

, wherein the sulfur atom is connected to R^1. In some embodiments, L is 

, or 

, wherein the oxygen atom is connected to R^1.

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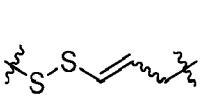
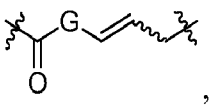
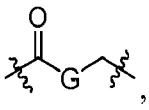
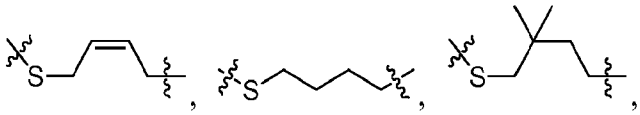
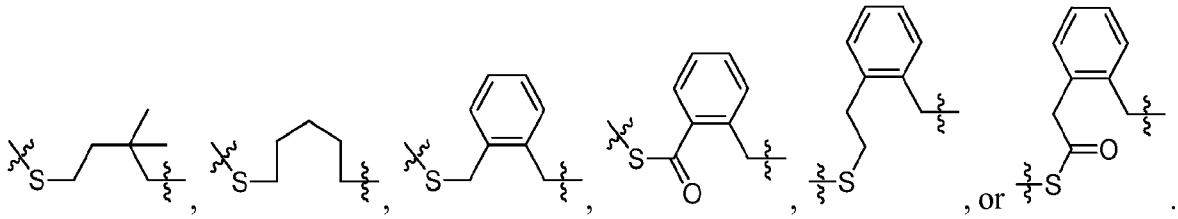
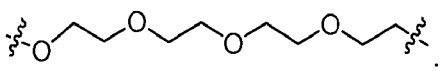
[00324] In some embodiments, L is , , or , wherein G is as defined above and described herein.

[00325] In some embodiments, L is $-S-R^{L3}-$ or $-S-C(O)-R^{L3}-$, wherein R^{L3} is an optionally substituted, linear or branched, C_1-C_9 alkylene, wherein one or more methylene units are optionally and independently replaced by an optionally substituted C_1-C_6 alkylene, C_1-C_6 alkenylene, $-C\equiv C-$, $-C(R')_2-$, $-Cy-$, $-O-$, $-S-$, $-S-S-$, $-N(R')-$, $-C(O)-$, $-C(S)-$, $-C(NR')-$, $-C(O)N(R')-$, $-N(R')C(O)N(R')-$, $-N(R')C(O)-$, $-N(R')C(O)O-$, $-OC(O)N(R')-$, $-S(O)-$, $-S(O)_2-$, $-S(O)_2N(R')-$, $-N(R')S(O)_2-$, $-SC(O)-$, $-C(O)S-$, $-OC(O)-$, or $-C(O)O-$, wherein each of R' and $-Cy-$ is independently as defined above and described herein. In some embodiments, L is $-S-R^{L3}-$ or $-S-C(O)-R^{L3}-$, wherein R^{L3} is an optionally substituted C_1-C_6 alkylene. In some embodiments, L is $-S-R^{L3}-$ or $-S-C(O)-R^{L3}-$, wherein R^{L3} is an optionally substituted C_1-C_6 alkenylene. In some embodiments, L is $-S-R^{L3}-$ or $-S-C(O)-R^{L3}-$, wherein R^{L3} is an optionally substituted C_1-C_6 alkylene wherein one or more methylene units are optionally and independently replaced by an optionally substituted C_1-C_6 alkenylene, arylene, or heteroarylene. In some embodiments, In some embodiments, R^{L3} is an optionally substituted $-S-(C_1-C_6$ alkenylene$)-$, $-S-(C_1-C_6$ alkylene$)-$, $-S-(C_1-C_6$ alkylene$)-$arylene$-(C_1-C_6$ alkylene$)-$, $-S-CO-$arylene$-(C_1-C_6$ alkylene$)-$, or $-S-CO-(C_1-C_6$ alkylene$)-$arylene$-(C_1-C_6$ alkylene$)-$.

[00326] In some embodiments, L is , , or .

[00327] In some embodiments, L is . In some embodiments, L is . In some embodiments, .

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[00328] In some embodiments, the sulfur atom in the L embodiments described above and herein is connected to X. In some embodiments, the sulfur atom in the L embodiments described above and herein is connected to R^1.

[00329] As defined generally above and herein, R^1 is halogen, R, or an optionally substituted C_1–C_{50} aliphatic wherein one or more methylene units are optionally and independently replaced by an optionally substituted C_1–C_6 alkylene, C_1–C_6 alkenylene, —C≡C—, –C(R′)$_2$–, –Cy–, –O–, –S–, –S–S–, –N(R′)–, –C(O)–, –C(S)–, –C(NR′)–, –C(O)N(R′)–, –N(R′)C(O)N(R′)-, –N(R′)C(O)–, –N(R′)C(O)O–, –OC(O)N(R′)-, –S(O)–, –S(O)$_2$–, –S(O)$_2$N(R′)–, –N(R′)S(O)$_2$–, –SC(O)–, –C(O)S–, –OC(O)–, or –C(O)O–, wherein each variable is independently as defined above and described herein. In some embodiments, R^1 is halogen, R, or an optionally substituted C_1–C_{10} aliphatic wherein one or more methylene units are optionally and independently replaced by an optionally substituted C_1–C_6 alkylene, C_1–C_6 alkenylene, —C≡C—, –C(R′)$_2$–, –Cy–, –O–, –S–, –S–S–, –N(R′)–, –C(O)–, –C(S)–, –C(NR′)–, –C(O)N(R′)–, –N(R′)C(O)N(R′)-, –N(R′)C(O)–, –N(R′)C(O)O–, –OC(O)N(R′)-, –S(O)–, –S(O)$_2$–, –S(O)$_2$N(R′)–, –N(R′)S(O)$_2$–, –SC(O)–, –C(O)S–, –OC(O)–, or –C(O)O–, wherein each variable is independently as defined above and described herein.

[00330] In some embodiments, R^1 is hydrogen. In some embodiments, R^1 is halogen. In some embodiments, R^1 is –F. In some embodiments, R^1 is –Cl. In some embodiments, R^1 is –Br. In some embodiments, R^1 is –I.

[00331] In some embodiments, R^1 is R wherein R is as defined above and described herein.

[00332] In some embodiments, R^1 is hydrogen. In some embodiments, R^1 is an optionally substituted group selected from C_1–C_{50} aliphatic, phenyl, carbocyclyl, aryl, heteroaryl, or heterocyclyl.

[00333] In some embodiments, R^1 is an optionally substituted C_1–C_{50} aliphatic. In some embodiments, R^1 is an optionally substituted C_1–C_{10} aliphatic. In some embodiments, R^1 is an optionally substituted C_1–C_6 aliphatic. In some embodiments, R^1 is an optionally substituted C_1–C_6 alkyl. In some embodiments, R^1 is optionally substituted, linear or branched hexyl. In some embodiments, R^1 is optionally substituted, linear or branched pentyl. In some embodiments, R^1 is optionally substituted, linear or branched butyl. In some embodiments, R^1 is optionally

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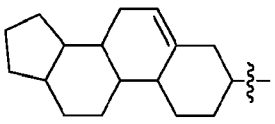
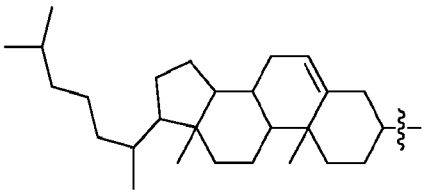
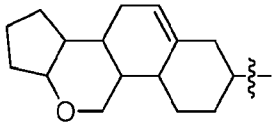
substituted, linear or branched propyl. In some embodiments, R^1 is optionally substituted ethyl. In some embodiments, R^1 is optionally substituted methyl.

[00334] In some embodiments, R^1 is optionally substituted phenyl. In some embodiments, R^1 is substituted phenyl. In some embodiments, R^1 is phenyl.

[00335] In some embodiments, R^1 is optionally substituted carbocyclyl. In some embodiments, R^1 is optionally substituted C_3-C_{10} carbocyclyl. In some embodiments, R^1 is optionally substituted monocyclic carbocyclyl. In some embodiments, R^1 is optionally substituted cycloheptyl. In some embodiments, R^1 is optionally substituted cyclohexyl. In some embodiments, R^1 is optionally substituted cyclopentyl. In some embodiments, R^1 is optionally substituted cyclobutyl. In some embodiments, R^1 is an optionally substituted cyclopropyl. In some embodiments, R^1 is optionally substituted bicyclic carbocyclyl.

[00336] In some embodiments, R^1 is an optionally substituted C_1–C_{50} polycyclic hydrocarbon. In some embodiments, R^1 is an optionally substituted C_1–C_{50} polycyclic hydrocarbon wherein one or more methylene units are optionally and independently replaced by an optionally substituted C_1–C_6 alkylene, C_1–C_6 alkenylene, —C≡C—, –C(R′)$_2$–, –Cy–, –O–, –S–, –S–S–, –N(R′)–, –C(O)–, –C(S)–, –C(NR′)–, –C(O)N(R′)–, –N(R′)C(O)N(R′)-, –N(R′)C(O)–, –N(R′)C(O)O–, –OC(O)N(R′)-, –S(O)–, –S(O)$_2$–, –S(O)$_2$N(R′)–, –N(R′)S(O)$_2$–, –SC(O)–, –C(O)S–, –OC(O)–, or –C(O)O–, wherein each variable is independently as defined above and described herein. In some embodiments, R^1 is optionally substituted . In some embodiments, R^1 is . In some embodiments, R^1 is optionally substituted .

[00337] In some embodiments, R^1 is an optionally substituted C_1–C_{50} aliphatic comprising one or more optionally substituted polycyclic hydrocarbon moieties. In some embodiments, R^1 is an optionally substituted C_1–C_{50} aliphatic comprising one or more optionally substituted polycyclic hydrocarbon moieties, wherein one or more methylene units are optionally and independently replaced by an optionally substituted C_1–C_6 alkylene, C_1–C_6 alkenylene, —C≡C—, –C(R′)$_2$–, –Cy–, –O–, –S–, –S–S–, –N(R′)–, –C(O)–, –C(S)–, –C(NR′)–, –

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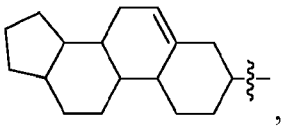
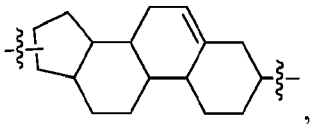
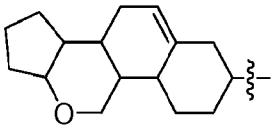
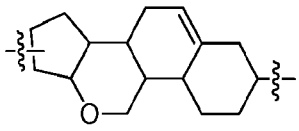
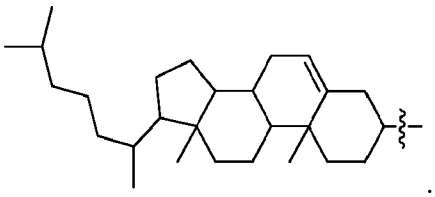
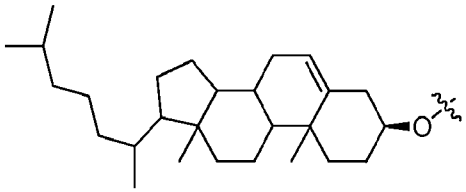
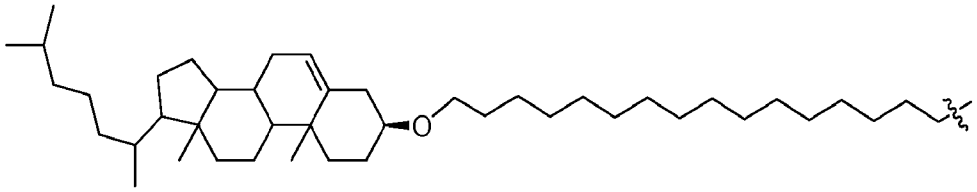
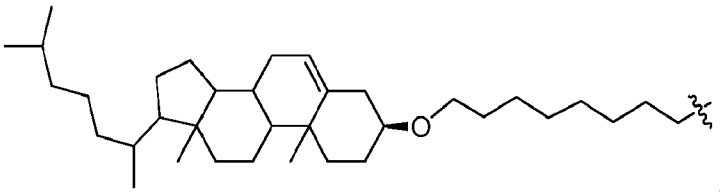
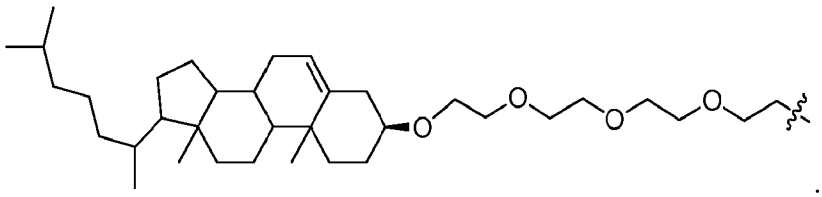
C(O)N(R′)–, –N(R′)C(O)N(R′)-, –N(R′)C(O)–, –N(R′)C(O)O–, –OC(O)N(R′)-, –S(O)–, –S(O)$_2$–, –S(O)$_2$N(R′)–, –N(R′)S(O)$_2$–, –SC(O)–, –C(O)S–, –OC(O)–, or –C(O)O–, wherein each variable is independently as defined above and described herein. In some embodiments, R^1 is an optionally substituted C_1–C_{50} aliphatic comprising one or more optionally substituted , , , or . In some embodiments, R^1 is . In some embodiments, R^1 is . In some embodiments, R^1 is . In some embodiments, R^1 is . In some embodiments, R^1 is .

[00338] In some embodiments, R^1 is an optionally substituted aryl. In some embodiments, R^1 is an optionally substituted bicyclic aryl ring.

[00339] In some embodiments, R^1 is an optionally substituted heteroaryl. In some embodiments, R^1 is an optionally substituted 5-6 membered monocyclic heteroaryl ring having 1-3 heteroatoms independently selected from nitrogen, sulfur, or oxygen. In some embodiments, R^1 is a substituted 5-6 membered monocyclic heteroaryl ring having 1-3 heteroatoms

independently selected from nitrogen, oxygen, or sulfur. In some embodiments, R^1 is an unsubstituted 5-6 membered monocyclic heteroaryl ring having 1-3 heteroatoms independently selected from nitrogen, sulfur, or oxygen.

[00340] In some embodiments, R^1 is an optionally substituted 5 membered monocyclic heteroaryl ring having 1-3 heteroatoms independently selected from nitrogen, oxygen or sulfur. In some embodiments, R^1 is an optionally substituted 6 membered monocyclic heteroaryl ring having 1-3 heteroatoms independently selected from nitrogen, oxygen, or sulfur.

[00341] In some embodiments, R^1 is an optionally substituted 5-membered monocyclic heteroaryl ring having 1 heteroatom selected from nitrogen, oxygen, or sulfur. In some embodiments, R^1 is selected from pyrrolyl, furanyl, or thienyl.

[00342] In some embodiments, R^1 is an optionally substituted 5-membered heteroaryl ring having 2 heteroatoms independently selected from nitrogen, oxygen, or sulfur. In certain embodiments, R^1 is an optionally substituted 5-membered heteroaryl ring having 1 nitrogen atom, and an additional heteroatom selected from sulfur or oxygen. Exemplary R^1 groups include optionally substituted pyrazolyl, imidazolyl, thiazolyl, isothiazolyl, oxazolyl or isoxazolyl.

[00343] In some embodiments, R^1 is a 6-membered heteroaryl ring having 1–3 nitrogen atoms. In other embodiments, R^1 is an optionally substituted 6-membered heteroaryl ring having 1–2 nitrogen atoms. In some embodiments, R^1 is an optionally substituted 6-membered heteroaryl ring having 2 nitrogen atoms. In certain embodiments, R^1 is an optionally substituted 6-membered heteroaryl ring having 1 nitrogen. Exemplary R^1 groups include optionally substituted pyridinyl, pyrimidinyl, pyrazinyl, pyridazinyl, triazinyl, or tetrazinyl.

[00344] In certain embodiments, R^1 is an optionally substituted 8–10 membered bicyclic heteroaryl ring having 1–4 heteroatoms independently selected from nitrogen, oxygen, or sulfur. In some embodiments, R^1 is an optionally substituted 5,6–fused heteroaryl ring having 1–4 heteroatoms independently selected from nitrogen, oxygen, or sulfur. In other embodiments, R^1 is an optionally substituted 5,6–fused heteroaryl ring having 1–2 heteroatoms independently selected from nitrogen, oxygen, or sulfur. In certain embodiments, R^1 is an optionally substituted 5,6–fused heteroaryl ring having 1 heteroatom independently selected from nitrogen, oxygen, or sulfur. In some embodiments, R^1 is an optionally substituted indolyl. In some embodiments, R^1 is an optionally substituted azabicyclo[3.2.1]octanyl. In certain embodiments,

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R^1 is an optionally substituted 5,6–fused heteroaryl ring having 2 heteroatoms independently selected from nitrogen, oxygen, or sulfur. In some embodiments, R^1 is an optionally substituted azaindolyl. In some embodiments, R^1 is an optionally substituted benzimidazolyl. In some embodiments, R^1 is an optionally substituted benzothiazolyl. In some embodiments, R^1 is an optionally substituted benzoxazolyl. In some embodiments, R^1 is an optionally substituted indazolyl. In certain embodiments, R^1 is an optionally substituted 5,6–fused heteroaryl ring having 3 heteroatoms independently selected from nitrogen, oxygen, or sulfur.

[00345] In certain embodiments, R^1 is an optionally substituted 6,6–fused heteroaryl ring having 1–4 heteroatoms independently selected from nitrogen, oxygen, or sulfur. In some embodiments, R^1 is an optionally substituted 6,6–fused heteroaryl ring having 1–2 heteroatoms independently selected from nitrogen, oxygen, or sulfur. In other embodiments, R^1 is an optionally substituted 6,6–fused heteroaryl ring having 1 heteroatom independently selected from nitrogen, oxygen, or sulfur. In some embodiments, R^1 is an optionally substituted quinolinyl. In some embodiments, R^1 is an optionally substituted isoquinolinyl. According to one aspect, R^1 is an optionally substituted 6,6–fused heteroaryl ring having 2 heteroatoms independently selected from nitrogen, oxygen, or sulfur. In some embodiments, R^1 is a quinazoline or a quinoxaline.

[00346] In some embodiments, R^1 is an optionally substituted heterocyclyl. In some embodiments, R^1 is an optionally substituted 3-7 membered saturated or partially unsaturated heterocyclic ring having 1-2 heteroatoms independently selected from nitrogen, oxygen, or sulfur. In some embodiments, R^1 is a substituted 3-7 membered saturated or partially unsaturated heterocyclic ring having 1-2 heteroatoms independently selected from nitrogen, oxygen, or sulfur. In some embodiments, R^1 is an unsubstituted 3-7 membered saturated or partially unsaturated heterocyclic ring having 1-2 heteroatoms independently selected from nitrogen, oxygen, or sulfur.

[00347] In some embodiments, R^1 is an optionally substituted heterocyclyl. In some embodiments, R^1 is an optionally substituted 6 membered saturated or partially unsaturated heterocyclic ring having 1-2 heteroatoms independently selected from nitrogen, oxygen, or sulfur. In some embodiments, R^1 is an optionally substituted 6 membered partially unsaturated heterocyclic ring having 2 heteroatoms independently selected from nitrogen, oxygen, or sulfur.

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In some embodiments, R^1 is an optionally substituted 6 membered partially unsaturated heterocyclic ring having 2 oxygen atoms.

[00348] In certain embodiments, R^1 is a 3-7 membered saturated or partially unsaturated heterocyclic ring having 1-2 heteroatoms independently selected from nitrogen, oxygen, or sulfur. In certain embodiments, R^1 is oxiranyl, oxetanyl, tetrahydrofuranyl, tetrahydropyranyl, oxepaneyl, aziridineyl, azetidineyl, pyrrolidinyl, piperidinyl, azepanyl, thiiranyl, thietanyl, tetrahydrothiophenyl, tetrahydrothiopyranyl, thiepanyl, dioxolanyl, oxathiolanyl, oxazolidinyl, imidazolidinyl, thiazolidinyl, dithiolanyl, dioxanyl, morpholinyl, oxathianyl, piperazinyl, thiomorpholinyl, dithianyl, dioxepanyl, oxazepanyl, oxathiepanyl, dithiepanyl, diazepanyl, dihydrofuranonyl, tetrahydropyranonyl, oxepanonyl, pyrolidinonyl, piperidinonyl, azepanonyl, dihydrothiophenonyl, tetrahydrothiopyranonyl, thiepanonyl, oxazolidinonyl, oxazinanonyl, oxazepanonyl, dioxolanonyl, dioxanonyl, dioxepanonyl, oxathiolinonyl, oxathianonyl, oxathiepanonyl, thiazolidinonyl, thiazinanonyl, thiazepanonyl, imidazolidinonyl, tetrahydropyrimidinonyl, diazepanonyl, imidazolidinedionyl, oxazolidinedionyl, thiazolidinedionyl, dioxolanedionyl, oxathiolanedionyl, piperazinedionyl, morpholinedionyl, thiomorpholinedionyl, tetrahydropyranyl, tetrahydrofuranyl, morpholinyl, thiomorpholinyl, piperidinyl, piperazinyl, pyrrolidinyl, tetrahydrothiophenyl, or tetrahydrothiopyranyl. In some embodiments, R^1 is an optionally substituted 5 membered saturated or partially unsaturated heterocyclic ring having 1-2 heteroatoms independently selected from nitrogen, oxygen, or sulfur.

[00349] In certain embodiments, R^1 is an optionally substituted 5–6 membered partially unsaturated monocyclic ring having 1–2 heteroatoms independently selected from nitrogen, oxygen, or sulfur. In certain embodiments, R^1 is an optionally substituted tetrahydropyridinyl, dihydrothiazolyl, dihydrooxazolyl, or oxazolinyl group.

[00350] In some embodiments, R^1 is an optionally substituted 8-10 membered bicyclic saturated or partially unsaturated heterocyclic ring having 1-4 heteroatoms independently selected from nitrogen, oxygen, or sulfur. In some embodiments, R^1 is an optionally substituted indolinyl. In some embodiments, R^1 is an optionally substituted isoindolinyl. In some embodiments, R^1 is an optionally substituted 1, 2, 3, 4-tetrahydroquinoline. In some embodiments, R^1 is an optionally substituted 1, 2, 3, 4-tetrahydroisoquinoline.

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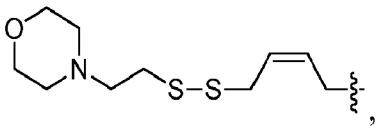
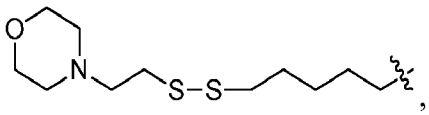
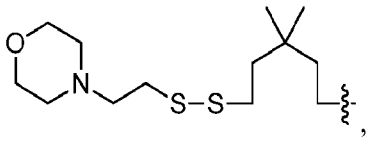
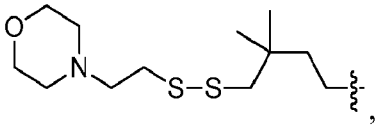
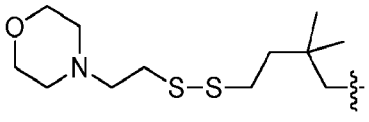
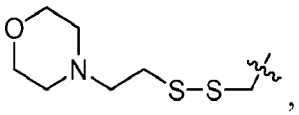
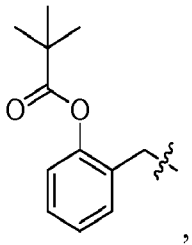
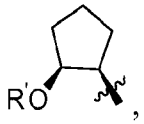
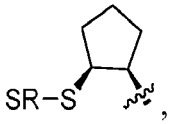
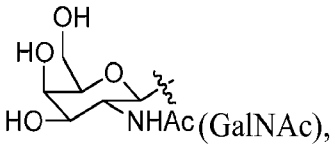
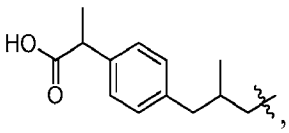
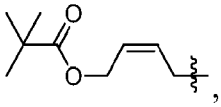
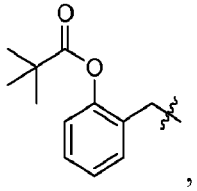
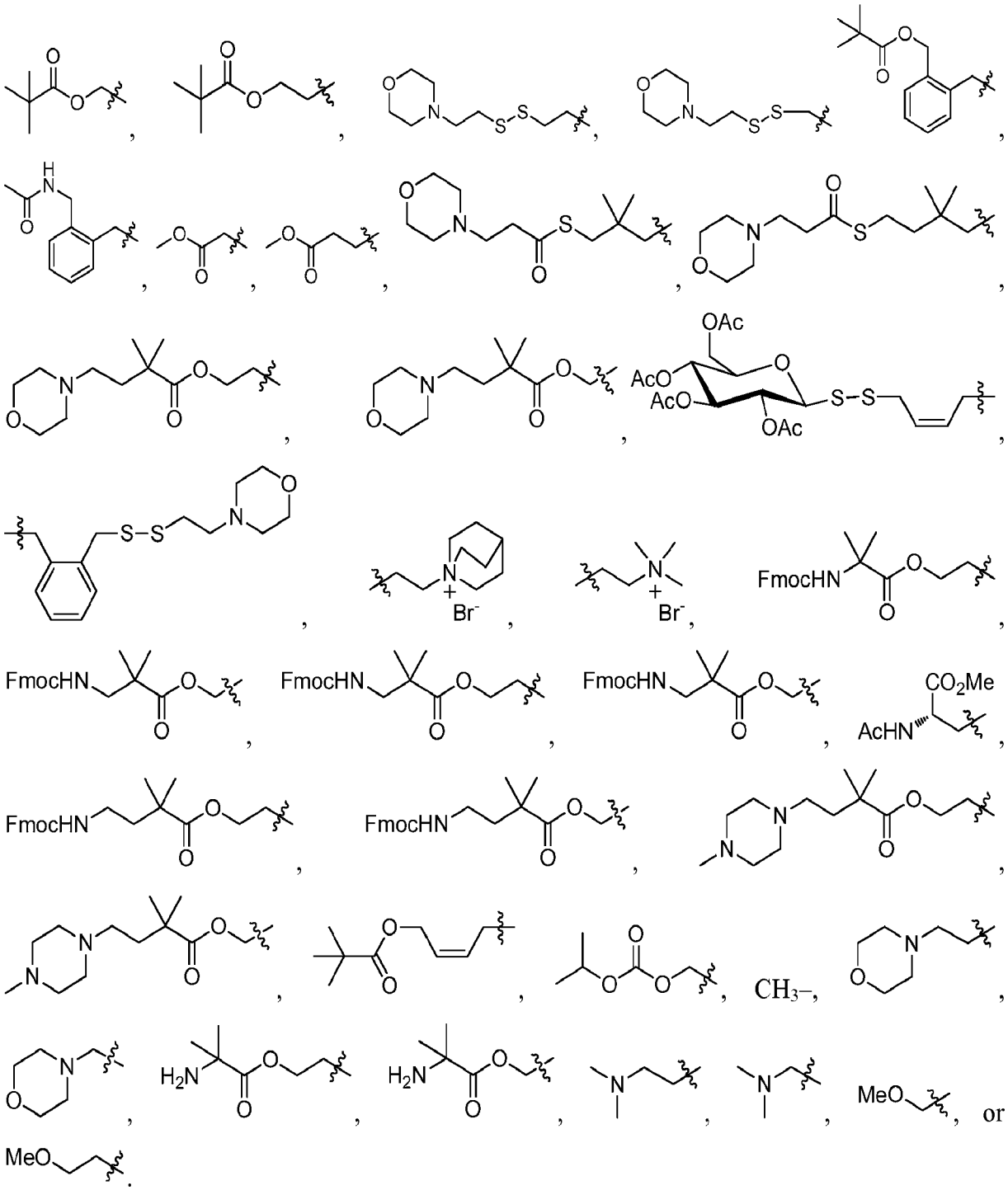
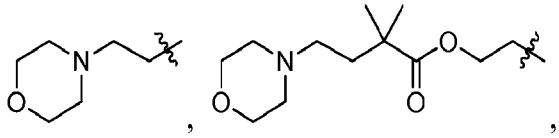
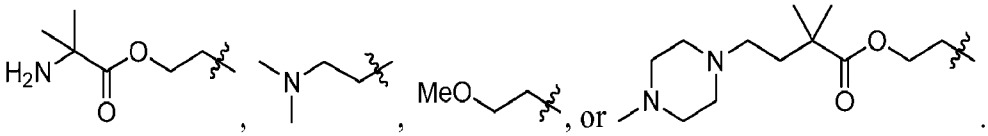
[00351] In some embodiments, R^1 is an optionally substituted C_1–C_{10} aliphatic wherein one or more methylene units are optionally and independently replaced by an optionally substituted C_1–C_6 alkylene, C_1–C_6 alkenylene, —C≡C—, –C(R′)$_2$–, –Cy–, –O–, –S–, –S–S–, –N(R′)–, –C(O)–, –C(S)–, –C(NR′)–, –C(O)N(R′)–, –N(R′)C(O)N(R′)-, –N(R′)C(O)–, –N(R′)C(O)O–, –OC(O)N(R′)-, –S(O)–, –S(O)$_2$–, –S(O)$_2$N(R′)–, –N(R′)S(O)$_2$–, –SC(O)–, –C(O)S–, –OC(O)–, or –C(O)O–, wherein each variable is independently as defined above and described herein. In some embodiments, R^1 is an optionally substituted C_1–C_{10} aliphatic wherein one or more methylene units are optionally and independently replaced by an optionally–Cy–, –O–, –S–, –S–S–, –N(R′)–, –C(O)–, –C(S)–, –C(NR′)–, –C(O)N(R′)–, –N(R′)C(O)N(R′)-, –N(R′)C(O)–, –N(R′)C(O)O–, –OC(O)N(R′)-, –S(O)–, –S(O)$_2$–, –S(O)$_2$N(R′)–, –N(R′)S(O)$_2$–, –OC(O)–, or –C(O)O–, wherein each R' is independently as defined above and described herein. In some embodiments, R^1 is an optionally substituted C_1–C_{10} aliphatic wherein one or more methylene units are optionally and independently replaced by an optionally–Cy–, –O–, –S–, –S–S–, –N(R′)–, –C(O)–, –OC(O)–, or –C(O)O–, wherein each R' is independently as defined above and described herein.

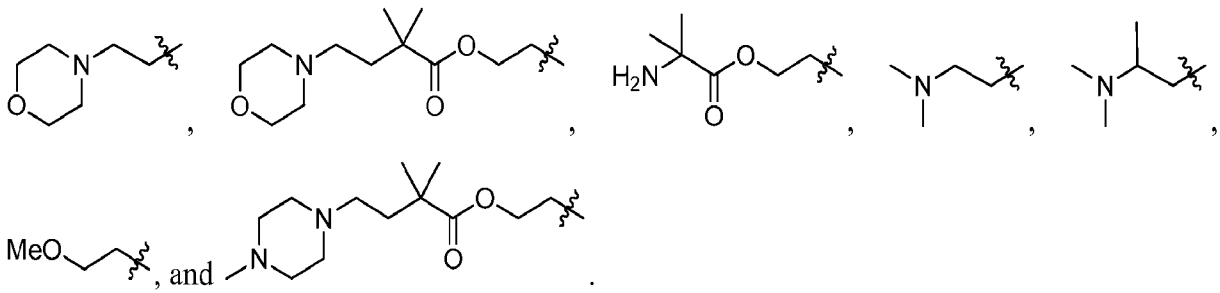
[00352] In some embodiments, R^1 is , , , , , , , , , , , , .



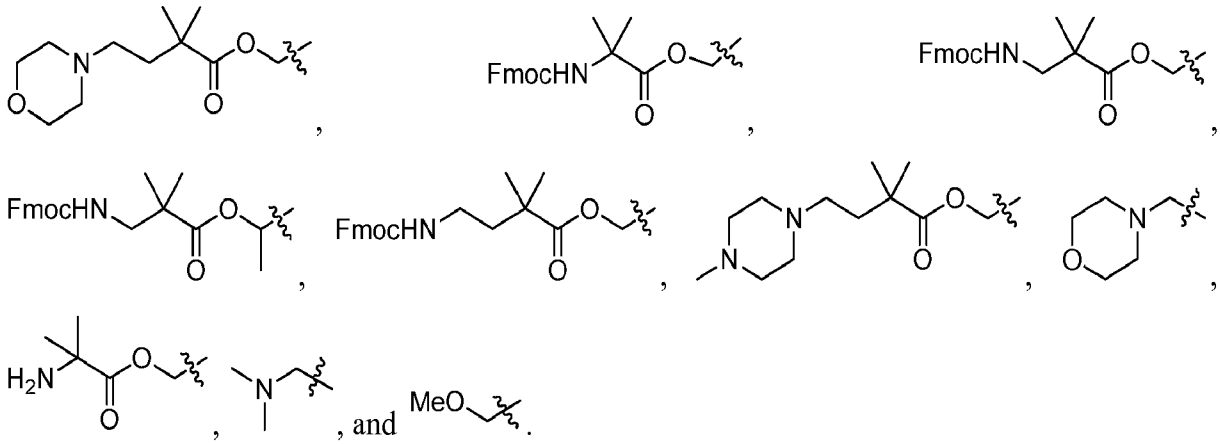
[00353] In some embodiments, R^1 is CH_3–, 



[00354] In some embodiments, R^1 comprises a terminal optionally substituted $-(CH_2)_2-$ moiety which is connected to L. Exemplary such R^1 groups are depicted below:



[00355] In some embodiments, R^1 comprises a terminal optionally substituted $-(CH_2)-$ moiety which is connected to L. Ecemplary such R^1 groups are depicted below:



[00356] In some embodiments, R^1 is $-S-R^{L2}$, wherein R^{L2} is an optionally substituted C_1-C_9 aliphatic wherein one or more methylene units are optionally and independently replaced by an optionally substituted C_1-C_6 alkylene, C_1-C_6 alkenylene, $-C\equiv C-$, $-C(R')_2-$, $-Cy-$, $-O-$, $-S-$, $-S-S-$, $-N(R')-$, $-C(O)-$, $-C(S)-$, $-C(NR')-$, $-C(O)N(R')-$, $-N(R')C(O)N(R')-$, $-N(R')C(O)-$, $-N(R')C(O)O-$, $-OC(O)N(R')-$, $-S(O)-$, $-S(O)_2-$, $-S(O)_2N(R')-$, $-N(R')S(O)_2-$, $-SC(O)-$, $-C(O)S-$, $-OC(O)-$, or $-C(O)O-$, and each of R' and $-Cy-$ is independently as defined above and described herein. In some embodiments, R^1 is $-S-R^{L2}$, wherein the sulfur atom is connected with the sulfur atom in L group.

[00357] In some embodiments, R^1 is $-C(O)-R^{L2}$, wherein R^{L2} is an optionally substituted C_1-C_9 aliphatic wherein one or more methylene units are optionally and independently replaced by an optionally substituted C_1-C_6 alkylene, C_1-C_6 alkenylene, $-C\equiv C-$, $-C(R')_2-$, $-Cy-$, $-O-$, $-S-$, $-S-S-$, $-N(R')-$, $-C(O)-$, $-C(S)-$, $-C(NR')-$, $-C(O)N(R')-$, $-N(R')C(O)N(R')-$, $-N(R')C(O)-$, $-N(R')C(O)O-$, $-OC(O)N(R')-$, $-S(O)-$, $-S(O)_2-$, $-S(O)_2N(R')-$, $-N(R')S(O)_2-$, $-SC(O)-$, $-C(O)S-$, $-OC(O)-$, or $-C(O)O-$, and each of R' and $-Cy-$ is independently as defined

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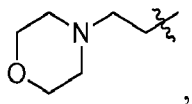
above and described herein. In some embodiments, R^1 is $-C(O)-R^{L2}$, wherein the carbonyl group is connected with G in L group. In some embodiments, R^1 is $-C(O)-R^{L2}$, wherein the carbonyl group is connected with the sulfur atom in L group.

[00358] In some embodiments, R^{L2} is optionally substituted C_1–C_9 aliphatic. In some embodiments, R^{L2} is optionally substituted C_1–C_9 alkyl. In some embodiments, R^{L2} is optionally substituted C_1–C_9 alkenyl. In some embodiments, R^{L2} is optionally substituted C_1–C_9 alkynyl. In some embodiments, R^{L2} is an optionally substituted C_1–C_9 aliphatic wherein one or more methylene units are optionally and independently replaced by –Cy– or –C(O)–. In some embodiments, R^{L2} is an optionally substituted C_1–C_9 aliphatic wherein one or more methylene units are optionally and independently replaced by –Cy–. In some embodiments, R^{L2} is an optionally substituted C_1–C_9 aliphatic wherein one or more methylene units are optionally and independently replaced by an optionally substituted heterocycylene. In some embodiments, R^{L2} is an optionally substituted C_1–C_9 aliphatic wherein one or more methylene units are optionally and independently replaced by an optionally substituted arylene. In some embodiments, R^{L2} is an optionally substituted C_1–C_9 aliphatic wherein one or more methylene units are optionally and independently replaced by an optionally substituted heteroarylene. In some embodiments, R^{L2} is an optionally substituted C_1–C_9 aliphatic wherein one or more methylene units are optionally and independently replaced by an optionally substituted C_3-C_{10} carbocyclylene. In some embodiments, R^{L2} is an optionally substituted C_1–C_9 aliphatic wherein two methylene units are optionally and independently replaced by –Cy– or –C(O)–. In some embodiments, R^{L2} is an optionally substituted C_1–C_9 aliphatic wherein two methylene units are optionally and independently replaced by –Cy– or –C(O)–. Exemplary R^{L2} groups are depicted below: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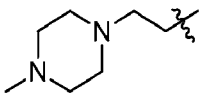
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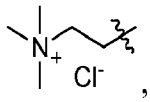
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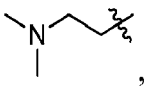
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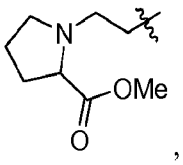
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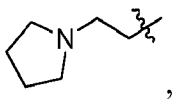
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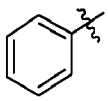
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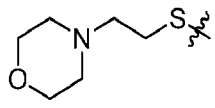
[00359] In some embodiments, R^1 is hydrogen, or an optionally substituted group selected from

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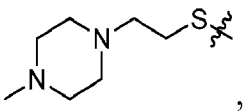
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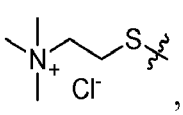
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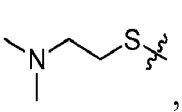
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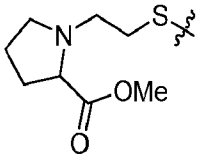
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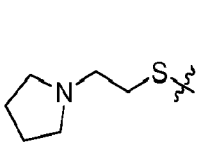
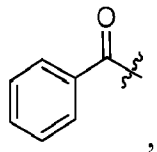
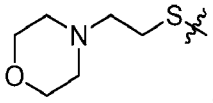
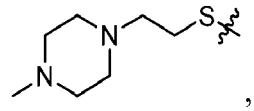


, , –S–(C_1–C_{10} aliphatic), C_1–C_{10} aliphatic, aryl, C_1–C_6 heteroalkyl, heteroaryl and heterocyclyl. In some embodiments, R^1 is 

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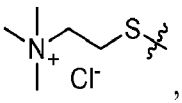
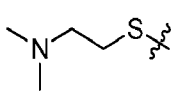
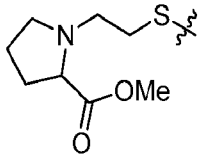
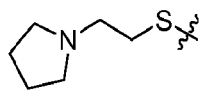
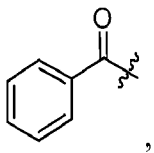
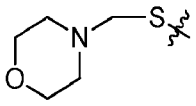
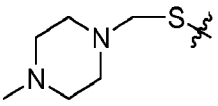
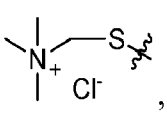





, or –S–(C_1–C_{10} aliphatic). In some embodiments, R^1 is 


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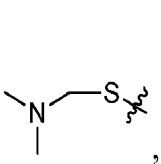
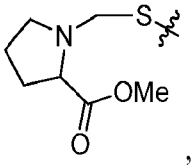
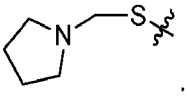
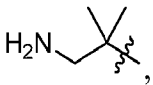
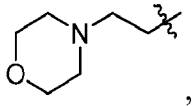
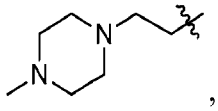


, or 
.

[00360] In some embodiments, R^1 is an optionally substituted group selected from –S–(C_1-C_6 aliphatic), C_1-C_{10} aliphatic, C_1-C_6 heteroaliphatic, aryl, heterocyclyl and heteroaryl.

[00361] In some embodiments, R^1 is 


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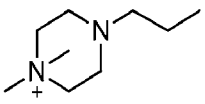
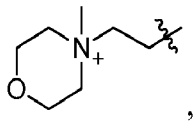
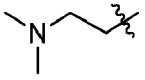
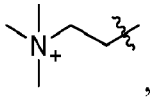
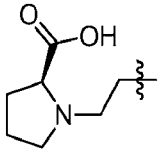
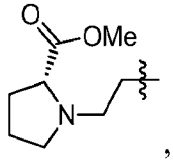
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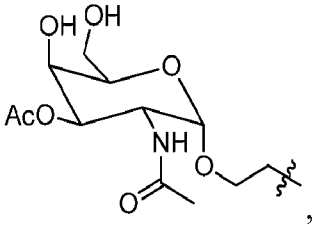
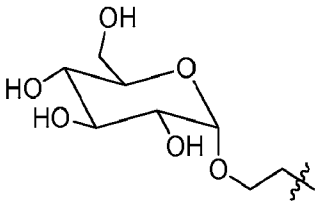
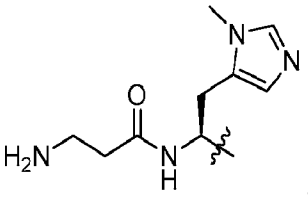



, or .

[00362] In some embodiments, the sulfur atom in the R^1 embodiments described above and herein is connected with the sulfur atom, G, E, or –C(O)– moiety in the L embodiments described above and herein. In some embodiments, the –C(O)– moiety in the R^1 embodiments described above and herein is connected with the sulfur atom, G, E, or –C(O)– moiety in the L embodiments described above and herein.

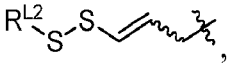
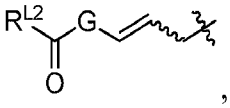
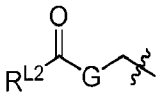
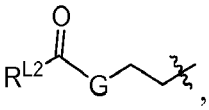
[00363] In some embodiments, $-L-R^1$ is any combination of the L embodiments and R^1 embodiments described above and herein.

[00364] In some embodiments, $-L-R^1$ is $-L^3-G-R^1$ wherein each variable is independently as defined above and described herein.

[00365] In some embodiments, $-L-R^1$ is $-L^4-G-R^1$ wherein each variable is independently as defined above and described herein.

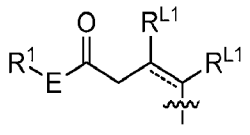
[00366] In some embodiments, $-L-R^1$ is $-L^3-G-S-R^{L2}$, wherein each variable is independently as defined above and described herein.

[00367] In some embodiments, $-L-R^1$ is $-L^3-G-C(O)-R^{L2}$, wherein each variable is independently as defined above and described herein.

[00368] In some embodiments, $-L-R^1$ is , , , or , wherein R^{L2} is an optionally substituted C_1–C_9 aliphatic wherein one or more methylene units are optionally and independently replaced by an optionally substituted C_1–C_6 alkylene, C_1–C_6 alkenylene, —C≡C—, $-C(R')_2-$, $-Cy-$, $-O-$, $-S-$, $-S-S-$, $-N(R')-$, $-C(O)-$, $-C(S)-$, $-C(NR')-$, $-C(O)N(R')-$, $-N(R')C(O)N(R')-$, $-N(R')C(O)-$, $-N(R')C(O)O-$, $-OC(O)N(R')-$, $-S(O)-$, $-S(O)_2-$, $-S(O)_2N(R')-$, $-N(R')S(O)_2-$, $-SC(O)-$, $-C(O)S-$, $-OC(O)-$, or $-C(O)O-$, and each G is independently as defined above and described herein.

[00369] In some embodiments, $-L-R^1$ is $-R^{L3}-S-S-R^{L2}$, wherein each variable is independently as defined above and described herein. In some embodiments, $-L-R^1$ is $-R^{L3}-C(O)-S-S-R^{L2}$, wherein each variable is independently as defined above and described herein.

[00370] In some embodiments, $-L-R^1$ has the structure of:



wherein each variable is independently as defined above and described herein.

[00371] In some embodiments, $-L-R^1$ has the structure of:

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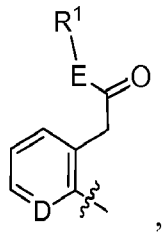
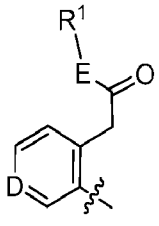


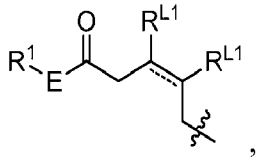
wherein each variable is independently as defined above and described herein.

[00372] In some embodiments, –L–R^1 has the structure of:



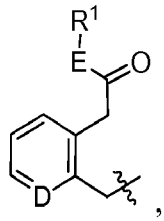
wherein each variable is independently as defined above and described herein.

[00373] In some embodiments, –L–R^1 has the structure of:



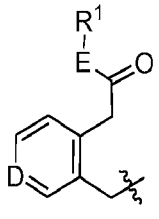
wherein each variable is independently as defined above and described herein.

[00374] In some embodiments, –L–R^1 has the structure of:



wherein each variable is independently as defined above and described herein.

[00375] In some embodiments, –L–R^1 has the structure of:



wherein each variable is independently as defined above and described herein.

[00376] In some embodiments, –L–R^1 has the structure of:

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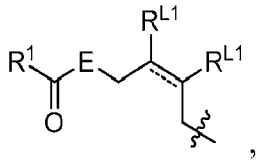
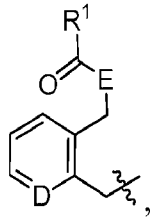


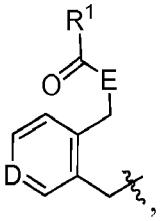
wherein each variable is independently as defined above and described herein.

[00377] In some embodiments, –L–R^1 has the structure of:



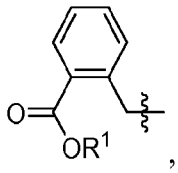
wherein each variable is independently as defined above and described herein.

[00378] In some embodiments, –L–R^1 has the structure of:



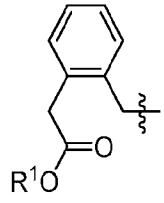
wherein each variable is independently as defined above and described herein.

[00379] In some embodiments, –L–R^1 has the structure of:



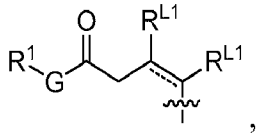
wherein each variable is independently as defined above and described herein.

[00380] In some embodiments, –L–R^1 has the structure of:



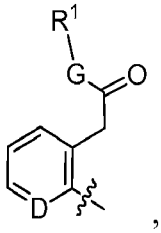
wherein each variable is independently as defined above and described herein.

[00381] In some embodiments, –L–R^1 has the structure of:



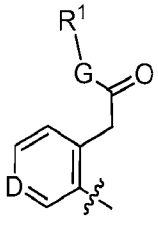
wherein each variable is independently as defined above and described herein.

[00382] In some embodiments, –L–R^1 has the structure of:



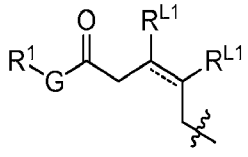
wherein each variable is independently as defined above and described herein.

[00383] In some embodiments, –L–R^1 has the structure of:



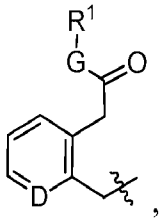
wherein each variable is independently as defined above and described herein.

[00384] In some embodiments, –L–R^1 has the structure of:



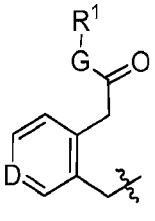
wherein each variable is independently as defined above and described herein.

[00385] In some embodiments, –L–R^1 has the structure of:



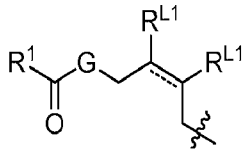
wherein each variable is independently as defined above and described herein.

[00386] In some embodiments, –L–R^1 has the structure of:



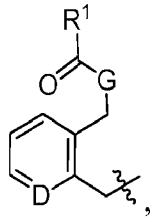
wherein each variable is independently as defined above and described herein.

[00387] In some embodiments, –L–R^1 has the structure of:



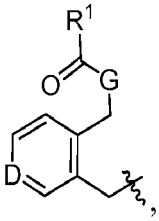
wherein each variable is independently as defined above and described herein.

[00388] In some embodiments, –L–R^1 has the structure of:



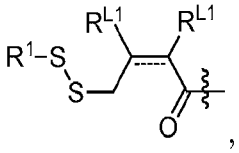
wherein each variable is independently as defined above and described herein.

[00389] In some embodiments, –L–R^1 has the structure of:



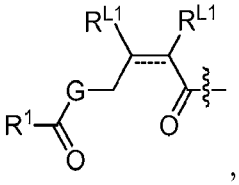
wherein each variable is independently as defined above and described herein.

[00390] In some embodiments, –L–R^1 has the structure of:



wherein each variable is independently as defined above and described herein.

[00391] In some embodiments, L has the structure of:



wherein each variable is independently as defined above and described herein.

[00392] In some embodiments, –X–L–R^1 has the structure of:

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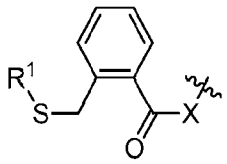
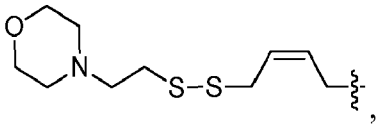
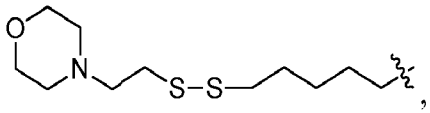
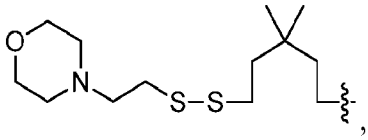
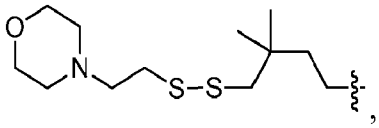
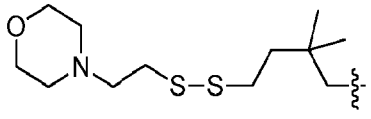
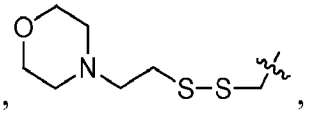
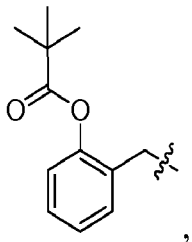
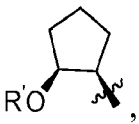
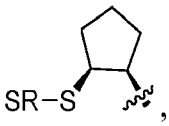
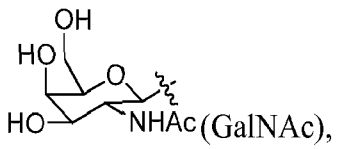
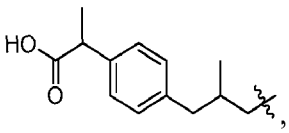
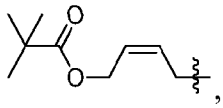
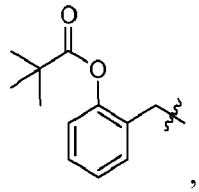
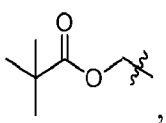
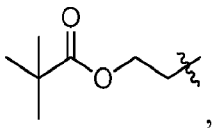
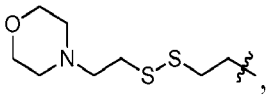
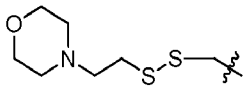
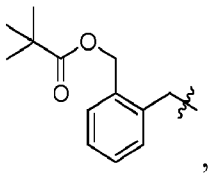
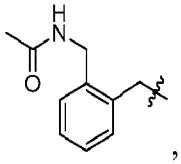
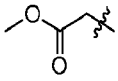
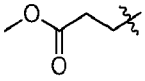
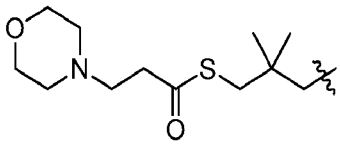
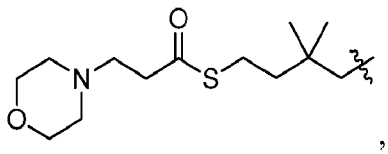
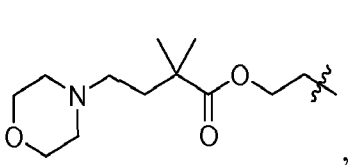
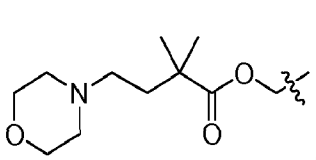
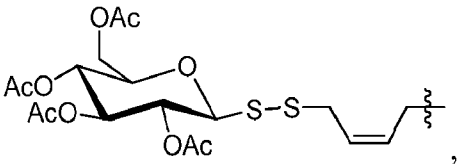
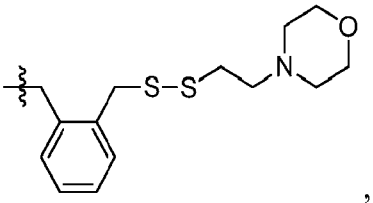
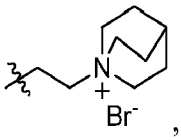
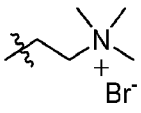
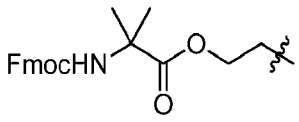
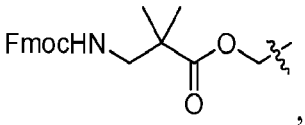
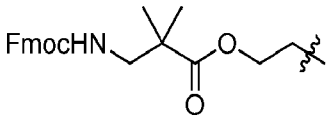
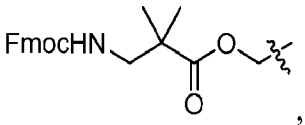
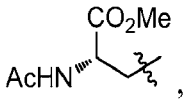
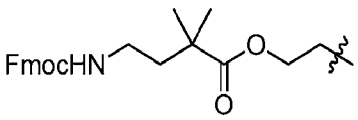
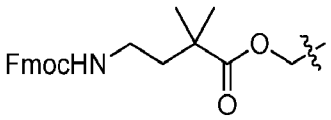
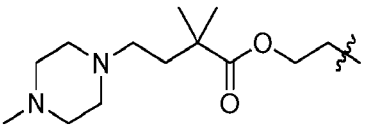
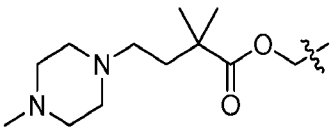
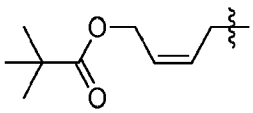
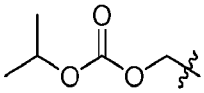


wherein:

the phenyl ring is optionally substituted, and

each of R^1 and X is independently as defined above and described herein.

[00393] In some embodiments, $-L-R^1$ is , , , , , , , , , , , , , , , , , , , , , , , , , .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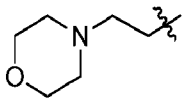
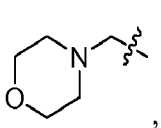
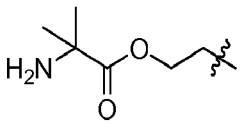
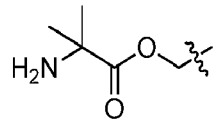
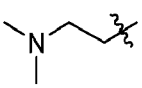
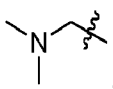
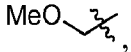
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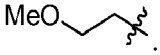
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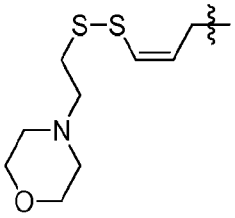
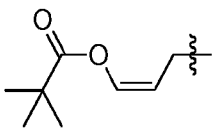
[00394] In some embodiments, –L–R^1 is: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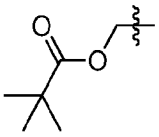
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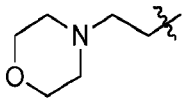
[00395] In some embodiments, –L–R^1 is CH_3–,

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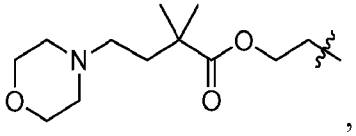
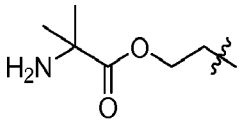
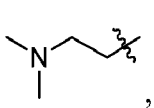
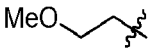
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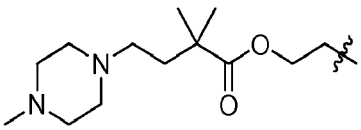
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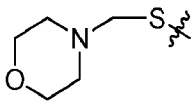
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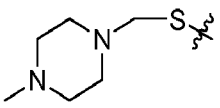
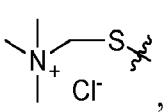
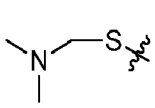
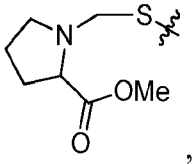
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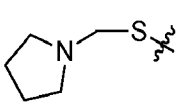
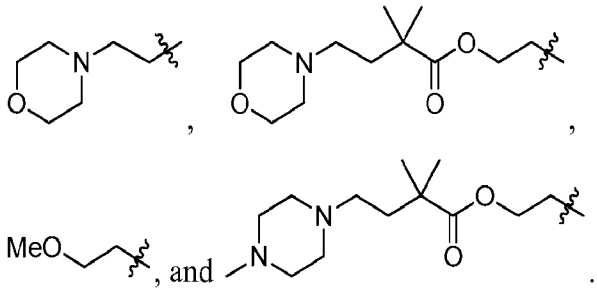
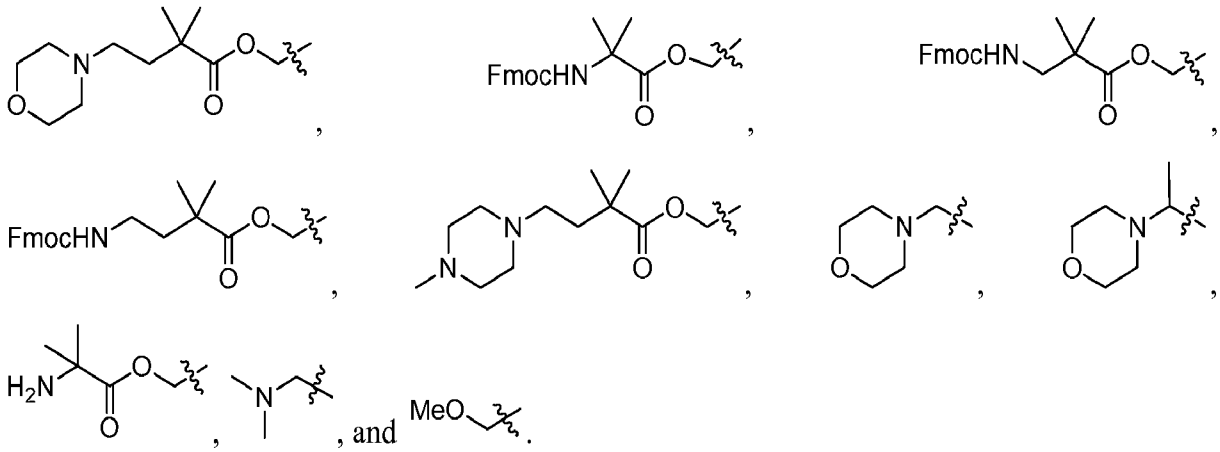

.

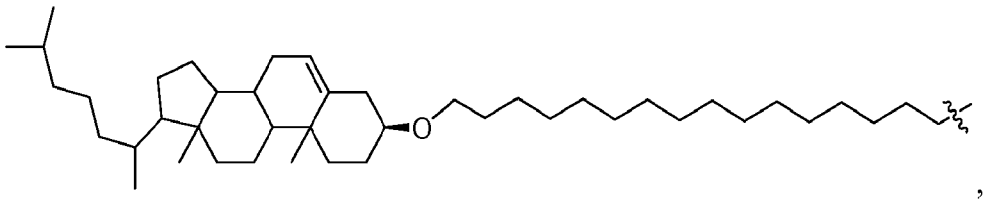
[00396] In some embodiments, –L–R^1 comprises a terminal optionally substituted –$(CH_2)_2$– moiety which is connected to X. In some embodiments, –L–R^1 comprises a terminal –$(CH_2)_2$– moiety which is connected to X. Exemplary such –L–R^1 moieties are depicted below:

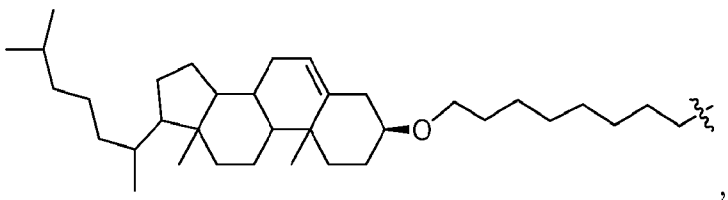


[00397] In some embodiments, –L–R^1 comprises a terminal optionally substituted –(CH_2)– moiety which is connected to X. In some embodiments, –L–R^1 comprises a terminal –(CH_2)– moiety which is connected to X. Exemplary such –L–R^1 moieties are depicted below:

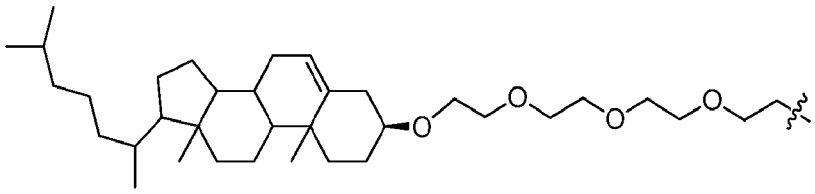


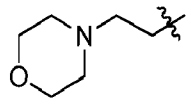
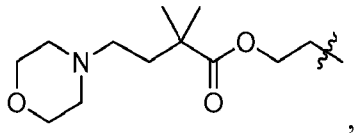
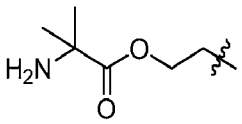
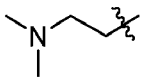
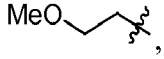
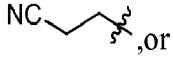
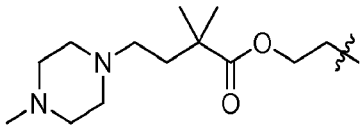
[00398] In some embodiments, –L–R^1 is

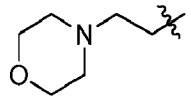
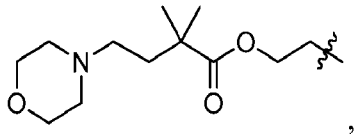
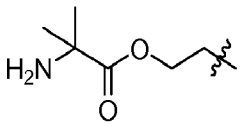
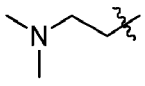
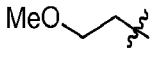
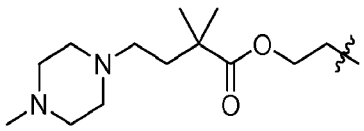


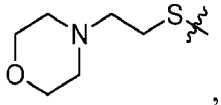
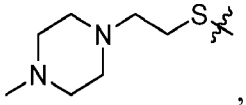
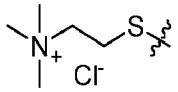
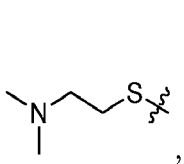
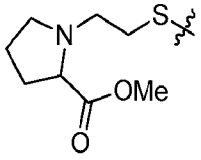
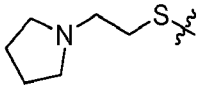
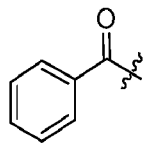


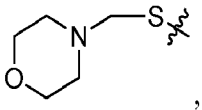
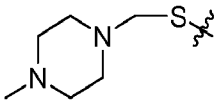
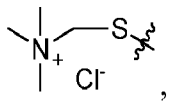
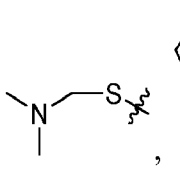
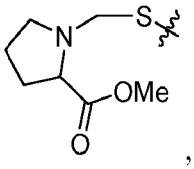
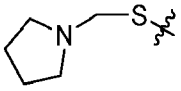
or

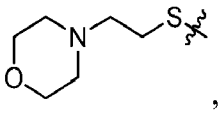
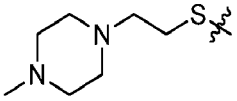
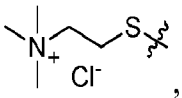
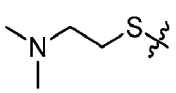
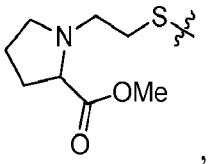
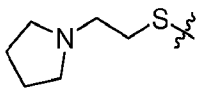
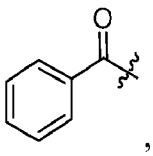


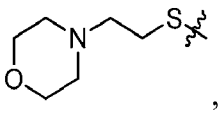
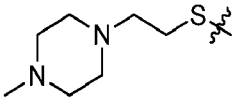
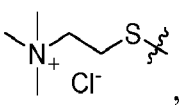
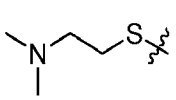
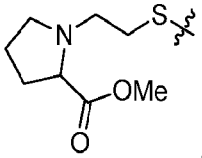
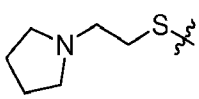
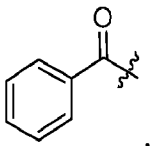
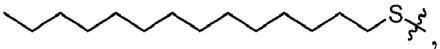
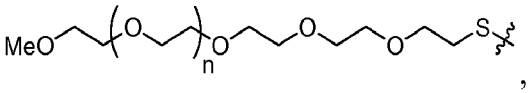
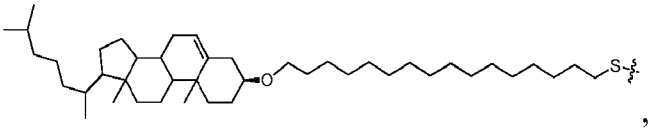
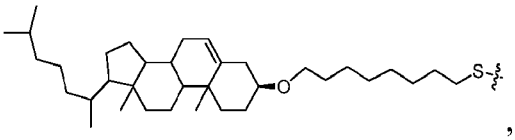
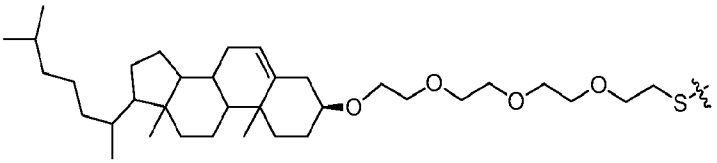
[00399] In some embodiments, –L–R^1 is CH_3–, , , , , , ,or ; and X is –S–.

[00400] In some embodiments, –L–R^1 is CH_3–, , , , , , or , X is –S–, W is O, Y is –O–, and Z is –O–.

[00401] In some embodiments, R^1 is , , , , , , , or –S–(C_1–C_{10} aliphatic).

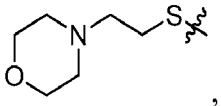
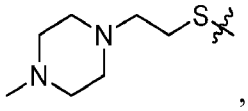
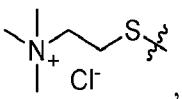
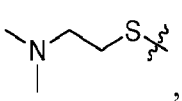
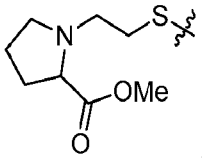
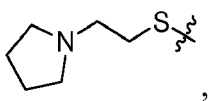
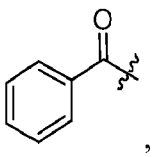
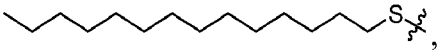
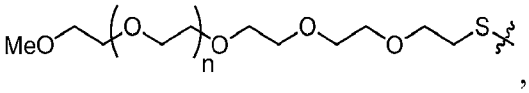
[00402] In some embodiments, R^1 is , , , , , or .

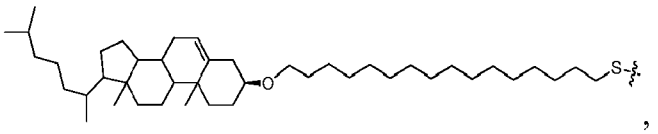
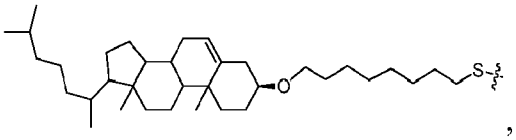
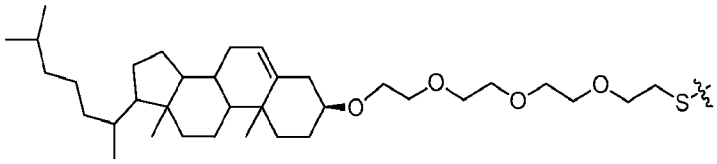
[00403] In some embodiments, X is –O– or –S–, and R^1 is , , , , , , , or –S–(C_1–C_{10} aliphatic).

[00404] In some embodiments, X is –O– or –S–, and R^1 is , , , , , , , , , , , , –S–(C_1–C_{10} aliphatic) or –S–(C_1–C_{50} aliphatic).

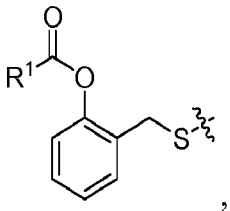
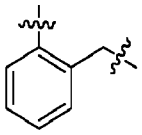
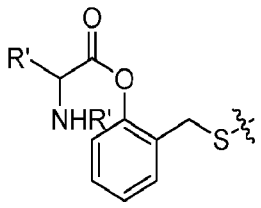
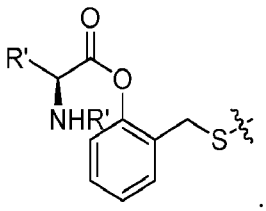
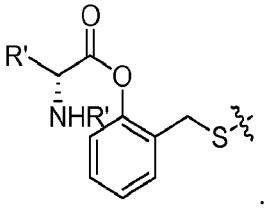
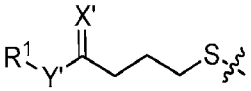
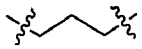
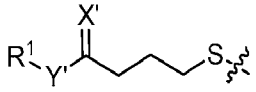
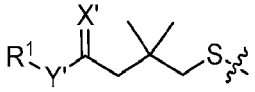
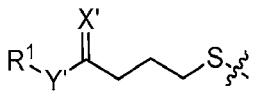
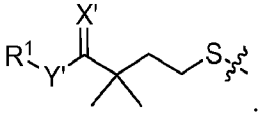
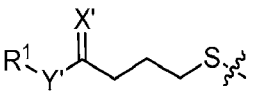
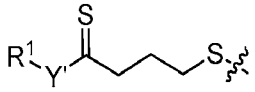
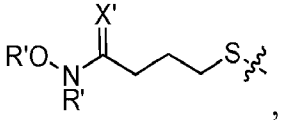
[00405] In some embodiments, L is a covalent bond and –L–R^1 is R^1.

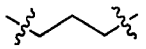
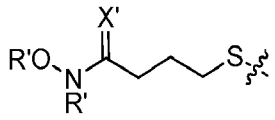
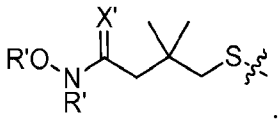
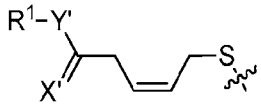
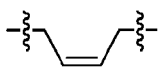
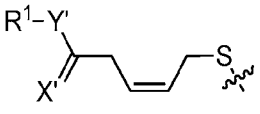
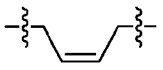
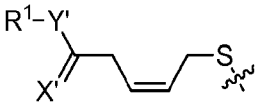
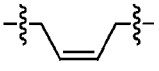
[00406] In some embodiments, –L–R^1 is not hydrogen.

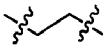
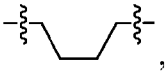
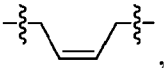
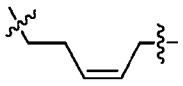
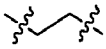
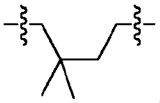
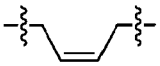
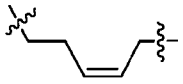
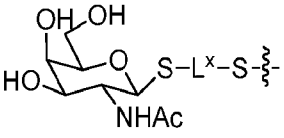
[00407] In some embodiments, –X–L–R^1 is R^1 is , , , , , , , , , , ,

, –S–(C_1–C_{10} aliphatic) or –S–(C_1–C_{50} aliphatic).

[00408] In some embodiments, –X–L–R^1 has the structure of , wherein the  moiety is optionally substituted. In some embodiments, –X–L–R^1 is . In some embodiments, –X–L–R^1 is . In some embodiments, –X–L–R^1 is . In some embodiments, –X–L–R^1 has the structure of , wherein X' is O or S, Y' is –O–, –S– or –NR'–, and the  moiety is optionally substituted. In some embodiments, Y' is –O–, –S– or –NH–. In some embodiments,  is . In some embodiments,  is . In some embodiments,  is . In some embodiments, –X–L–R^1 has the structure of ,

wherein X' is O or S, and the  moiety is optionally substituted. In some embodiments,  is . In some embodiments, –X–L–R^1 is , wherein the  is optionally substituted. In some embodiments, –X–L–R^1 is , wherein the  is substituted. In some embodiments, –X–L–R^1 is , wherein the  is unsubstituted.

[00409] In some embodiments, –X–L–R^1 is R^1–C(O)–S–L^x–S–, wherein L^x is an optionally substituted group selected from , , , , and . In some embodiments, L^x is , , , , and . In some embodiments, –X–L–R^1 is $(CH_3)_3C$–S–S–L^x–S–. In some embodiments, –X–L–R^1 is R^1–C(=X')–Y'–$C(R)_2$–S–L^x–S–. In some embodiments, –X–L–R^1 is R–C(=X')–Y'–CH_2–S–L^x–S–. In some embodiments, –X–L–R^1 is .

[00410] As will be appreciated by a person skilled in the art, many of the –X–L–R^1 groups described herein are cleavable and can be converted to $–X^-$ after administration to a subject. In some embodiments, –X–L–R^1 is cleavable. In some embodiments, –X–L–R^1 is –S–L–R^1, and is converted to $–S^-$ after administration to a subject. In some embodiments, the conversion is promoted by an enzyme of a subject. As appreciated by a person skilled in the art, methods of determining whether the –S–L–R^1 group is converted to $–S^-$ after administration is widely known and practiced in the art, including those used for studying drug metabolism and pharmacokinetics.

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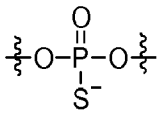
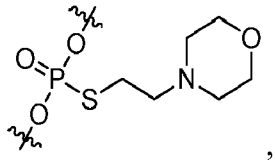
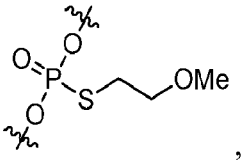
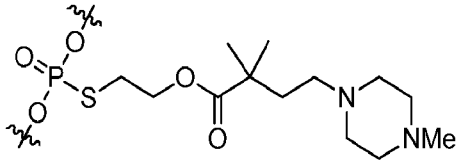
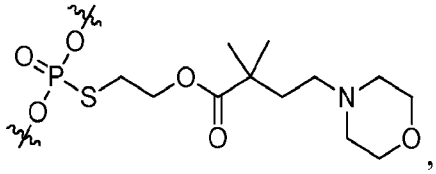
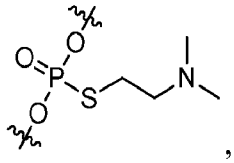
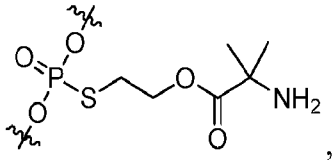
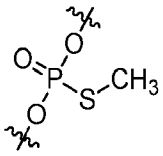
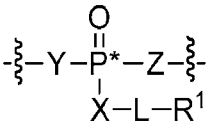
[00411] In some embodiments, the internucleotidic linkage having the structure of formula **I** is , , , , , , , or .

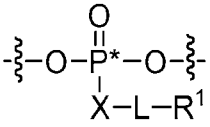
[00412] In some embodiments, the internucleotidic linkage of formula **I** has the structure of formula **I-a**:



(I-a)

wherein each variable is independently as defined above and described herein.

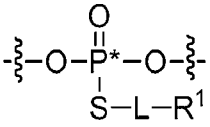
[00413] In some embodiments, the internucleotidic linkage of formula **I** has the structure of formula **I-b**:



(I-b)

wherein each variable is independently as defined above and described herein.

[00414] In some embodiments, the internucleotidic linkage of formula **I** is an phosphorothioate triester linkage having the structure of formula **I-c**:



(I-c)

wherein:

P* is an asymmetric phosphorus atom and is either *R*p or *S*p;

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L is a covalent bond or an optionally substituted, linear or branched C_1–C_{10} alkylene, wherein one or more methylene units of L are optionally and independently replaced by an optionally substituted C_1–C_6 alkylene, C_1–C_6 alkenylene, —C≡C—, –C(R′)$_2$–, –Cy–, –O–, –S–, –S–S–, –N(R′)–, –C(O)–, –C(S)–, –C(NR′)–, –C(O)N(R′)–, –N(R′)C(O)N(R′)-, –N(R′)C(O)–, –N(R′)C(O)O–, –OC(O)N(R′)-, –S(O)–, –S(O)$_2$–, –S(O)$_2$N(R′)–, –N(R′)S(O)$_2$–, –SC(O)–, –C(O)S–, –OC(O)–, or –C(O)O–;

R^1 is halogen, R, or an optionally substituted C_1–C_{50} aliphatic wherein one or more methylene units are optionally and independently replaced by an optionally substituted C_1–C_6 alkylene, C_1–C_6 alkenylene, —C≡C—, –C(R′)$_2$–, –Cy–, –O–, –S–, –S–S–, –N(R′)–, –C(O)–, –C(S)–, –C(NR′)–, –C(O)N(R′)–, –N(R′)C(O)N(R′)-, –N(R′)C(O)–, –N(R′)C(O)O–, –OC(O)N(R′)-, –S(O)–, –S(O)$_2$–, –S(O)$_2$N(R′)–, –N(R′)S(O)$_2$–, –SC(O)–, –C(O)S–, –OC(O)–, or –C(O)O–;

each R′ is independently –R, -C(O)R, -CO_2R, or –SO_2R, or:

two R′ on the same nitrogen are taken together with their intervening atoms to form an optionally substituted heterocyclic or heteroaryl ring, or

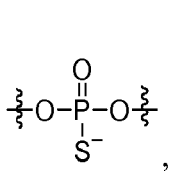
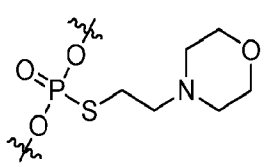
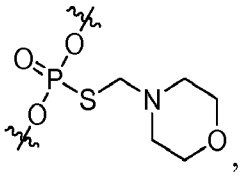
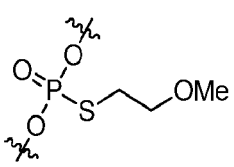
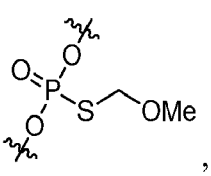
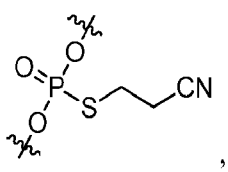
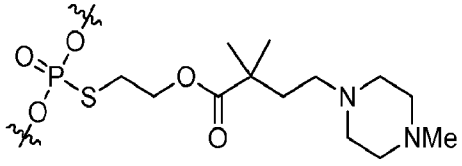
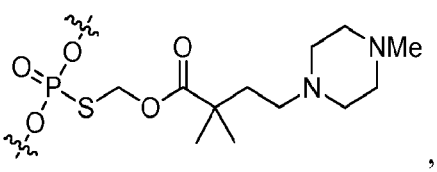
two R′ on the same carbon are taken together with their intervening atoms to form an optionally substituted aryl, carbocyclic, heterocyclic, or heteroaryl ring;

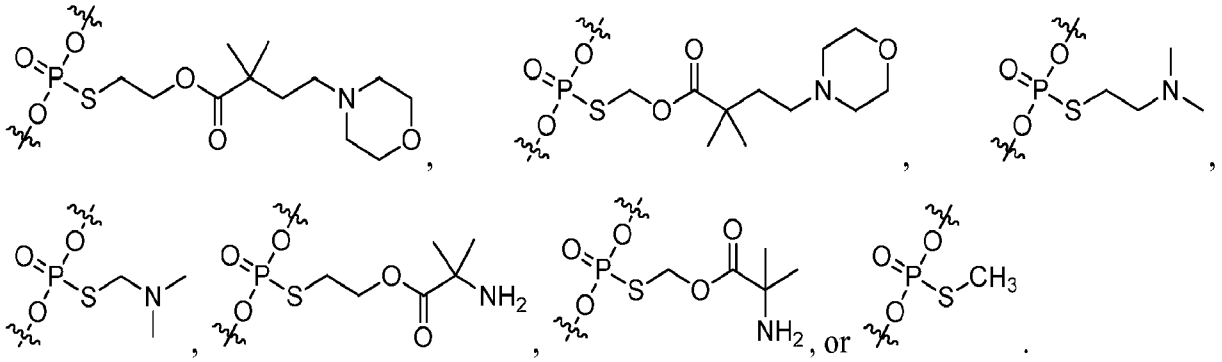
–Cy– is an optionally substituted bivalent ring selected from phenylene, carbocyclylene, arylene, heteroarylene, or heterocyclylene;

each R is independently hydrogen, or an optionally substituted group selected from C_1–C_6 aliphatic, phenyl, carbocyclyl, aryl, heteroaryl, or heterocyclyl;

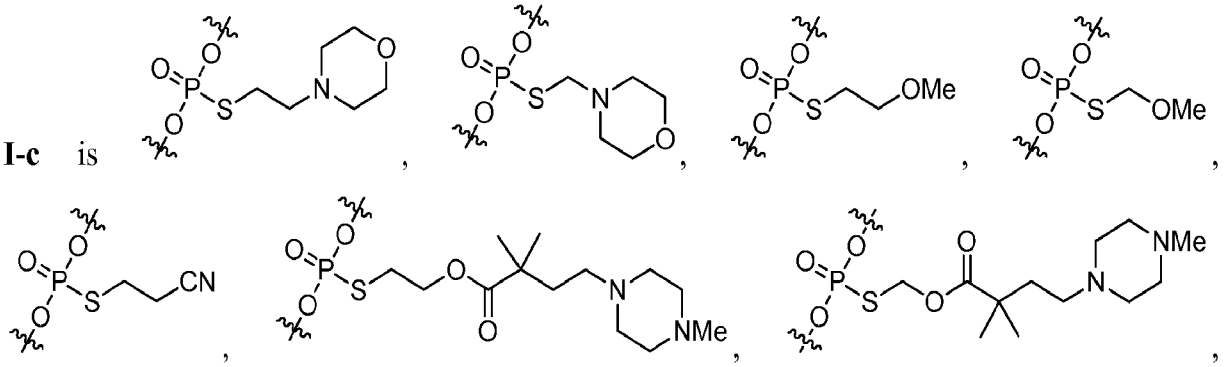
each ~ independently represents a connection to a nucleoside; and

R^1 is not –H when L is a covalent bond.

[00415] In some embodiments, the internucleotidic linkage having the structure of formula **I** is , , , , , , , ,



[00416] In some embodiments, the internucleotidic linkage having the structure of formula **I-c** is



[00417] In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising one or more phosphate diester linkages, and one or more modified internucleotide linkages having the formula of **I-a**, **I-b**, or **I-c**.

[00418] In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising at least one phosphate diester internucleotidic linkage and at least one phosphorothioate triester linkage having the structure of formula **I-c**. In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising at least one phosphate diester internucleotidic linkage and at least two phosphorothioate triester linkages having the structure of formula **I-c**. In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising at least one phosphate diester internucleotidic linkage and at least three phosphorothioate triester linkages having the structure of formula **I-c**.

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In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising at least one phosphate diester internucleotidic linkage and at least four phosphorothioate triester linkages having the structure of formula **I-c**. In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising at least one phosphate diester internucleotidic linkage and at least five phosphorothioate triester linkages having the structure of formula **I-c**.

[00419] In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising a sequence found in any of the Appendixes of the application. In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising a sequence found in Appendix A. In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising a sequence found in Appendix B. In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising a sequence found in Appendix C. In some embodiments, the present invention provides a chirally controlled oligonucleotide having a sequence found in any of the Appendixes of the application. In some embodiments, the present invention provides a chirally controlled oligonucleotide having a sequence found in Appendix A. In some embodiments, the present invention provides a chirally controlled oligonucleotide having a sequence found in Appendix B. In some embodiments, the present invention provides a chirally controlled oligonucleotide having a sequence found in Appendix C.

[00420] In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising a sequence found in GCCTCAGTCTGCTTCGCACC. In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising a sequence found in GCCTCAGTCTGCTTCGCACC, wherein the said sequence has over 50% identity with GCCTCAGTCTGCTTCGCACC. In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising a sequence found in GCCTCAGTCTGCTTCGCACC, wherein the said sequence has over 60% identity with GCCTCAGTCTGCTTCGCACC. In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising a sequence found in GCCTCAGTCTGCTTCGCACC, wherein the said sequence has over 70% identity with GCCTCAGTCTGCTTCGCACC. In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising a sequence found in

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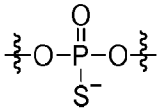
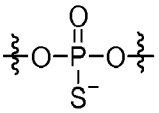
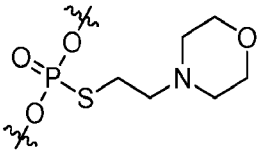
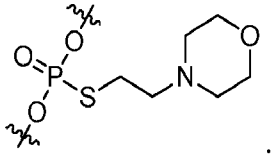
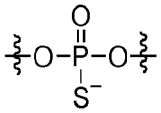
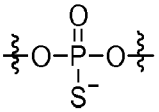
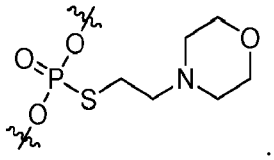
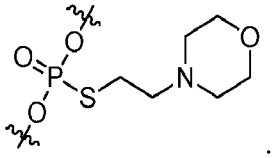
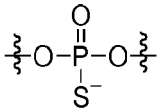
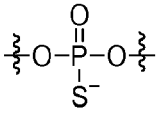
GCCTCAGTCTGCTTCGCACC, wherein the said sequence has over 80% identity with GCCTCAGTCTGCTTCGCACC. In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising a sequence found in GCCTCAGTCTGCTTCGCACC, wherein the said sequence has over 90% identity with GCCTCAGTCTGCTTCGCACC. In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising a sequence found in GCCTCAGTCTGCTTCGCACC, wherein the said sequence has over 95% identity with GCCTCAGTCTGCTTCGCACC. In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising the sequence of GCCTCAGTCTGCTTCGCACC. In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC.

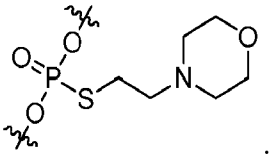
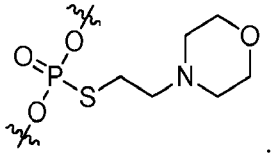
[00421] In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising a sequence found in GCCTCAGTCTGCTTCGCACC, wherein at least one internucleotidic linkage has a chiral linkage phosphorus. In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising a sequence found in GCCTCAGTCTGCTTCGCACC, wherein at least one internucleotidic linkage has the structure of formula **I**. In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising a sequence found in GCCTCAGTCTGCTTCGCACC, wherein each internucleotidic linkage has the structure of formula **I**. In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising a sequence found in GCCTCAGTCTGCTTCGCACC, wherein at least one internucleotidic linkage has the structure of formula **I-c**. In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising a sequence found in GCCTCAGTCTGCTTCGCACC, wherein each internucleotidic linkage has the structure of formula **I-c**. In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising a sequence found in GCCTCAGTCTGCTTCGCACC, wherein at least one internucleotidic linkage is . In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising a sequence found in GCCTCAGTCTGCTTCGCACC, wherein each internucleotidic

linkage is  . In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising a sequence found in GCCTCAGTCTGCTTCGCACC, wherein at least one internucleotidic linkage is  . In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising a sequence found in GCCTCAGTCTGCTTCGCACC, wherein each internucleotidic linkage is  .

[00422] In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising the sequence of GCCTCAGTCTGCTTCGCACC, wherein at least one internucleotidic linkage has a chiral linkage phosphorus. In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising the sequence of GCCTCAGTCTGCTTCGCACC, wherein at least one internucleotidic linkage has the structure of formula **I**. In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising the sequence of GCCTCAGTCTGCTTCGCACC, wherein each internucleotidic linkage has the structure of formula **I**. In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising the sequence of GCCTCAGTCTGCTTCGCACC, wherein at least one internucleotidic linkage has the structure of formula **I-c**. In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising the sequence of GCCTCAGTCTGCTTCGCACC, wherein each internucleotidic linkage has the structure of formula **I-c**. In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising the sequence of GCCTCAGTCTGCTTCGCACC, wherein at least one internucleotidic linkage is  . In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising the sequence of GCCTCAGTCTGCTTCGCACC, wherein each internucleotidic linkage is  . In some embodiments, the present invention provides a chirally

controlled oligonucleotide comprising the sequence of GCCTCAGTCTGCTTCGCACC, wherein at least one internucleotidic linkage is . In some embodiments, the present invention provides a chirally controlled oligonucleotide comprising the sequence of GCCTCAGTCTGCTTCGCACC, wherein each internucleotidic linkage is .

[00423] In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC, wherein at least one internucleotidic linkage has a chiral linkage phosphorus. In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC, wherein at least one internucleotidic linkage has the structure of formula **I**. In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC, wherein each internucleotidic linkage has the structure of formula **I**. In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC, wherein at least one internucleotidic linkage has the structure of formula **I-c**. In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC, wherein each internucleotidic linkage has the structure of formula **I-c**. In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC, wherein at least one internucleotidic linkage is . In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC, wherein each internucleotidic linkage is . In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC, wherein at least one

internucleotidic linkage is . In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC, wherein each internucleotidic linkage is .

[00424] In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC, wherein at least one linkage phosphorus is *R*p. It is understood by a person of ordinary skill in the art that in certain embodiments wherein the chirally controlled oligonucleotide comprises an RNA sequence, each T is independently and optionally replaced with U. In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC, wherein each linkage phosphorus is *R*p. In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC, wherein at least one linkage phosphorus is *S*p. In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC, wherein each linkage phosphorus is *S*p. In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC, wherein the oligonucleotide is a blockmer. In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC, wherein the oligonucleotide is a stereoblockmer. In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC, wherein the oligonucleotide is a P-modification blockmer. In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC, wherein the oligonucleotide is a linkage blockmer. In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC, wherein the oligonucleotide is an altmer. In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC, wherein the oligonucleotide is a stereoaltmer. In

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some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC, wherein the oligonucleotide is a P-modification altmer. In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC, wherein the oligonucleotide is a linkage altmer. In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC, wherein the oligonucleotide is a unimer. In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC, wherein the oligonucleotide is a stereounimer. In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC, wherein the oligonucleotide is a P-modification unimer. In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC, wherein the oligonucleotide is a linkage unimer. In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC, wherein the oligonucleotide is a gapmer. In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC, wherein the oligonucleotide is a skipmer.

[00425] In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC, wherein each cytosine is optionally and independently replaced by 5-methylcytosine. In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC, wherein at least one cytosine is optionally and independently replaced by 5-methylcytosine. In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of GCCTCAGTCTGCTTCGCACC, wherein each cytosine is optionally and independently replaced by 5-methylcytosine. Exemplary chirally controlled oligonucleotides having the sequence of GCCTCAGTCTGCTTCGCACC are depicted in Table 2, below:

[00426] Table 2. Exemplary chirally controlled oligonucleotides.

Oligo	Stereochemistry/Sequence	Description
101	All-(*R*p)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	All-R
102	All-(*S*p)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	All-S

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103	(*Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	5R-9S-5R
104	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	5S-9R-5S
105	(*Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	1S-17R-1S
106	(*Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	1R-17S-1R
107	(*Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	$(R/S)_9R$
108	(*Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	$(S/R)_9S$
109	(*Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*)d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	3S-13R-3S
110	(*Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	3R-13S-3R
111	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	$18S/R^{19}$
112	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Sp Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	$18S/R^{9}$
113	(*Sp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	$18S/R^{2}$
114	(*Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	$(RRS)_6$-R
115	(*Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	S-$(RRS)_6$
116	(*Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*)d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	RS-$(RRS)_5$-RR
122	All-(*Rp*)-d[Gs1Cs1Cs1Ts1Cs1As1Gs1Ts1Cs1Ts1Gs1Cs1Ts1Ts1Cs1Gs1Cs1As1Cs1C]	All-R
123	(*Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*)-d[Gs1Cs1Cs1Ts1Cs1As1Gs1Ts1Cs1Ts1Gs1Cs1Ts1Ts1Cs1Gs1Cs1As1Cs1C]	1S-17R-1S
124	All-(*Sp*)-d[Gs1Cs1Cs1Ts1Cs1As1Gs1Ts1Cs1Ts1Gs1Cs1Ts1Ts1Cs1Gs1Cs1As1Cs1C]	All-S
125	All-(*Rp*)-d[5mCs1As1Ts1G]	All-R
126	All-(*Rp*)-d[Cs2As2Gs2T]	All-R
127	All-(*Rp*)-d[Cs3As3Gs3T]	All-R
128	All-(*Sp*)-d[Cs4As4Gs4T]	All-S
129	All-(*Sp*)-d[Cs5As5Gs5T]	All-S
130	All-(*Sp*)-d[Cs6As6Gs6T]	All-S
131	All-(*Rp*)-d[Gs7Cs7Cs7Ts7Cs7As7Gs7Ts7Cs7Ts7Gs7Cs7Ts7Ts7Cs7Gs7Cs7As7Cs7C]	All-R
132	All-(*Sp*)-d[Gs7Cs7Cs7Ts7Cs7As7Gs7Ts7Cs7Ts7Gs7Cs7Ts7Ts7Cs7Gs7Cs7As7Cs7C]	All-S

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133	(*Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*)-d[Gs15mCs15mCs1Ts15mCs1As1Gs1Ts15mCs1Ts1 Gs15mCs1Ts1Ts15mCs1Gs15mCs1As15mCs15mC]	5R-9S-5R
134	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[Gs15mCs15mCs1Ts15mCs1As1Gs1Ts15mCs1Ts1 Gs15mCs1Ts1Ts15mCs1Gs15mCs1As15mCs15mC]	5S-9R-5S
135	All-(*Rp*)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G]	All-R
136	All-(*Sp*)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G]	All-S
137	(*Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G]	1S-9R-1S
138	(*Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G]	2S-7R-2S
139	(*Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G]	1R-9S-1R
140	(*Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G]	2R-7S-2R
141	(*Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G]	3S-5R-3S
142	(*Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G]	3R-5S-3R
143	(*Sp*, *Sp*, *Rp*, *Sp*, *Sp*, *Rp*, *Sp*, *Sp*, *Rp*, *Sp*, *Sp*)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G]	$(SSR)_3$-SS
144	(*Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G]	$(RRS)_3$-RR
145	All-(*Rp*)-d[5mCs1Ts15mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1 Ts15mCs1Gs15mC]	All-R
146	All-(*Rp*)-d[Gs15mCs1Ts1G]	All-R
147	All-(*Rp*)-d[5mCs1As1Gs1T]	All-R
148	All-(*Rp*)-d[5mCs2As2Gs2Ts25mCs2Ts2Gs25mCs2Ts2Ts25mCs2G]	All-R
149	All-(*Rp*)-d[5mCs4As4Gs4Ts45mCs4Ts4Gs45mCs4Ts4Ts45mCs4G]	All-R
150	All-(*Rp*)-d[TsCs1AsT]	All-R
151	All-(*Sp*)-d[Cs1AsGs1T]	All-S
152	All-(*Sp*)-d[Cs1AGs1T]	All-S
153	All-(*Sp*)-d[CAs1GsT]	All-S
154	All-(*Rp*)-d[Ts1Cs1As1T]	All-R
155	All-(*Rp*)-d[Ts2Gs2As2C]	All-R
156	All-(*Sp*)-d[Gs15mCs1Ts1G]	All-S
157	All-(*Sp*)-d[5mCs1As1Gs1T]	All-S
158	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCs1GsCsACsC]	5S-9R-4S
159	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[Gs1Cs1Cs1Ts1CsAsGsTsCsTsGsCsTsTsCs1GsCs2As2Cs2C]	5S-9R-5S
160	All-(*Rp*)-$(Gs5mCs5mCsTs5mCs)_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs] $(Gs5mCsAs5mCs5mC)_{MOE}$	All-R

161	All-(*Sp*)-(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs](Gs5mCsAs5mCs5mC)$_{MOE}$	All-S
162	(*Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*)-(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs](Gs5mCsAs5mCs5mC)$_{MOE}$	5R-9S-5R
163	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*)-(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs](Gs5mCsAs5mCs5mC)$_{MOE}$	5S-9R-5S
164	(*Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*)-(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs](Gs5mCsAs5mCs5mC)$_{MOE}$	1S-17R-1S
165	(*Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*)-(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs](Gs5mCsAs5mCs5mC)$_{MOE}$	1R-17S-1R
166	(*Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*)-(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs](Gs5mCsAs5mCs5mC)$_{MOE}$	(R/S)$_9$R
167	(*Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*)-(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs](Gs5mCsAs5mCs5mC)$_{MOE}$	(S/R)$_9$S
168	(*Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*)(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs](Gs5mCsAs5mCs5mC)$_{MOE}$	3S-13R-3S
169	(*Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*)-(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs](Gs5mCsAs5mCs5mC)$_{MOE}$	3R-13S-3R
170	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*)-(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs](Gs5mCsAs5mCs5mC)$_{MOE}$	18S/R^{19}
171	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Sp Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*)-(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs](Gs5mCsAs5mCs5mC)$_{MOE}$	18S/R^9
172	(*Sp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*)-(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs](Gs5mCsAs5mCs5mC)$_{MOE}$	18S/R^2
173	(*Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*)-(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs](Gs5mCsAs5mCs5mC)$_{MOE}$	(RRS)$_6$-R
174	(*Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*)-(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs](Gs5mCsAs5mCs5mC)$_{MOE}$	S-(RRS)$_6$
175	(*Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*)(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs](Gs5mCsAs5mCs5mC)$_{MOE}$	RS-(RRS)$_5$-RR

176	(*Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*)(Gs15mCs15mCs1Ts15mCs1)$_{MOE}$d[As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1] (Gs15mCs1As15mCs15mC)$_{MOE}$	RS-(RRS)$_5$-RR
177	(*Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*)(Gs15mCs15mCs1Ts15mCs1)$_{MOE}$d[AGT5mCTG5mCTT5mC] (Gs25mCs2As25mCs25mC)$_{MOE}$	RS-(RRS)$_5$-RR
178	(*Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*)-(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs] (Gs5mCsAs5mCs5mC)$_F$ (F: 2-fluorodeoxyribose)	S-(RRS)$_6$
179	(*Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*)d[Gs8Cs8Cs8Ts8Cs8As8Gs8Ts8Cs8Ts8Gs8Cs8Ts8Ts8Cs8Gs8Cs8As8Cs8C]	RS-(RRS)$_5$-RR
180	(*Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*)d[Gs9Cs9Cs9Ts9Cs9As9Gs9Ts9Cs9Ts9Gs9Cs9Ts9Ts9Cs9Gs9Cs9As9Cs9C]	RS-(RRS)$_5$-RR
181	(*Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*)d[Gs10Cs10Cs10Ts10Cs10As10Gs10Ts10Cs10Ts10Gs10Cs10Ts10Ts10Cs10Gs10Cs10As10Cs10C]	RS-(RRS)$_5$-RR
182	(*Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*)d[Gs11Cs11Cs11Ts11Cs11As11Gs11Ts11Cs11Ts11Gs11Cs11Ts11Ts11Cs11Gs11Cs11As11Cs11C]	RS-(RRS)$_5$-RR
183	(*Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*)d[Gs12Cs12Cs12Ts12Cs12As12Gs12Ts12Cs12Ts12Gs12Cs12Ts12Ts12Cs12Gs12Cs12As12Cs12C]	RS-(RRS)$_5$-RR
184	(*Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*)d[Gs13Cs13Cs13Ts13Cs13As13Gs13Ts13Cs13Ts13Gs13Cs13Ts13Ts13Cs13Gs13Cs13As13Cs13C]	RS-(RRS)$_5$-RR
185	(*Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*)d[Gs14Cs14Cs14Ts14Cs14As14Gs14Ts14Cs14Ts14Gs14Cs14Ts14Ts14Cs14Gs14Cs14As14Cs14C]	RS-(RRS)$_5$-RR
186	(*Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*)d[Gs15Cs15Cs15Ts15Cs15As15Gs15Ts15Cs15Ts15Gs15Cs15Ts15Ts15Cs15Gs15Cs15As15Cs15C]	RS-(RRS)$_5$-RR
187	(*Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*)d[GsCsCs1TsCsAs]GsUs2CsUsGsd[CsTs3TsCsGs]CsAs4CsC	RS-(RRS)$_5$-RR
188	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsACsC]	5S-9R-4S
189	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[Gs1Cs1Cs1Ts1Cs1As1Gs1Ts1Cs1Ts1Gs1Cs1Ts1Ts1Cs1Gs1CsACs1C]	5S-9R-4S
190	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[Gs8Cs8Cs8Ts8Cs8As8Gs8Ts8Cs8Ts8Gs8Cs8Ts8Ts8Cs8Gs8Cs1ACs8C]	5S-9R-4S
191	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[Gs9Cs9Cs9Ts9Cs9As9Gs9Ts9Cs9Ts9Gs9Cs9Ts9Ts9Cs9Gs9Cs1ACs9C]	5S-9R-4S

192	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[Gs10Cs10Cs10Ts10Cs10As10Gs10Ts10Cs10Ts10Gs10Cs10Ts10Ts10Cs10Gs10Cs1ACs10C]	5S-9R-4S
193	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[Gs11Cs11Cs11Ts11Cs11As11Gs11Ts11Cs11Ts11Gs11Cs11Ts11Ts11Cs11Gs11Cs1ACs11C]	5S-9R-4S
194	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[Gs12Cs12Cs12Ts12Cs12As12Gs12Ts12Cs12Ts12Gs12Cs12Ts12Ts12Cs12Gs12Cs1ACs12C]	5S-9R-4S
195	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[Gs13Cs13Cs13Ts13Cs13As13Gs13Ts13Cs13Ts13Gs13Cs13Ts13Ts13Cs13Gs13Cs1ACs13C]	5S-9R-4S
196	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[Gs14Cs14Cs14Ts14Cs14As14Gs14Ts14Cs14Ts14Gs14Cs14Ts14Ts14Cs14Gs14Cs1ACs14C]	5S-9R-4S
197	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[Gs15Cs15Cs15Ts15Cs15As15Gs15Ts15Cs15Ts15Gs15Cs15Ts15Ts15Cs15Gs15Cs1ACs15C]	5S-9R-4S
198	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-GsCsCsUsCsAsGsUsCsUsGsCsUsUsCsGsCsACsC	5S-9R-4S
199	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-Gs1Cs1Cs1Us1Cs1As1Gs1Us1Cs1Us1Gs1Cs1Us1Us1Cs1Gs1CsACs1C	5S-9R-4S
200	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-Gs8Cs8Cs8Us8Cs8As8Gs8Us8Cs8Us8Gs8Cs8Us8Us8Cs8Gs8Cs1ACs8C	5S-9R-4S
201	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-Gs9Cs9Cs9Us9Cs9As9Gs9Us9Cs9Us9Gs9Cs9Us9Us9Cs9Gs9Cs1ACs9C	5S-9R-4S
202	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-Gs10Cs10Cs10Us10Cs10As10Gs10Us10Cs10Us10Gs10Cs10Us10Us10Cs10Gs10Cs1ACs10C	5S-9R-4S
203	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-Gs11Cs11Cs11Us11Cs11As11Gs11Us11Cs11Us11Gs11Cs11Us11Us11Cs11Gs11Cs1ACs11C	5S-9R-4S
204	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-Gs12Cs12Cs12Us12Cs12As12Gs12Us12Cs12Us12Gs12Cs12Us12Us12Cs12Gs12Cs1ACs12C	5S-9R-4S
205	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-Gs13Cs13Cs13Us13Cs13As13Gs13Us13Cs13Us13Gs13Cs13Us13Us13Cs13Gs13Cs1ACs13C	5S-9R-4S
206	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-Gs14Cs14Cs14Us14Cs14As14Gs14Us14Cs14Us14Gs14Cs14Us14Us14Cs14Gs14Cs1ACs14C	5S-9R-4S
207	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-Gs15Cs15Cs15Us15Cs15As15Gs15Us15Cs15Us15Gs15Cs15Us15Us	5S-9R-4S

	15Cs15Gs15Cs1ACs15C	

[00427] In some embodiments, a chirally controlled oligonucleotide is designed such that one or more nucleotides comprise a phosphorus modification prone to "autorelease" under certain conditions. That is, under certain conditions, a particular phosphorus modification is designed such that it self-cleaves from the oligonucleotide to provide, e.g., a phosphate diester such as those found in naturally occurring DNA and RNA. In some embodiments, such a phosphorus modification has a structure of $-O-L-R^1$, wherein each of L and R^1 is independently as defined above and described herein. In some embodiments, an autorelease group comprises a morpholino group. In some embodiments, an autorelease group is characterized by the ability to deliver an agent to the internucleotidic phosphorus linker, which agent facilitates further modification of the phosphorus atom such as, e.g., desulfurization. In some embodiments, the agent is water and the further modification is hydrolysis to form a phosphate diester as is found in naturally occurring DNA and RNA.

[00428] In some embodiments, a chirally controlled oligonucleotide is designed such that the resulting pharmaceutical properties are improved through one or more particular modifications at phosphorus. It is well documented in the art that certain oligonucleotides are rapidly degraded by nucleases and exhibit poor cellular uptake through the cytoplasmic cell membrane (Poijarvi-Virta *et al.*, Curr. Med. Chem. (2006), 13(28);3441-65; Wagner *et al.*, Med. Res. Rev. (2000), 20(6):417-51; Peyrottes *et al.*, Mini Rev. Med. Chem. (2004), 4(4):395-408; Gosselin *et al.,* (1996), 43(1):196-208; Bologna *et al.*, (2002), Antisense & Nucleic Acid Drug Development 12:33-41). For instance, Vives *et al.,* (Nucleic Acids Research (1999), 27(20):4071-76) found that tert-butyl SATE pro-oligonucleotides displayed markedly increased cellular penetration compared to the parent oligonucleotide.

[00429] In some embodiments, a modification at a linkage phosphorus is characterized by its ability to be transformed to a phosphate diester, such as those present in naturally occurring DNA and RNA, by one or more esterases, nucleases, and/or cytochrome P450 enzymes, including but not limited to, those listed in Table 3, below.

Table 3. Exemplary enzymes.

Family	Gene

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CYP1	CYP1A1, CYP1A2, CYP1B1
CYP2	CYP2A6, CYP2A7, CYP2A13, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP2F1, CYP2J2, CYP2R1, CYP2S1, CYP2U1, CYP2W1
CYP3	CYP3A4, CYP3A5, CYP3A7, CYP3A43
CYP4	CYP4A11, CYP4A22, CYP4B1, CYP4F2, CYP4F3, CYP4F8, CYP4F11, CYP4F12, CYP4F22, CYP4V2, CYP4X1, CYP4Z1
CYP5	CYP5A1
CYP7	CYP7A1, CYP7B1
CYP8	CYP8A1 (prostacyclin synthase), CYP8B1 (bile acid biosynthesis)
CYP11	CYP11A1, CYP11B1, CYP11B2
CYP17	CYP17A1
CYP19	CYP19A1
CYP20	CYP20A1
CYP21	CYP21A2
CYP24	CYP24A1
CYP26	CYP26A1, CYP26B1, CYP26C1
CYP27	CYP27A1 (bile acid biosynthesis), CYP27B1 (vitamin D3 1-alpha hydroxylase, activates vitamin D3), CYP27C1 (unknown function)

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CYP39	CYP39A1
CYP46	CYP46A1
CYP51	CYP51A1 (lanosterol 14-alpha demethylase)

[00430] In some embodiments, a modification at phosphorus results in a P-modification moiety characterized in that it acts as a pro-drug, e.g., the P-modification moiety facilitates delivery of an oligonucleotide to a desired location prior to removal. For instance, in some embodiments, a P-modification moiety results from PEGylation at the linkage phosphorus. One of skill in the relevant arts will appreciate that various PEG chain lengths are useful and that the selection of chain length will be determined in part by the result that is sought to be achieved by PEGylation. For instance, in some embodiments, PEGylation is effected in order to reduce RES uptake and extend *in vivo* circulation lifetime of an oligonucleotide.

[00431] In some embodiments, a PEGylation reagent for use in accordance with the present invention is of a molecular weight of about 300 g/mol to about 100,000 g/mol. In some embodiments, a PEGylation reagent is of a molecular weight of about 300 g/mol to about 10,000 g/mol. In some embodiments, a PEGylation reagent is of a molecular weight of about 300 g/mol to about 5,000 g/mol. In some embodiments, a PEGylation reagent is of a molecular weight of about 500 g/mol. In some embodiments, a PEGylation reagent of a molecular weight of about 1000 g/mol. In some embodiments, a PEGylation reagent is of a molecular weight of about 3000 g/mol. In some embodiments, a PEGylation reagent is of a molecular weight of about 5000 g/mol.

[00432] In certain embodiments, a PEGylation reagent is PEG500. In certain embodiments, a PEGylation reagent is PEG1000. In certain embodiments, a PEGylation reagent is PEG3000. In certain embodiments, a PEGylation reagent is PEG5000.

[00433] In some embodiments, a P-modification moiety is characterized in that it acts as a PK enhancer, e.g., lipids, PEGylated lipids, etc.

[00434] In some embodiments, a P-modification moiety is characterized in that it acts as an agent which promotes cell entry and/or endosomal escape, such as a membrane-disruptive lipid or peptide.

[00435] In some embodiments, a P-modification moiety is characterized in that it acts as a targeting agent. In some embodiments, a P-modification moiety is or comprises a targeting agent. The phrase "targeting agent," as used herein, is an entity that is associates with a payload of interest (e.g., with an oligonucleotide or oligonucleotide composition) and also interacts with a target site of interest so that the payload of interest is targeted to the target site of interest when associated with the targeting agent to a materially greater extent than is observed under otherwise comparable conditions when the payload of interest is not associated with the targeting agent. A targeting agent may be, or comprise, any of a variety of chemical moieties, including, for example, small molecule moieties, nucleic acids, polypeptides, carbohydrates, etc. Targeting agents are described further by Adarsh et al., "Organelle Specific Targeted Drug Delivery – A Review," International Journal of Research in Pharmaceutical and Biomedical Sciences, 2011, p. 895.

[00436] Exemplary such targeting agents include, but are not limited to, proteins (e.g. Transferrin), oligopeptides (e.g., cyclic and acylic RGD-containing oligopedptides), antibodies (monoclonal and polyclonal antibodies, e.g. IgG, IgA, IgM, IgD, IgE antibodies), sugars / carbohydrates (e.g., monosaccharides and/or oligosaccharides (mannose, mannose-6-phosphate, galactose, and the like)), vitamins (e.g., folate), or other small biomolecules. In some embodiments, a targeting moiety is a steroid molecule (e.g., bile acids including cholic acid, deoxycholic acid, dehydrocholic acid; cortisone; digoxigenin; testosterone; cholesterol; cationic steroids such as cortisone having a trimethylaminomethyl hydrazide group attached via a double bond at the 3-position of the cortisone ring, etc.). In some embodiments, a targeting moiety is a lipophilic molecule (e.g., alicyclic hydrocarbons, saturated and unsaturated fatty acids, waxes, terpenes, and polyalicyclic hydrocarbons such as adamantine and buckminsterfullerenes). In some embodiments, a lipophilic molecule is a terpenoid such as vitamin A, retinoic acid, retinal, or dehydroretinal. In some embodiments, a targeting moiety is a peptide.

[00437] In some embodiments, a P-modification moiety is a targeting agent of formula –-X-L-R^1 wherein each of X, L, and R^1 are as defined in Formula **I** above.

[00438] In some embodiments, a P-modification moiety is characterized in that it facilitates cell specific delivery.

[00439] In some embodiments, a P-modification moiety is characterized in that it falls into one or more of the above-described categories. For instance, in some embodiments, a P-

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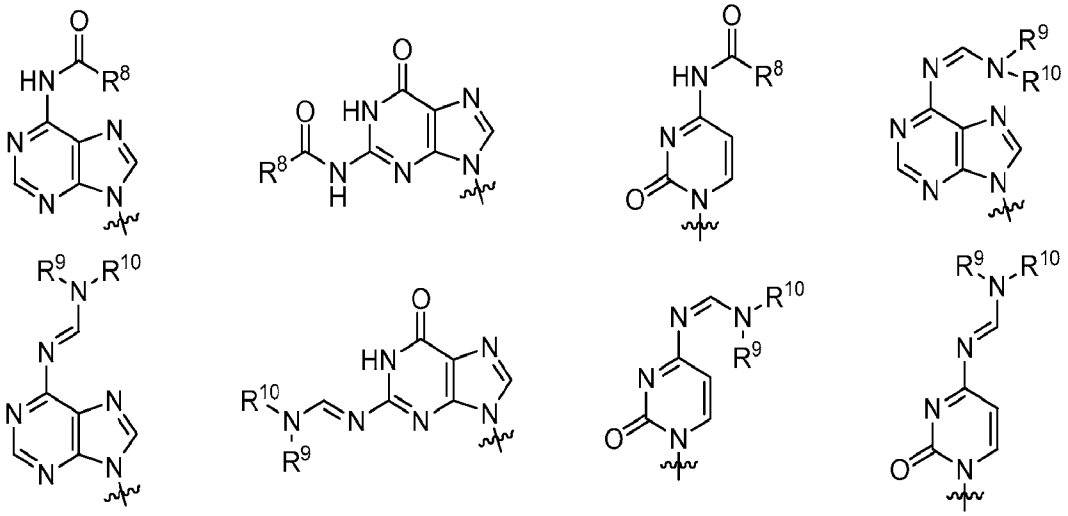
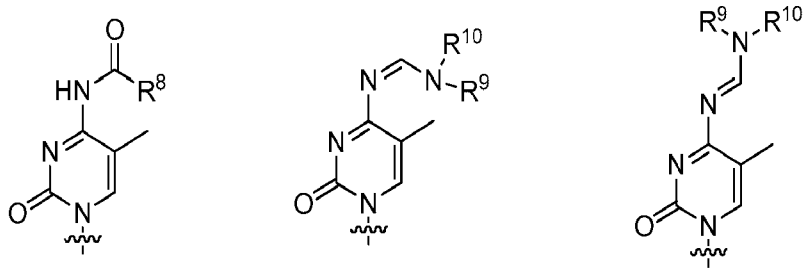
modification moiety acts as a PK enhancer and a targeting ligand. In some embodiments, a P-modification moiety acts as a pro-drug and an endosomal escape agent. One of skill in the relevant arts would recognize that numerous other such combinations are possible and are contemplated by the present invention.

Nucleobases

[00440] In some embodiments, a nucleobase present in a provided oligonucleotide is a natural nucleobase or a modified nucleobase derived from a natural nucleobase. Examples include, but are not limited to, uracil, thymine, adenine, cytosine, and guanine having their respective amino groups protected by acyl protecting groups, 2-fluorouracil, 2-fluorocytosine, 5-bromouracil, 5-iodouracil, 2,6-diaminopurine, azacytosine, pyrimidine analogs such as pseudoisocytosine and pseudouracil and other modified nucleobases such as 8-substituted purines, xanthine, or hypoxanthine (the latter two being the natural degradation products). Exemplary modified nucleobases are disclosed in Chiu and Rana, *RNA*, **2003**, *9*, 1034-1048, Limbach *et al. Nucleic Acids Research*, **1994**, *22*, 2183-2196 and Revankar and Rao, *Comprehensive Natural Products Chemistry*, vol. 7, 313.

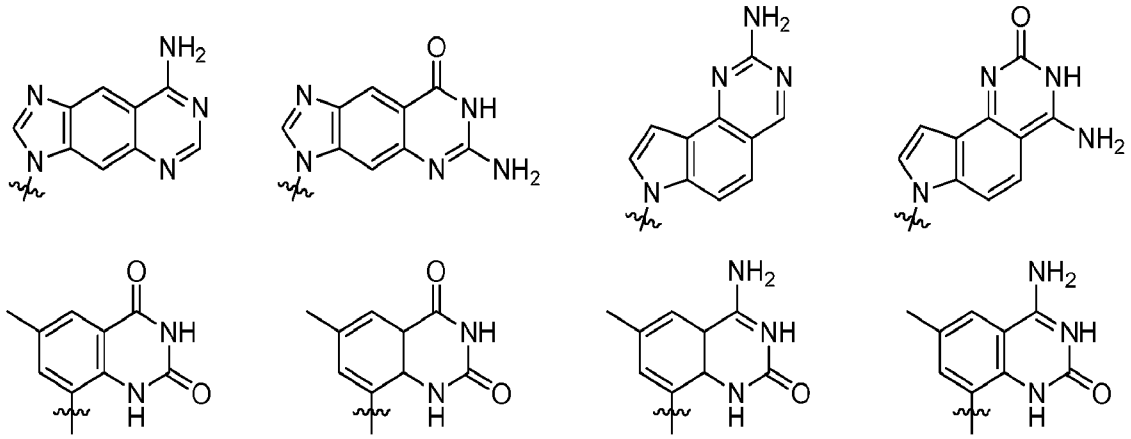
[00441] Compounds represented by the following general formulae are also contemplated as modified nucleobases:

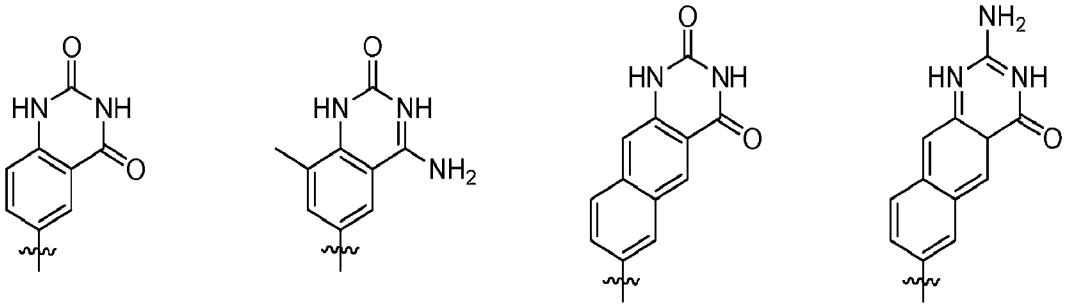




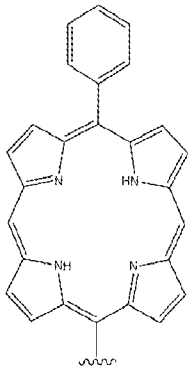
wherein R^8 is an optionally substituted, linear or branched group selected from aliphatic, aryl, aralkyl, aryloxylalkyl, carbocyclyl, heterocyclyl or heteroaryl group having 1 to 15 carbon atoms, including, by way of example only, a methyl, isopropyl, phenyl, benzyl, or phenoxymethyl group; and each of R^9 and R^{10} is independently an optionally substituted group selected from linear or branched aliphatic, carbocyclyl, aryl, heterocyclyl and heteroaryl.

[00442] Modified nucleobases also include expanded-size nucleobases in which one or more aryl rings, such as phenyl rings, have been added. Nucleic base replacements described in the Glen Research catalog (www.glenresearch.com); Krueger AT *et al*, *Acc. Chem. Res.*, **2007**, *40*, 141-150; Kool, ET, *Acc. Chem. Res.*, **2002**, *35*, 936-943; Benner S.A., *et al.*, *Nat. Rev. Genet.*, **2005**, *6*, 553-543; Romesberg, F.E., *et al.*, *Curr. Opin. Chem. Biol.*, **2003**, *7*, 723-733; Hirao, I., *Curr. Opin. Chem. Biol.*, **2006**, *10*, 622-627, are contemplated as useful for the synthesis of the nucleic acids described herein. Some examples of these expanded-size nucleobases are shown below:

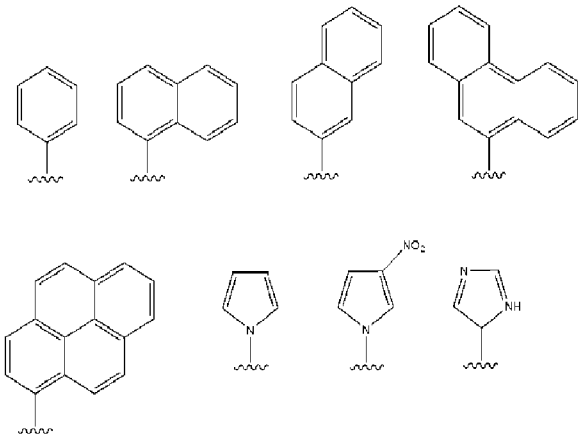




[00443] Herein, modified nucleobases also encompass structures that are not considered nucleobases but are other moieties such as, but not limited to, corrin- or porphyrin-derived rings. Porphyrin-derived base replacements have been described in Morales-Rojas, H and Kool, ET, *Org. Lett.*, 2002, *4*, 4377-4380. Shown below is an example of a porphyrin-derived ring which can be used as a base replacement:



[00444] In some embodiments, modified nucleobases are of any one of the following structures, optionally substituted:



[00445] In some embodiments, a modified nucleobase is fluorescent. Exemplary such fluorescent modified nucleobases include phenanthrene, pyrene, stillbene, isoxanthine, isozanthopterin, terphenyl, terthiophene, benzoterthiophene, coumarin, lumazine, tethered stillbene, benzo-uracil, and naphtho-uracil, as shown below:

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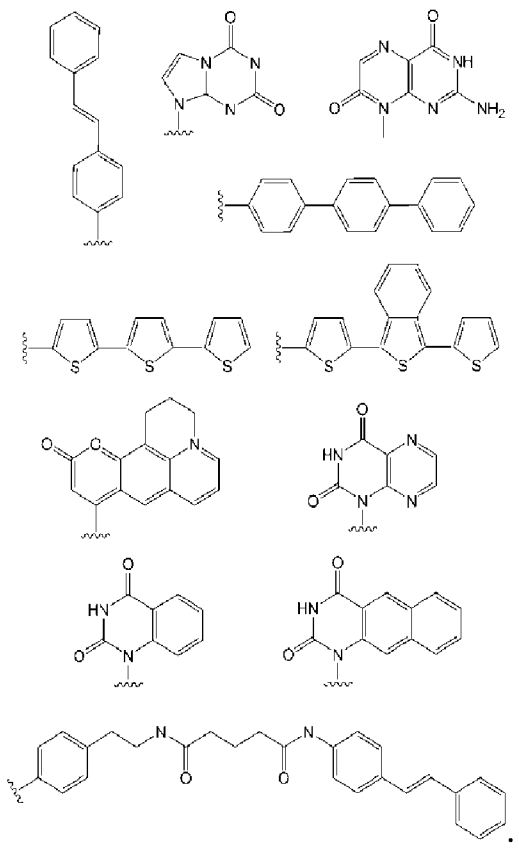

[00446] In some embodiments, a modified nucleobase is unsubstituted. In some embodiments, a modified nucleobase is substituted. In some embodiments, a modified nucleobase is substituted such that it contains, e.g., heteroatoms, alkyl groups, or linking moieties connected to fluorescent moieties, biotin or avidin moieties, or other protein or peptides. In some embodiments, a modified nucleobase is a "universal base" that is not a nucleobase in the most classical sense, but that functions similarly to a nucleobase. One representative example of such a universal base is 3-nitropyrrole.

[00447] In some embodiments, other nucleosides can also be used in the process disclosed herein and include nucleosides that incorporate modified nucleobases, or nucleobases covalently bound to modified sugars. Some examples of nucleosides that incorporate modified nucleobases include 4-acetylcytidine; 5-(carboxyhydroxylmethyl)uridine; 2′-*O*-methylcytidine; 5-carboxymethylaminomethyl-2-thiouridine; 5-carboxymethylaminomethyluridine; dihydrouridine; 2′-*O*-methylpseudouridine; beta,D-galactosylqueosine; 2′-*O*-methylguanosine; N^6-isopentenyladenosine; 1-methyladenosine; 1-methylpseudouridine; 1-methylguanosine; 1-methylinosine; 2,2-dimethylguanosine; 2-methyladenosine; 2-methylguanosine; N^7-methylguanosine; 3-methyl-cytidine; 5-methylcytidine; 5-hydroxymethylcytidine; 5-

formylcytosine; 5-carboxylcytosine; N^6-methyladenosine; 7-methylguanosine; 5-methylaminoethyluridine; 5-methoxyaminomethyl-2-thiouridine; beta,D-mannosylqueosine; 5-methoxycarbonylmethyluridine; 5-methoxyuridine; 2-methylthio-N^6-isopentenyladenosine; *N*-((9-beta,D-ribofuranosyl-2-methylthiopurine-6-yl)carbamoyl)threonine; *N*-((9-beta,D-ribofuranosylpurine-6-yl)-*N*-methylcarbamoyl)threonine; uridine-5-oxyacetic acid methylester; uridine-5-oxyacetic acid (v); pseudouridine; queosine; 2-thiocytidine; 5-methyl-2-thiouridine; 2-thiouridine; 4-thiouridine; 5-methyluridine; 2′-*O*-methyl-5-methyluridine; and 2′-*O*-methyluridine.

[00448] In some embodiments, nucleosides include 6′-modified bicyclic nucleoside analogs that have either (*R*) or (*S*)-chirality at the 6′-position and include the analogs described in US Patent No. 7,399,845. In other embodiments, nucleosides include 5′-modified bicyclic nucleoside analogs that have either (*R*) or (*S*)-chirality at the 5′-position and include the analogs described in US Patent Application Publication No. 20070287831.

[00449] In some embodiments, a nucleobase or modified nucleobase comprises one or more biomolecule binding moieties such as e.g., antibodies, antibody fragments, biotin, avidin, streptavidin, receptor ligands, or chelating moieties. In other embodiments, a nucleobase or modified nucleobase is 5-bromouracil, 5-iodouracil, or 2,6-diaminopurine. In some embodiments, a nucleobase or modified nucleobase is modified by substitution with a fluorescent or biomolecule binding moiety. In some embodiments, the substituent on a nucleobase or modified nucleobase is a fluorescent moiety. In some embodiments, the substituent on a nucleobase or modified nucleobase is biotin or avidin.

[00450] Representative U.S. patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. Pat. No. 3,687,808, as well as U.S. Pat. Nos. 4,845,205; 5,130,30; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,457,191; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,681,941; 5,750,692; 6,015,886; 6,147,200; 6,166,197; 6,222,025; 6,235,887; 6,380,368; 6,528,640; 6,639,062; 6,617,438; 7,045,610; 7,427,672; and 7,495,088, each of which is herein incorporated by reference in its entirety.

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Sugars

[00451] The most common naturally occurring nucleotides are comprised of ribose sugars linked to the nucleobases adenosine (A), cytosine (C), guanine (G), and thymine (T) or uracil (U). Also contemplated are modified nucleotides wherein a phosphate group or linkage phosphorus in the nucleotides can be linked to various positions of a sugar or modified sugar. As non-limiting examples, the phosphate group or linkage phosphorus can be linked to the 2′, 3′, 4′ or 5′ hydroxyl moiety of a sugar or modified sugar. Nucleotides that incorporate modified nucleobases as described herein are also contemplated in this context. In some embodiments, nucleotides or modified nucleotides comprising an unprotected –OH moiety are used in accordance with methods of the present invention.

[00452] Other modified sugars can also be incorporated within a provided oligonucleotide. In some embodiments, a modified sugar contains one or more substituents at the 2′ position including one of the following: –F; $–CF_3$, –CN, $–N_3$, –NO, $–NO_2$, –OR', –SR', or $–N(R')_2$, wherein each R' is independently as defined above and described herein; $–O–(C_1–C_{10}$ alkyl), $–S–(C_1–C_{10}$ alkyl), $–NH–(C_1–C_{10}$ alkyl), or $–N(C_1–C_{10}$ alkyl)$_2$; $–O–(C_2–C_{10}$ alkenyl), $–S–(C_2–C_{10}$ alkenyl), $–NH–(C_2–C_{10}$ alkenyl), or $–N(C_2–C_{10}$ alkenyl)$_2$; $–O–(C_2–C_{10}$ alkynyl), $–S–(C_2–C_{10}$ alkynyl), $–NH–(C_2–C_{10}$ alkynyl), or $–N(C_2–C_{10}$ alkynyl)$_2$; or $–O—(C_1–C_{10}$ alkylene)$–O—(C_1–C_{10}$ alkyl), $–O–(C_1–C_{10}$ alkylene)$–NH–(C_1–C_{10}$ alkyl) or $–O–(C_1–C_{10}$ alkylene)$–NH(C_1–C_{10}$ alkyl)$_2$, $–NH–(C_1–C_{10}$ alkylene)$–O–(C_1–C_{10}$ alkyl), or $–N(C_1–C_{10}$ alkyl)$–(C_1–C_{10}$ alkylene)$–O–(C_1–C_{10}$ alkyl), wherein the alkyl, alkylene, alkenyl and alkynyl may be substituted or unsubstituted. Examples of substituents include, and are not limited to, $–O(CH_2)_nOCH_3$, and $–O(CH_2)_nNH_2$, wherein n is from 1 to about 10, MOE, DMAOE, DMAEOE. Also contemplated herein are modified sugars described in WO 2001/088198; and Martin *et al.*, *Helv. Chim. Acta*, **1995**, *78*, 486-504. In some embodiments, a modified sugar comprises one or more groups selected from a substituted silyl group, an RNA cleaving group, a reporter group, a fluorescent label, an intercalator, a group for improving the pharmacokinetic properties of a nucleic acid, a group for improving the pharmacodynamic properties of a nucleic acid, or other substituents having similar properties. In some embodiments, modifications are made at one or more of the the 2′, 3′, 4′, 5′, or 6′ positions of the sugar or modified sugar, including the 3′ position of the sugar on the 3′-terminal nucleotide or in the 5′ position of the 5′-terminal nucleotide.

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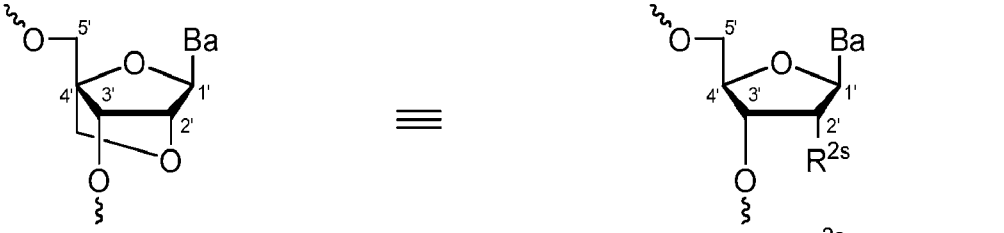
[00453] In some embodiments, the 2'-OH of a ribose is replaced with a substituent including one of the following: –H, –F; $–CF_3$, –CN, $–N_3$, –NO, $–NO_2$, –OR', –SR', or $–N(R')_2$, wherein each R' is independently as defined above and described herein; $–O–(C_1–C_{10}$ alkyl), $–S–(C_1–C_{10}$ alkyl), $–NH–(C_1–C_{10}$ alkyl), or $–N(C_1–C_{10}$ alkyl$)_2$; $–O–(C_2–C_{10}$ alkenyl), $–S–(C_2–C_{10}$ alkenyl), $–NH–(C_2–C_{10}$ alkenyl), or $–N(C_2–C_{10}$ alkenyl$)_2$; $–O–(C_2–C_{10}$ alkynyl), $–S–(C_2–C_{10}$ alkynyl), $–NH–(C_2–C_{10}$ alkynyl), or $–N(C_2–C_{10}$ alkynyl$)_2$; or $–O—(C_1–C_{10}$ alkylene$)–O—(C_1–C_{10}$ alkyl), $–O–(C_1–C_{10}$ alkylene$)–NH–(C_1–C_{10}$ alkyl) or $–O–(C_1–C_{10}$ alkylene$)–NH(C_1–C_{10}$ alkyl$)_2$, $–NH–(C_1–C_{10}$ alkylene$)–O–(C_1–C_{10}$ alkyl), or $–N(C_1–C_{10}$ alkyl$)–(C_1–C_{10}$ alkylene$)–O–(C_1–C_{10}$ alkyl), wherein the alkyl, alkylene, alkenyl and alkynyl may be substituted or unsubstituted. In some embodiments, the 2'–OH is replaced with –H (deoxyribose). In some embodiments, the 2'–OH is replaced with –F. In some embodiments, the 2'–OH is replaced with –OR'. In some embodiments, the 2'–OH is replaced with –OMe. In some embodiments, the 2'–OH is replaced with $–OCH_2CH_2OMe$.

[00454] Modified sugars also include locked nucleic acids (LNAs). In some embodiments, the locked nucleic acid has the structure indicated below. A locked nucleic acid of the structure below is indicated, wherein Ba represents a nucleobase or modified nucleobase as described herein, and wherein R^{2s} is $–OCH_2C4'–$.



C2'OCH_2C4' = LNA (Locked Nucleic Acid)

R^{2s} = OCH_2C4'

[00455] In some embodiments, a modified sugar is an ENA such as those described in, e.g., Seth et al., J Am Chem Soc. 2010 October 27; 132(42): 14942–14950. In some embodiments, a modified sugar is any of those found in an XNA (xenonucleic acid), for instance, arabinose, anhydrohexitol, threose, 2'fluoroarabinose, or cyclohexene.

[00456] Modified sugars include sugar mimetics such as cyclobutyl or cyclopentyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, US Patent Nos.: 4,981,957; 5,118,800; 5,319,080 ; and 5,359,044. Some modified sugars that are contemplated include sugars in which the oxygen atom within the ribose ring is replaced by nitrogen, sulfur,

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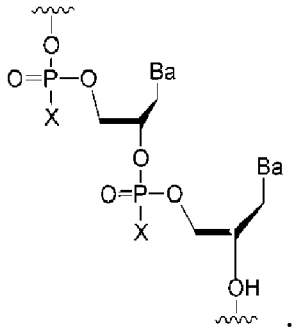
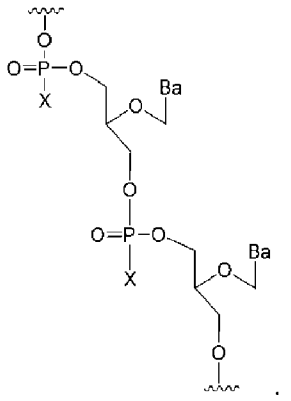
selenium, or carbon. In some embodiments, a modified sugar is a modified ribose wherein the oxygen atom within the ribose ring is replaced with nitrogen, and wherein the nitrogen is optionally substituted with an alkyl group (e.g., methyl, ethyl, isopropyl, etc).

[00457] Non-limiting examples of modified sugars include glycerol, which form glycerol nucleic acid (GNA) analogues. One example of a GNA analogue is shown below and is described in Zhang, R *et al.*, *J. Am. Chem. Soc.*, 2008, *130*, 5846-5847; Zhang L, *et al.*, *J. Am. Chem. Soc.*, 2005, *127*, 4174-4175 and Tsai CH *et al.*, *PNAS*, 2007, 14598-14603 (X = O^-):



[00458] Another example of a GNA derived analogue, flexible nucleic acid (FNA) based on the mixed acetal aminal of formyl glycerol, is described in Joyce GF *et al.*, *PNAS*, 1987, *84*, 4398-4402 and Heuberger BD and Switzer C, *J. Am. Chem. Soc.*, 2008, *130*, 412-413, and is shown below:



[00459] Additional non-limiting examples of modified sugars include hexopyranosyl (6' to 4'), pentopyranosyl (4' to 2'), pentopyranosyl (4' to 3'), or tetrofuranosyl (3' to 2') sugars. In some embodiments, a hexopyranosyl (6' to 4') sugar is of any one of the following formulae:

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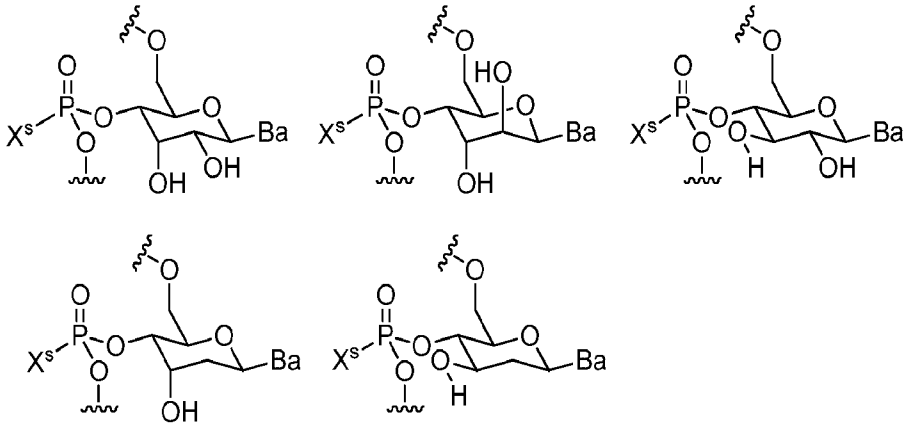
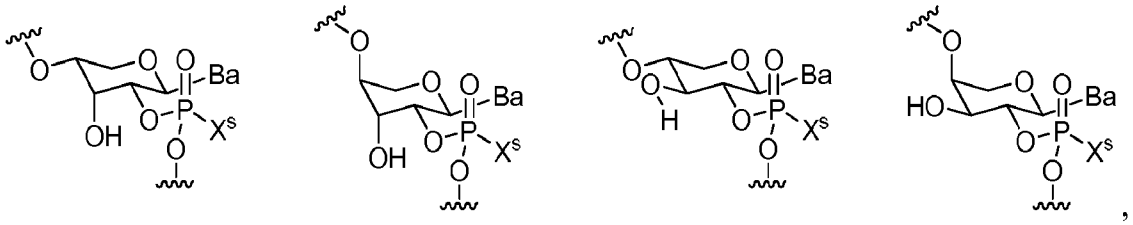


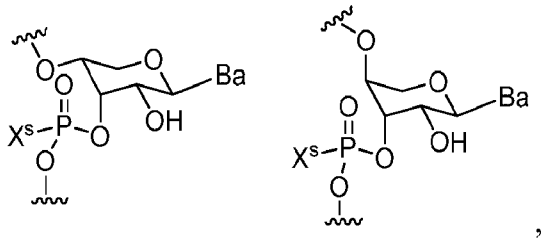
wherein X^s corresponds to the P-modification group "-XLR^1" described herein and Ba is as defined herein.

[00460] In some embodiments, a pentopyranosyl (4' to 2') sugar is of any one of the following formulae:



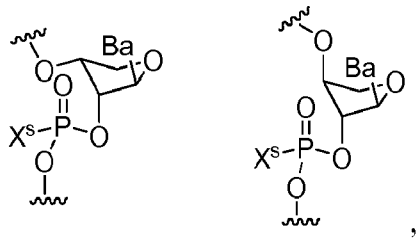
wherein X^s corresponds to the P-modification group "-XLR^1" described herein and Ba is as defined herein.

[00461] In some embodiments, a pentopyranosyl (4' to 3') sugar is of any one of the following formulae:



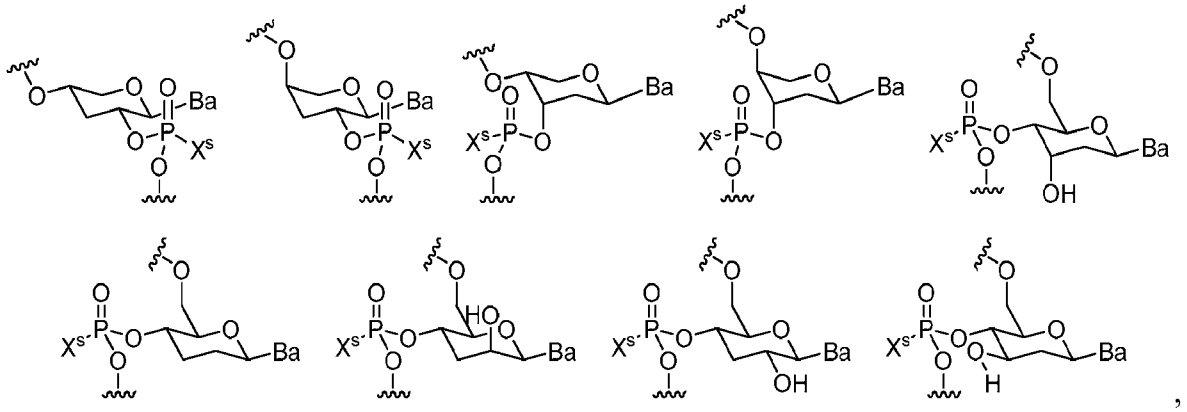
wherein X^s corresponds to the P-modification group "-XLR^1" described herein and Ba is as defined herein.

[00462] In some embodiments, a tetrofuranosyl (3' to 2') sugar is of either of the following formulae:



wherein X^s corresponds to the P-modification group "-XLR^1" described herein and Ba is as defined herein.

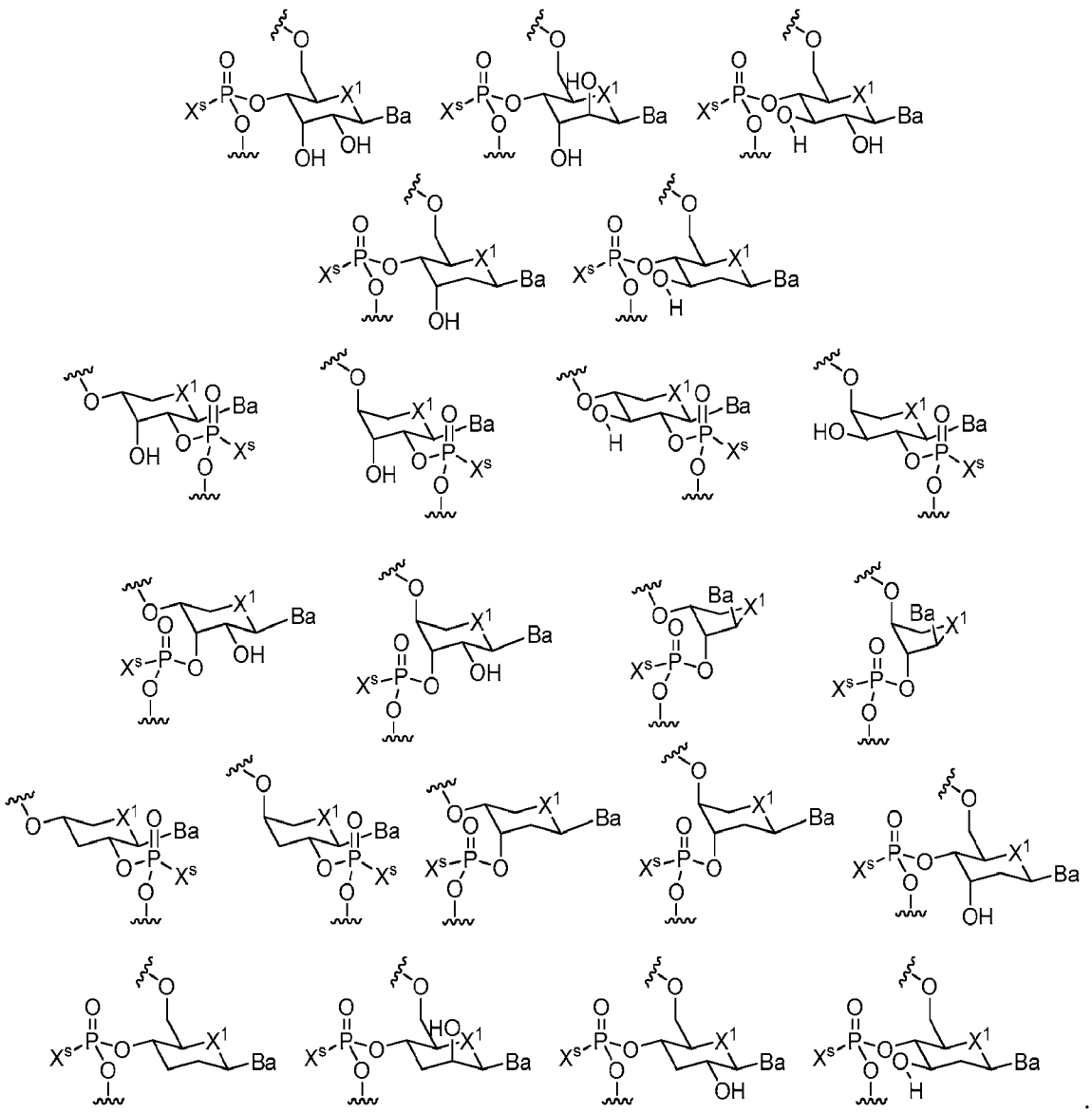
[00463] In some embodiments, a modified sugar is of any one of the following formulae:



wherein X^s corresponds to the P-modification group "-XLR^1" described herein and Ba is as defined herein.

[00464] In some embodiments, one or more hydroxyl group in a sugar moiety is optionally and independently replaced with halogen, R' $-N(R')_2$, $-OR'$, or $-SR'$, wherein each R' is independently as defined above and described herein.

[00465] In some embodiments, a sugar mimetic is as illustrated below, wherein X^s corresponds to the P-modification group "-XLR^1" described herein, Ba is as defined herein, and X^1 is selected from –S–, –Se–, $-CH_2-$, –NMe–, –NEt– or –N*i*Pr–.



[00466] In some embodiments, at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50% or more (e.g., 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more), inclusive, of the sugars in a chirally controlled oligonucleotide composition are modified. In some embodiments, only purine residues are modified (e.g., about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50% or more [e.g., 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more] of the purine residues

are modified). In some embodiments, only pyrimidine residues are modified (e.g., about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50% or more [e.g., 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more] of the pyridimine residues are modified). In some embodiments, both purine and pyrimidine residues are modified.

[00467] Modified sugars and sugar mimetics can be prepared by methods known in the art, including, but not limited to: A. Eschenmoser, Science (1999), 284:2118; M. Bohringer *et al,* Helv. Chim. Acta (1992), 75:1416-1477; M. Egli *et al*, J. Am. Chem. Soc. (2006), 128(33):10847-56; A. Eschenmoser in *Chemical Synthesis: Gnosis to Prognosis,* C. Chatgilialoglu and V. Sniekus, Ed., (Kluwer Academic, Netherlands, 1996), p.293; K.-U. Schoning *et al,* Science (2000), 290:1347-1351; A. Eschenmoser *et al,* Helv. Chim. Acta (1992), 75:218; J. Hunziker *et al,* Helv. Chim. Acta (1993), 76:259; G. Otting *et al,* Helv. Chim. Acta (1993), 76:2701; K. Groebke *et al,* Helv. Chim. Acta (1998), 81:375; and A. Eschenmoser, Science (1999), 284:2118. Modifications to the 2' modifications can be found in Verma, S. *et al. Annu. Rev. Biochem.* **1998,** 67, 99-134 and all references therein. Specific modifications to the ribose can be found in the following references: 2'-fluoro (Kawasaki et. al., *J. Med. Chem.,* **1993,** 36, 831- 841), 2'-MOE (Martin, P. *Helv. Chim. Acta* **1996,** 79, 1930-1938), "LNA" (Wengel, J. *Acc. Chem. Res.* **1999,** *32,* 301-310). In some embodiments, a modified sugar is any of those described in PCT Publication No. WO2012/030683, incorporated herein by reference, and depicted in the Figures 26-30 of the present application.

Oligonucleotides

[00468] In some embodiments, the present invention provides oligonucleotides and oligonucleotide compositions that are chirally controlled. For instance, in some embodiments, a provided composition contains predetermined levels of one or more individual oligonucleotide types, wherein an oligonucleotide type is defined by: 1) base sequence; 2) pattern of backbone linkages; 3) pattern of backbone chiral centers; and 4) pattern of backbone P-modifications.

[00469] In some embodiments, a provided oligonucleotide is a unimer. In some embodiments, a provided oligonucleotide is a P-modification unimer. In some embodiments, a provided oligonucleotide is a stereounimer. In some embodiments, a provided oligonucleotide is

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a stereounimer of configuration *R*p. In some embodiments, a provided oligonucleotide is a stereounimer of configuration *S*p.

[00470] In some embodiments, a provided oligonucleotide is an altmer. In some embodiments, a provided oligonucleotide is a P-modification altmer. In some embodiments, a provided oligonucleotide is a stereoaltmer.

[00471] In some embodiments, a provided oligonucleotide is a blockmer. In some embodiments, a provided oligonucleotide is a P-modification blockmer. In some embodiments, a provided oligonucleotide is a stereoblockmer.

[00472] In some embodiments, a provided oligonucleotide is a gapmer.

[00473] In some embodiments, a provided oligonucleotide is a skipmer.

[00474] In some embodiments, a provided oligonucleotide is a combination of one or more of unimer, altmer, blockmer, gapmer, and skipmer. For instance, in some embodiments, a provided oligonucleotide is both an altmer and a gapmer. In some embodiments, a provided nucleotide is both a gapmer and a skipmer. One of skill in the chemical and synthetic arts will recognize that numerous other combinations of patterns are available and are limited only by the commercial availability and / or synthetic accessibility of constituent parts required to synthesize a provided oligonucleotide in accordance with methods of the present invention.

[00475] In some embodiments, a provided oligonucleotide comprises one or more optionally substituted nucleotides. In some embodiments, a provided oligonucleotide comprises one or more modified nucleotides. In some embodiments, a provided oligonucleotide comprises one or more optionally substituted nucleosides. In some embodiments, a provided oligonucleotide comprises one or more modified nucleosides. In some embodiments, a provided oligonucleotide comprises one or more optionally substituted LNAs.

[00476] In some embodiments, a provided oligonucleotide comprises one or more optionally substituted nucleobases. In some embodiments, a provided oligonucleotide comprises one or more optionally substituted natural nucleobases. In some embodiments, a provided oligonucleotide comprises one or more optionally substituted modified nucleobases. In some embodiments, a provided oligonucleotide comprises one or more 5-methylcytidine; 5-hydroxymethylcytidine, 5-formylcytosine, or 5-carboxylcytosine. In some embodiments, a provided oligonucleotide comprises one or more 5-methylcytidine.

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[00477] In some embodiments, a provided oligonucleotide comprises one or more optionally substituted sugars. In some embodiments, a provided oligonucleotide comprises one or more optionally substituted sugars found in naturally occurring DNA and RNA. In some embodiments, a provided oligonucleotide comprises one or more optionally substituted ribose or deoxyribose. In some embodiments, a provided oligonucleotide comprises one or more optionally substituted ribose or deoxyribose, wherein one or more hydroxyl groups of the ribose or deoxyribose moiety is optionally and independently replaced by halogen, R', $-N(R')_2$, –OR', or –SR', wherein each R' is independently as defined above and described herein. In some embodiments, a provided oligonucleotide comprises one or more optionally substituted deoxyribose, wherein the 2' position of the deoxyribose is optionally and independently substituted with halogen, R', $-N(R')_2$, –OR', or –SR', wherein each R' is independently as defined above and described herein. In some embodiments, a provided oligonucleotide comprises one or more optionally substituted deoxyribose, wherein the 2' position of the deoxyribose is optionally and independently substituted with halogen. In some embodiments, a provided oligonucleotide comprises one or more optionally substituted deoxyribose, wherein the 2' position of the deoxyribose is optionally and independently substituted with one or more –F. halogen. In some embodiments, a provided oligonucleotide comprises one or more optionally substituted deoxyribose, wherein the 2' position of the deoxyribose is optionally and independently substituted with –OR', wherein each R' is independently as defined above and described herein. In some embodiments, a provided oligonucleotide comprises one or more optionally substituted deoxyribose, wherein the 2' position of the deoxyribose is optionally and independently substituted with –OR', wherein each R' is independently an optionally substituted C_1–C_6 aliphatic. In some embodiments, a provided oligonucleotide comprises one or more optionally substituted deoxyribose, wherein the 2' position of the deoxyribose is optionally and independently substituted with –OR', wherein each R' is independently an optionally substituted C_1–C_6 alkyl. In some embodiments, a provided oligonucleotide comprises one or more optionally substituted deoxyribose, wherein the 2' position of the deoxyribose is optionally and independently substituted with –OMe. In some embodiments, a provided oligonucleotide comprises one or more optionally substituted deoxyribose, wherein the 2' position of the deoxyribose is optionally and independently substituted with –O–methoxyethyl.

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[00478] In some embodiments, a provided oligonucleotide is single-stranded oligonucleotide.

[00479] In some embodiments, a provided oligonucleotide is a hybridized oligonucleotide strand. In certain embodiments, a provided oligonucleotide is a partially hydridized oligonucleotide strand. In certain embodiments, a provided oligonucleotide is a completely hydridized oligonucleotide strand. In certain embodiments, a provided oligonucleotide is a double-stranded oligonucleotide. In certain embodiments, a provided oligonucleotide is a triple-stranded oligonucleotide (e.g., a triplex).

[00480] In some embodiments, a provided oligonucleotide is chimeric. For example, in some embodiments, a provided oligonucleotide is DNA-RNA chimera, DNA-LNA chimera, etc.

[00481] In some embodiments, any one of the structures comprising an oligonucleotide depicted in WO2012/030683 can be modified in accordance with methods of the present invention to provide chirally controlled variants thereof. For example, in some embodiments the chirally controlled variants comprise a stereochemical modification at any one or more of the linkage phosphorus and/or a P-modification at any one or more of the linkage phosphorus. For example, in some embodiments, a particular nucleotide unit of a oligonucleotide of WO2012/030683 is preselected to be stereochemically modified at the linkage phosphorus of that nucleotide unit and/or P-modified at the linkage phosphorus of that nucleotide unit. In some embodiments, a chirally controlled oligonucleotide is of any one of the structures depicted in Figures 26-30. In some embodiments, a chirally controlled oligonucleotide is a variant (e.g., modified version) of any one of the structures depicted in Figures 26-30. The disclosure of WO2012/030683 is herein incorporated by reference in its entirety.

[00482] In some embodiments, a provided oligonucleotide is a therapeutic agent.

[00483] In some embodiments, a provided oligonucleotide is an antisense oligonucleotide.

[00484] In some embodiments, a provided oligonucleotide is an antigene oligonucleotide.

[00485] In some embodiments, a provided oligonucleotide is a decoy oligonucleotide.

[00486] In some embodiments, a provided oligonucleotide is part of a DNA vaccine.

[00487] In some embodiments, a provided oligonucleotide is an immunomodulatory oligonucleotide, e.g., immunostimulatory oligonucleotide and immunoinhibitory oligonucleotide.

[00488] In some embodiments, a provided oligonucleotide is an adjuvant.

[00489] In some embodiments, a provided oligonucleotide is an aptamer.

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[00490] In some embodiments, a provided oligonucleotide is a ribozyme.

[00491] In some embodiments, a provided oligonucleotide is a deoxyribozyme (DNAzymes or DNA enzymes).

[00492] In some embodiments, a provided oligonucleotide is an siRNA.

[00493] In some embodiments, a provided oligonucleotide is a microRNA, or miRNA.

[00494] In some embodiments, a provided oligonucleotide is a ncRNA (non-coding RNAs), including a long non-coding RNA (lncRNA) and a small non-coding RNA, such as piwi-interacting RNA (piRNA).

[00495] In some embodiments, a provided oligonucleotide is complementary to a structural RNA, e.g., tRNA.

[00496] In some embodiments, a provided oligonucleotide is a nucleic acid analog, e.g., GNA, LNA, PNA, TNA and Morpholino.

[00497] In some embodiments, a provided oligonucleotide is a P-modified prodrug.

[00498] In some embodiments, a provided oligonucleotide is a primer. In some embodiments, a primers is for use in polymerase-based chain reactions (i.e., PCR) to amplify nucleic acids. In some embodiments, a primer is for use in any known variations of PCR, such as reverse transcription PCR (RT-PCR) and real-time PCR.

[00499] In some embodiments, a provided oligonucleotide is characterized as having the ability to modulate RNase H activation. For example, in some embodiments, RNase H activation is modulated by the presence of stereocontrolled phosphorothioate nucleic acid analogs, with natural DNA/RNA being more or equally susceptible than the *R*p stereoisomer, which in turn is more susceptible than the corresponding *S*p stereoisomer.

[00500] In some embodiments, a provided oligonucleotide is characterized as having the ability to indirectly or directly increase or decrease activity of a protein or inhibition or promotion of the expression of a protein. In some embodiments, a provided oligonucleotide is characterized in that it is useful in the control of cell proliferation, viral replication, and/or any other cell signaling process.

[00501] In some embodiments, a provided oligonucleotide is from about 2 to about 200 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 2 to about 180 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 2 to about 160 nucleotide units in length. In some embodiments, a provided

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oligonucleotide is from about 2 to about 140 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 2 to about 120 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 2 to about 100 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 2 to about 90 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 2 to about 80 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 2 to about 70 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 2 to about 60 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 2 to about 50 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 2 to about 40 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 2 to about 30 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 2 to about 29 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 2 to about 28 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 2 to about 27 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 2 to about 26 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 2 to about 25 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 2 to about 24 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 2 to about 23 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 2 to about 22 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 2 to about 21 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 2 to about 20 nucleotide units in length.

[00502] In some embodiments, a provided oligonucleotide is from about 4 to about 200 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 4 to about 180 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 4 to about 160 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 4 to about 140 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 4 to about 120 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 4 to about 100 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 4 to about 90 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 4 to about 80 nucleotide

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units in length. In some embodiments, a provided oligonucleotide is from about 4 to about 70 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 4 to about 60 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 4 to about 50 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 4 to about 40 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 4 to about 30 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 4 to about 29 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 4 to about 28 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 4 to about 27 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 4 to about 26 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 4 to about 25 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 4 to about 24 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 4 to about 23 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 4 to about 22 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 4 to about 21 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 4 to about 20 nucleotide units in length.

[00503] In some embodiments, a provided oligonucleotide is from about 5 to about 10 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 10 to about 30 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 15 to about 25 nucleotide units in length. In some embodiments, a provided oligonucleotide is from about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotide units in length.

[00504] In some embodiments, the oligonucleotide is at least 2 nucleotide units in length. In some embodiments, the oligonucleotide is at least 3 nucleotide units in length. In some embodiments, the oligonucleotide is at least 4 nucleotide units in length. In some embodiments, the oligonucleotide is at least 5 nucleotide units in length. In some embodiments, the oligonucleotide is at least 6 nucleotide units in length. In some embodiments, the oligonucleotide is at least 7 nucleotide units in length. In some embodiments, the oligonucleotide is at least 8 nucleotide units in length. In some embodiments, the oligonucleotide is at least 9 nucleotide units in length. In some embodiments, the

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oligonucleotide is at least 10 nucleotide units in length. In some embodiments, the oligonucleotide is at least 11 nucleotide units in length. In some embodiments, the oligonucleotide is at least 12 nucleotide units in length. In some embodiments, the oligonucleotide is at least 15 nucleotide units in length. In some embodiments, the oligonucleotide is at least 20 nucleotide units in length. In some embodiments, the oligonucleotide is at least 25 nucleotide units in length. In some other embodiments, the oligonucleotide is at least 30 nucleotide units in length. In some other embodiments, the oligonucleotide is a duplex of complementary strands of at least 18 nucleotide units in length. In some other embodiments, the oligonucleotide is a duplex of complementary strands of at least 21 nucleotide units in length.

[00505] In some embodiments, the 5'-end and/or the 3'-end of a provided oligonucleotide is modified. In some embodiments, the 5'-end and/or the 3'-end of a provided oligonucleotide is modified with a terminal cap moiety. Exemplary such modifications, including terminal cap moieties are extensively described herein and in the art, for example but not limited to those described in US Patent Application Publication US 2009/0023675A1.

Species of oligonucleotides

[00506] In certain embodiments, an oligonucleotide of formula **I** is of any one of the structures shown in Table **2**, above and those described in the examples.

[00507] In some embodiments, a provided chirally controlled oligonucleotide comprises the sequence of, or part of the sequence of mipomersen. Mipomersen is based on the following base sequence GCCT/UCAGT/UCT/UGCT/UT/UCGCACC. In some embodiments, one or more of any of the nucleotide or linkages may be modified in accordance of the present invention. In some embodiments, the present invention provides a chirally controlled oligonucleotide having the sequence of G*-C*-C*-U*-C*-dA-dG-dT-dC-dT-dG-dmC-dT-dT-dmC-G*-C*-A*-C*-C* [d = 2'-deoxy, * = 2'-O-(2-methoxyethyl)] with 3'→5' phosphorothioate linkages. Exemplary modified mipomersen sequences are described throughout the application, including but not limited to those in Table 4.

[00508] In certain embodiments, a provided oligonucleotide is a mipomersen unimer. In certain embodiments, a provided oligonucleotide is a mipomersen unimer of configuration *R*p. In certain embodiments, a provided oligonucleotide is a mipomersen unimer of configuration *S*p.

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[00509] Exempary chirally controlled oligonucleotides comprising the sequence of, or part of the sequence of mipomersen is depicted in Table **4**, below.

[00510] Table 4. Exemplary Mipomersen related sequences.

Oligo	Stereochemistry/Sequence	Description
101	All-(*Rp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	All-R
102	All-(*Sp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	All-S
103	(*Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	5R-9S-5R
104	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	5S-9R-5S
105	(*Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	1S-17R-1S
106	(*Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	1R-17S-1R
107	(*Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	$(R/S)_9R$
108	(*Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	$(S/R)_9S$
109	(*Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*)d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	3S-13R-3S
110	(*Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	3R-13S-3R
111	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	$18S/R^{19}$
112	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Sp Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	$18S/R^{9}$
113	(*Sp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	$18S/R^{2}$
114	(*Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	$(RRS)_6$-R
115	(*Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	S-$(RRS)_6$
116	(*Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*)d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	RS-$(RRS)_5$-RR
122	All-(*Rp*)-d[Gs1Cs1Cs1Ts1Cs1As1Gs1Ts1Cs1Ts1Gs1Cs1Ts1Ts1Cs1Gs1Cs1As1Cs1C]	All-R
123	(*Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*)-d[Gs1Cs1Cs1Ts1Cs1As1Gs1Ts1Cs1Ts1Gs1Cs1Ts1Ts1Cs1Gs1Cs1As1Cs1C]	1S-17R-1S
124	All-(*Sp*)-d[Gs1Cs1Cs1Ts1Cs1As1Gs1Ts1Cs1Ts1Gs1Cs1Ts1Ts1Cs1Gs1Cs1As1Cs1C]	All-S
126	All-(*Rp*)-d[Cs2As2Gs2T]	All-R
127	All-(*Rp*)-d[Cs3As3Gs3T]	All-R

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128	All-(*Sp*)-d[Cs4As4Gs4T]	All-S
129	All-(*Sp*)-d[Cs5As5Gs5T]	All-S
130	All-(*Sp*)-d[Cs6As6Gs6T]	All-S
131	All-(*Rp*)-d[Gs7Cs7Cs7Ts7Cs7As7Gs7Ts7Cs7Ts7Gs7 Cs7Ts7Ts7Cs7Gs7Cs7As7Cs7C]	All-R
132	All-(*Sp*)-d[Gs7Cs7Cs7Ts7Cs7As7Gs7Ts7Cs7Ts7Gs7 Cs7Ts7Ts7Cs7Gs7Cs7As7Cs7C]	All-S
133	(*Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*)-d[Gs15mCs15mCs1Ts15mCs1As1Gs1Ts15mCs1Ts1 Gs15mCs1Ts1Ts15mCs1Gs15mCs1As15mCs15mC]	5R-9S-5R
134	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[Gs15mCs15mCs1Ts15mCs1As1Gs1Ts15mCs1Ts1 Gs15mCs1Ts1Ts15mCs1Gs15mCs1As15mCs15mC]	5S-9R-5S
135	All-(*Rp*)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G]	All-R
136	All-(*Sp*)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G]	All-S
137	(*Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G]	1S-9R-1S
138	(*Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G]	2S-7R-2S
139	(*Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G]	1R-9S-1R
140	(*Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G]	2R-7S-2R
141	(*Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G]	3S-5R-3S
142	(*Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G]	3R-5S-3R
143	(*Sp*, *Sp*, *Rp*, *Sp*, *Sp*, *Rp*, *Sp*, *Sp*, *Rp*, *Sp*, *Sp*)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G]	$(SSR)_3$-SS
144	(*Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G]	$(RRS)_3$-RR
145	All-(*Rp*)-d[5mCs1Ts15mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1 Gs15mC]	All-R
146	All-(*Rp*)-d[Gs15mCs1Ts1G]	All-R
147	All-(*Rp*)-d[5mCs1As1Gs1T]	All-R
148	All-(*Rp*)-d[5mCs2As2Gs2Ts25mCs2Ts2Gs25mCs2Ts2Ts25mCs2G]	All-R
149	All-(*Rp*)-d[5mCs4As4Gs4Ts45mCs4Ts4Gs45mCs4Ts4Ts45mCs4G]	All-R
151	All-(*Sp*)-d[Cs1AsGs1T]	All-S
152	All-(*Sp*)-d[Cs1AGs1T]	All-S
153	All-(*Sp*)-d[CAs1GsT]	All-S
157	All-(*Sp*)-d[5mCs1As1Gs1T]	All-S
158	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCs1GsCsACsC]	5S-9R-4S
159	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*,	5S-9R-5S

	Sp)-d[Gs1Cs1Cs1Ts1CsAsGsTsCsTsGsCsTsTsCs1GsCs2As2Cs2C]	
160	All-(*Rp*)-(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs](Gs5mCsAs5mCs5mC)$_{MOE}$	All-R
161	All-(*Sp*)-(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs](Gs5mCsAs5mCs5mC)$_{MOE}$	All-S
162	(*Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*)-(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs](Gs5mCsAs5mCs5mC)$_{MOE}$	5R-9S-5R
163	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*)-(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs](Gs5mCsAs5mCs5mC)$_{MOE}$	5S-9R-5S
164	(*Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*)-(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs](Gs5mCsAs5mCs5mC)$_{MOE}$	1S-17R-1S
165	(*Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*)-(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs](Gs5mCsAs5mCs5mC)$_{MOE}$	1R-17S-1R
166	(*Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*)-(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs](Gs5mCsAs5mCs5mC)$_{MOE}$	(R/S)$_9$R
167	(*Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*)-(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs](Gs5mCsAs5mCs5mC)$_{MOE}$	(S/R)$_9$S
168	(*Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*)(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs](Gs5mCsAs5mCs5mC)$_{MOE}$	3S-13R-3S
169	(*Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*)-(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs](Gs5mCsAs5mCs5mC)$_{MOE}$	3R-13S-3R
170	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*)-(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs](Gs5mCsAs5mCs5mC)$_{MOE}$	18S/R^{19}
171	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Sp Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*)-(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs](Gs5mCsAs5mCs5mC)$_{MOE}$	18S/R^{9}
172	(*Sp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*)-(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs](Gs5mCsAs5mCs5mC)$_{MOE}$	18S/R^{2}
173	(*Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*)-(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs](Gs5mCsAs5mCs5mC)$_{MOE}$	(RRS)$_6$-R
174	(*Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*)-(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs]	S-(RRS)$_6$

	(Gs5mCsAs5mCs5mC)$_{MOE}$	
175	(*Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*)(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs] (Gs5mCsAs5mCs5mC)$_{MOE}$	RS-(RRS)$_5$-RR
176	(*Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*)(Gs15mCs15mCs1Ts15mCs1)$_{MOE}$d[As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1] (Gs15mCs1As15mCs15mC)$_{MOE}$	RS-(RRS)$_5$-RR
177	(*Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*)(Gs15mCs15mCs1Ts15mCs1)$_{MOE}$d[AGT5mCTG5mCTT5mC] (Gs25mCs2As25mCs25mC)$_{MOE}$	RS-(RRS)$_5$-RR
178	(*Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*)-(Gs5mCs5mCsTs5mCs)$_{MOE}$d[AsGsTs5mCsTsGs5mCsTsTs5mCs] (Gs5mCsAs5mCs5mC)$_F$ (F: 2-fluorodeoxyribose)	S-(RRS)$_6$
179	(*Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*)d[Gs8Cs8Cs8Ts8Cs8As8Gs8Ts8Cs8Ts8Gs8Cs8Ts8Ts8Cs8Gs8Cs8As8Cs8C]	RS-(RRS)$_5$-RR
180	(*Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*)d[Gs9Cs9Cs9Ts9Cs9As9Gs9Ts9Cs9Ts9Gs9Cs9Ts9Ts9Cs9Gs9Cs9As9Cs9C]	RS-(RRS)$_5$-RR
181	(*Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*)d[Gs10Cs10Cs10Ts10Cs10As10Gs10Ts10Cs10Ts10Gs10Cs10Ts10Ts10Cs10Gs10Cs10As10Cs10C]	RS-(RRS)$_5$-RR
182	(*Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*)d[Gs11Cs11Cs11Ts11Cs11As11Gs11Ts11Cs11Ts11Gs11Cs11Ts11Ts11Cs11Gs11Cs11As11Cs11C]	RS-(RRS)$_5$-RR
183	(*Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*)d[Gs12Cs12Cs12Ts12Cs12As12Gs12Ts12Cs12Ts12Gs12Cs12Ts12Ts12Cs12Gs12Cs12As12Cs12C]	RS-(RRS)$_5$-RR
184	(*Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*)d[Gs13Cs13Cs13Ts13Cs13As13Gs13Ts13Cs13Ts13Gs13Cs13Ts13Ts13Cs13Gs13Cs13As13Cs13C]	RS-(RRS)$_5$-RR
185	(*Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*)d[Gs14Cs14Cs14Ts14Cs14As14Gs14Ts14Cs14Ts14Gs14Cs14Ts14Ts14Cs14Gs14Cs14As14Cs14C]	RS-(RRS)$_5$-RR
186	(*Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*)d[Gs15Cs15Cs15Ts15Cs15As15Gs15Ts15Cs15Ts15Gs15Cs15Ts15Ts15Cs15Gs15Cs15As15Cs15C]	RS-(RRS)$_5$-RR
187	(*Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp Rp*)d[GsCsCs1TsCsAs]GsUs2CsUsGsd[CsTs3TsCsGs]CsAs4CsC	RS-(RRS)$_5$-RR
188	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsACsC]	5S-9R-4S
189	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[Gs1Cs1Cs1Ts1Cs1As1Gs1Ts1Cs1Ts1Gs1Cs1Ts1Ts1Cs1Gs1CsACs1C]	5S-9R-4S
190	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[Gs8Cs8Cs8Ts8Cs8As8Gs8Ts8Cs8Ts8Gs8Cs8Ts8Ts8Cs8Gs8Cs1A	5S-9R-4S

	Cs8C]	
191	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[Gs9Cs9Cs9Ts9Cs9As9Gs9Ts9Cs9Ts9Gs9Cs9Ts9Ts9Cs9Gs9Cs1ACs9C]	5S-9R-4S
192	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[Gs10Cs10Cs10Ts10Cs10As10Gs10Ts10Cs10Ts10Gs10Cs10Ts10Ts10Cs10Gs10Cs1ACs10C]	5S-9R-4S
193	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[Gs11Cs11Cs11Ts11Cs11As11Gs11Ts11Cs11Ts11Gs11Cs11Ts11Ts11Cs11Gs11Cs1ACs11C]	5S-9R-4S
194	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[Gs12Cs12Cs12Ts12Cs12As12Gs12Ts12Cs12Ts12Gs12Cs12Ts12Ts12Cs12Gs12Cs1ACs12C]	5S-9R-4S
195	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[Gs13Cs13Cs13Ts13Cs13As13Gs13Ts13Cs13Ts13Gs13Cs13Ts13Ts13Cs13Gs13Cs1ACs13C]	5S-9R-4S
196	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[Gs14Cs14Cs14Ts14Cs14As14Gs14Ts14Cs14Ts14Gs14Cs14Ts14Ts14Cs14Gs14Cs1ACs14C]	5S-9R-4S
197	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[Gs15Cs15Cs15Ts15Cs15As15Gs15Ts15Cs15Ts15Gs15Cs15Ts15Ts15Cs15Gs15Cs1ACs15C]	5S-9R-4S
198	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-GsCsCsUsCsAsGsUsCsUsGsCsUsUsCsGsCsACsC	5S-9R-4S
199	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-Gs1Cs1Cs1Us1Cs1As1Gs1Us1Cs1Us1Gs1Cs1Us1Us1Cs1Gs1CsACs1C	5S-9R-4S
200	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-Gs8Cs8Cs8Us8Cs8As8Gs8Us8Cs8Us8Gs8Cs8Us8Us8Cs8Gs8Cs1ACs8C	5S-9R-4S
201	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-Gs9Cs9Cs9Us9Cs9As9Gs9Us9Cs9Us9Gs9Cs9Us9Us9Cs9Gs9Cs1ACs9C	5S-9R-4S
202	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-Gs10Cs10Cs10Us10Cs10As10Gs10Us10Cs10Us10Gs10Cs10Us10Us10Cs10Gs10Cs1ACs10C	5S-9R-4S
203	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-Gs11Cs11Cs11Us11Cs11As11Gs11Us11Cs11Us11Gs11Cs11Us11Us11Cs11Gs11Cs1ACs11C	5S-9R-4S
204	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-Gs12Cs12Cs12Us12Cs12As12Gs12Us12Cs12Us12Gs12Cs12Us12Us12Cs12Gs12Cs1ACs12C	5S-9R-4S
205	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-Gs13Cs13Cs13Us13Cs13As13Gs13Us13Cs13Us13Gs13Cs13Us13Us13Cs13Gs13Cs1ACs13C	5S-9R-4S
206	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-	5S-9R-4S

	Gs14Cs14Cs14Us14Cs14As14Gs14Us14Cs14Us14Gs14Cs14Us14Us 14Cs14Gs14Cs1ACs14C	
207	(*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-Gs15Cs15Cs15Us15Cs15As15Gs15Us15Cs15Us15Gs15Cs15Us15Us 15Cs15Gs15Cs1ACs15C	5S-9R-4S

Oligonucleotide compositions

[00511] The present invention provides compositions comprising or consisting of a plurality of provided oligonucleotides (e.g., chirally controlled oligonucleotide compositions). In some embodiments, all such provided oligonucleotides are of the same type, i.e., all have the same base sequence, pattern of backbone linkages (i.e., pattern of internucleotidic linkage types, for example, phosphate, phosphorothioate, etc), pattern of backbone chiral centers (i.e. pattern of linkage phosphorus stereochemistry (*Rp*/*Sp*)), and pattern of backbone phosphorus modifications (e.g., pattern of "-XLR^1" groups in formula **I**). In many embodiments, however, provided compositions comprise a plurality of oligonucleotides types, typically in pre-determined relative amounts.

[00512] In some embodiments, a provided chirally controlled oligonucleotide composition is a chirally pure mipomersen composition. That is to say, in some embodiments, a provided chirally controlled oligonucleotide composition provides mipomersen as a single diastereomer with respect to the configuration of the linkage phosphorus.

[00513] In some embodiments, a provided chirally controlled oligonucleotide composition is a chirally uniform mipomersen composition. That is to say, in some embodiments, every linkage phosphorus of mipomersen is in the *Rp* configuration or every linkage phosphorus of mipomersen is in the *Sp* configuration.

[00514] In some embodiments, a provided chirally controlled oligonucleotide composition comprises a combination of one or more provided oligonucleotide types. One of skill in the chemical and medicinal arts will recognize that the selection and amount of each of the one or more types of provided oligonucleotides in a provided composition will depend on the intended use of that composition. That is to say, one of skill in the relevant arts would design a provided chirally controlled oligonucleotide composition such that the amounts and types of provided oligonucleotides contained therein cause the composition as a whole to have certain desirable characteristics (e.g., biologically desirable, therapeutically desirable, etc.).

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[00515] In some embodiments, a provided chirally controlled oligonucleotide composition comprises a combination of two or more provided oligonucleotide types. In some embodiments, a provided chirally controlled oligonucleotide composition comprises a combination of three or more provided oligonucleotide types. In some embodiments, a provided chirally controlled oligonucleotide composition comprises a combination of four or more provided oligonucleotide types. In some embodiments, a provided chirally controlled oligonucleotide composition comprises a combination of five or more provided oligonucleotide types. In some embodiments, a provided chirally controlled oligonucleotide composition comprises a combination of six or more provided oligonucleotide types. In some embodiments, a provided chirally controlled oligonucleotide composition comprises a combination of seven or more provided oligonucleotide types. In some embodiments, a provided chirally controlled oligonucleotide composition comprises a combination of eight or more provided oligonucleotide types. In some embodiments, a provided chirally controlled oligonucleotide composition comprises a combination of nine or more provided oligonucleotide types. In some embodiments, a provided chirally controlled oligonucleotide composition comprises a combination of ten or more provided oligonucleotide types. In some embodiments, a provided chirally controlled oligonucleotide composition comprises a combination of fifteen or more provided oligonucleotide types.

[00516] In some embodiments, a provided chirally controlled oligonucleotide composition is a combination of an amount of chirally uniform mipomersen of the *R*p configuration and an amount of chirally uniform mipomersen of the *S*p configuration.

[00517] In some embodiments, a provided chirally controlled oligonucleotide composition is a combination of an amount of chirally uniform mipomersen of the *R*p configuration, an amount of chirally uniform mipomersen of the *S*p configuration, and an amount of one or more chirally pure mipomersen of a desired diastereomeric form.

Methods for Making Chirally Controlled Oligonucleotides and Compositions Thereof

[00518] The present invention provides methods for making chirally controlled oligonucleotides and chirally controlled compositions comprising one or more specific nucleotide types. As noted above, the phrase "oligonucleotide type," as used herein, defines an oligonucleotide that has a particular base sequence, pattern of backbone linkages, pattern of

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backbone chiral centers, and pattern of backbone phosphorus modifications (e.g., "-XLR^1" groups). Oligonucleotides of a common designated "type" are structurally identical to one another with respect to base sequence, pattern of backbone linkages, pattern of backbone chiral centers, and pattern of backbone phosphorus modifications.

[00519] In some embodiments, a provided chirally controlled oligonucleotide in the invention has properties different from those of the corresponding stereorandom oligonucleotide mixture. In some embodiments, a chirally controlled oligonucleotide has lipophilicity different from that of the stereorandom oligonucleotide mixture. In some embodiments, a chirally controlled oligonucleotide has different retention time on HPLC. In some embodiments, a chirally controlled oligonucleotide may have a peak retention time significantly different from that of the corresponding stereorandom oligonucleotide mixture. During oligonucleotide purification using HPLC as generally practiced in the art, certain chirally controlled oligonucleotides will be largely if not totally lost. During oligonucleotide purification using HPLC as generally practiced in the art, certain chirally controlled oligonucleotides will be largely if not totally lost. One of the consequences is that certain diastereomers of a stereorandom oligonucleotide mixture (certain chirally controlled oligonucleotides) are not tested in assays. Another consequence is that from batches to batches, due to the inevitable instrumental and human errors, the supposedly "pure" stereorandom oligonucleotide will have inconsistent compositions in that diastereomers in the composition, and their relative and absolute amounts, are different from batches to batches. The chirally controlled oligonucleotide and chirally controlled oligonucleotide composition provided in this invention overcome such problems, as a chirally controlled oligonucleotide is synthesized in a chirally controlled fashion as a single diastereomer, and a chirally controlled oligonucleotide composition comprise predetermined levels of one or more individual oligonucleotide types.

[00520] One of skill in the chemical and synthetic arts will appreciate that synthetic methods of the present invention provide for a degree of control during each step of the synthesis of a provided oligonucleotide such that each nucleotide unit of the oligonucleotide can be designed and/or selected in advance to have a particular stereochemistry at the linkage phosphorus and/or a particular modification at the linkage phosphorus, and/or a particular base, and/or a particular sugar. In some embodiments, a provided oligonucleotide is designed and/or

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selected in advance to have a particular combination of stereocenters at the linkage phosphorus of the internucleotidic linkage.

[00521] In some embodiments, a provided oligonucleotide made using methods of the present invention is designed and/or determined to have a particular combination of linkage phosphorus modifications. In some embodiments, a provided oligonucleotide made using methods of the present invention is designed and/or determined to have a particular combination of bases. In some embodiments, a provided oligonucleotide made using methods of the present invention is designed and/or determined to have a particular combination of sugars. In some embodiments, a provided oligonucleotide made using methods of the present invention is designed and/or determined to have a particular combination of one or more of the above structural characteristics.

[00522] Methods of the present invention exhibit a high degree of chiral control. For instance, methods of the present invention facilitate control of the stereochemical configuration of every single linkage phosphorus within a provided oligonucleotide. In some embodiments, methods of the present invention provide an oligonucleotide comprising one or more modified internucleotidic linkages independently having the structure of formula **I**.

[00523] In some embodiments, methods of the present invention provide an oligonucleotide which is a mipomersen unimer. In some embodiments, methods of the present invention provide an oligonucleotide which is a mipomersen unimer of configuration *R*p. In some embodiments, methods of the present invention provide an oligonucleotide which is a mipomersen unimer of configuration *S*p.

[00524] In some embodiments, methods of the present invention provide a chirally controlled oligonucleotide composition, i.e., an oligonucleotide composition that contains predetermined levels of individual oligonucleotide types. In some embodiments a chirally controlled oligonucleotide composition comprises one oligonucleotide type. In some embodiments, a chirally controlled oligonucleotide composition comprises more than one oligonucleotide type. In some embodiments, a chirally controlled oligonucleotide composition comprises a plurality of oligonucleotide types. Exemplary chirally controlled oligonucleotide compositions made in accordance with the present invention are described herein.

[00525] In some embodiments, methods of the present invention provide chirally pure mipomersen compositions with respect to the configuration of the linkage phosphorus. That is to

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say, in some embodiments, methods of the present invention provide compositions of mipomersen wherein mipomersen exists in the composition in the form of a single diastereomer with respect to the configuration of the linkage phosphorus.

[00526] In some embodiments, methods of the present invention provide chirally uniform mipomersen compositions with respect to the configuration of the linkage phosphorus. That is to say, in some embodiments, methods of the present invention provide compositions of mipomersen in which all nucleotide units therein have the same stereochemistry with respect to the configuration of the linkage phosphorus, e.g., all nucleotide units are of the *R*p configuration at the linkage phosphorus or all nucleotide units are of the *S*p configuration at the linkage phosphorus.

[00527] In some embodiments, a provided chirally controlled oligonucleotide is over 50% pure. In some embodiments, a provided chirally controlled oligonucleotide is over about 55% pure. In some embodiments, a provided chirally controlled oligonucleotide is over about 60% pure. In some embodiments, a provided chirally controlled oligonucleotide is over about 65% pure. In some embodiments, a provided chirally controlled oligonucleotide is over about 70% pure. In some embodiments, a provided chirally controlled oligonucleotide is over about 75% pure. In some embodiments, a provided chirally controlled oligonucleotide is over about 80% pure. In some embodiments, a provided chirally controlled oligonucleotide is over about 85% pure. In some embodiments, a provided chirally controlled oligonucleotide is over about 90% pure. In some embodiments, a provided chirally controlled oligonucleotide is over about 91% pure. In some embodiments, a provided chirally controlled oligonucleotide is over about 92% pure. In some embodiments, a provided chirally controlled oligonucleotide is over about 93% pure. In some embodiments, a provided chirally controlled oligonucleotide is over about 94% pure. In some embodiments, a provided chirally controlled oligonucleotide is over about 95% pure. In some embodiments, a provided chirally controlled oligonucleotide is over about 96% pure. In some embodiments, a provided chirally controlled oligonucleotide is over about 97% pure. In some embodiments, a provided chirally controlled oligonucleotide is over about 98% pure. In some embodiments, a provided chirally controlled oligonucleotide is over about 99% pure. In some embodiments, a provided chirally controlled oligonucleotide is over about 99.5% pure. In some embodiments, a provided chirally controlled oligonucleotide is over about 99.6% pure. In some embodiments, a provided chirally controlled oligonucleotide is over about 99.7%

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pure. In some embodiments, a provided chirally controlled oligonucleotide is over about 99.8% pure. In some embodiments, a provided chirally controlled oligonucleotide is over about 99.9% pure. In some embodiments, a provided chirally controlled oligonucleotide is over at least about 99% pure.

[00528] In some embodiments, a chirally controlled oligonucleotide composition is a composition designed to comprise a single oligonucleotide type. In certain embodiments, such compositions are about 50% diastereomerically pure. In some embodiments, such compositions are about 50% diastereomerically pure. In some embodiments, such compositions are about 50% diastereomerically pure. In some embodiments, such compositions are about 55% diastereomerically pure. In some embodiments, such compositions are about 60% diastereomerically pure. In some embodiments, such compositions are about 65% diastereomerically pure. In some embodiments, such compositions are about 70% diastereomerically pure. In some embodiments, such compositions are about 75% diastereomerically pure. In some embodiments, such compositions are about 80% diastereomerically pure. In some embodiments, such compositions are about 85% diastereomerically pure. In some embodiments, such compositions are about 90% diastereomerically pure. In some embodiments, such compositions are about 91% diastereomerically pure. In some embodiments, such compositions are about 92% diastereomerically pure. In some embodiments, such compositions are about 93% diastereomerically pure. In some embodiments, such compositions are about 94% diastereomerically pure. In some embodiments, such compositions are about 95% diastereomerically pure. In some embodiments, such compositions are about 96% diastereomerically pure. In some embodiments, such compositions are about 97% diastereomerically pure. In some embodiments, such compositions are about 98% diastereomerically pure. In some embodiments, such compositions are about 99% diastereomerically pure. In some embodiments, such compositions are about 99.5% diastereomerically pure. In some embodiments, such compositions are about 99.6% diastereomerically pure. In some embodiments, such compositions are about 99.7% diastereomerically pure. In some embodiments, such compositions are about 99.8% diastereomerically pure. In some embodiments, such compositions are about 99.9%

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diastereomerically pure. In some embodiments, such compositions are at least about 99% diastereomerically pure.

[00529] In some embodiments, a chirally controlled oligonucleotide composition is a composition designed to comprise multiple oligonucleotide types. In some embodiments, methods of the present invention allow for the generation of a library of chirally controlled oligonucleotides such that a pre-selected amount of any one or more chirally controlled oligonucleotide types can be mixed with any one or more other chirally controlled oligonucleotide types to create a chirally controlled oligonucleotide composition. In some embodiments, the pre-selected amount of an oligonucleotide type is a composition having any one of the above-described diastereomeric purities.

[00530] In some embodiments, the present invention provides methods for making a chirally controlled oligonucleotide comprising steps of:

(1) coupling;

(2) capping;

(3) modifying;

(4) deblocking; and

(5) repeating steps (1) – (4) until a desired length is achieved.

[00531] When describing the provided methods, the word "cycle" has its ordinary meaning as understood by a person of ordinary skill in the art. In some embodiments, one round of steps (1)-(4) is referred to as a cycle.

[00532] In some embodiments, the present invention provides methods for making chirally controlled oligonucleotide compositions, comprising steps of:

(a) providing an amount of a first chirally controlled oligonucleotide; and

(b) optionally providing an amount of one or more additional chirally controlled oligonucleotides.

[00533] In some embodiments, a first chirally controlled oligonucleotide is an oligonucleotide type, as described herein. In some embodiments, a one or more additional chirally controlled oligonucleotide is a one or more oligonucleotide type, as described herein.

[00534] One of skill in the relevant chemical and synthetic arts will recognize the degree of versatility and control over structural variation and stereochemical configuration of a provided oligonucleotide when synthesized using methods of the present invention. For instance, after a

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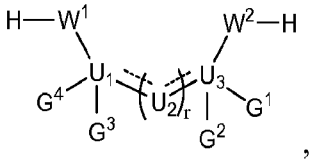
first cycle is complete, a subsequent cycle can be performed using a nucleotide unit individually selected for that subsequent cycle which, in some embodiments, comprises a nucleobase and/or a sugar that is different from the first cycle nucleobase and/or sugar. Likewise, the chiral auxiliary used in the coupling step of the subsequent cycle can be different from the chiral auxiliary used in the first cycle, such that the second cycle generates a phosphorus linkage of a different stereochemical configuration. In some embodiments, the stereochemistry of the linkage phosphorus in the newly formed internucleotidic linkage is controlled by using stereochemically pure phosphoramidites. Additionally, the modification reagent used in the modifying step of a subsequent cycle can be different from the modification reagent used in the first or former cycle. The cumulative effect of this iterative assembly approach is such that each component of a provided oligonucleotide can be structurally and configurationally tailored to a high degree. An additional advantage to this approach is that the step of capping minimizes the formation of "n-1" impurities that would otherwise make isolation of a provided oligonucleotide extremely challenging, and especially oligonucleotides of longer lengths.

[00535] In some embodiments, an exemplary cycle of the method for making chirally controlled oligonucleotides is illustrated in Scheme I. In Scheme I,  represents the solid support, and optionally a portion of the growing chirally controlled oligonucleotide attached to the solid support. The chiral auxiliary exemplified has the structure of formula 3-I:



Formula 3-I

which is further described below. "Cap" is any chemical moiety introduced to the nitrogen atom by the capping step, and in some embodiments, is an amino protecting group. One of ordinary skill in the art understands that in the first cycle, there may be only one nucleoside attached to the solid support when started, and cycle exit can be performed optionally before deblocking. As understood by a person of skill in the art, B^{PRO} is a protected base used in oligonucleotide synthesis. Each step of the above-depicted cycle of Scheme I is described further below.

[00536] **Scheme I.** Synthesis of chirally controlled oligonucleotide.

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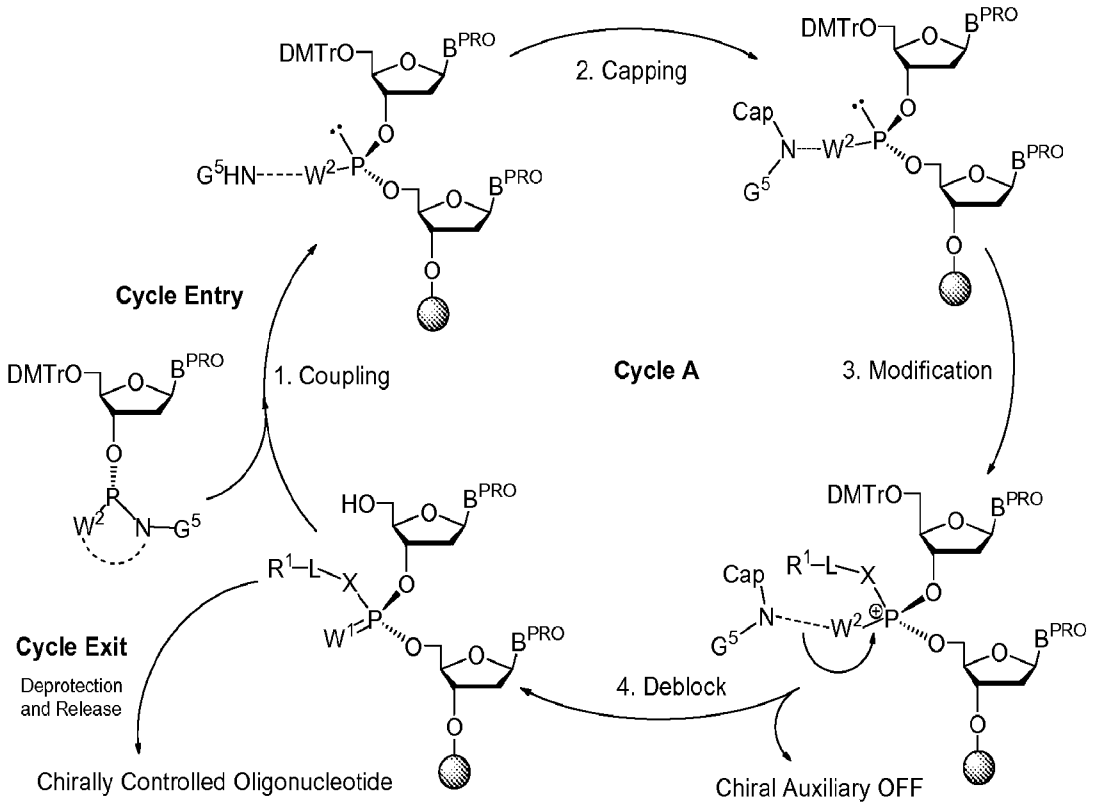

Synthesis on solid support

[00537] In some embodiments, the synthesis of a provided oligonucleotide is performed on solid phase. In some embodiments, reactive groups present on a solid support are protected. In some embodiments, reactive groups present on a solid support are unprotected. During oligonucleotide synthesis a solid support is treated with various reagents in several synthesis cycles to achieve the stepwise elongation of a growing oligonucleotide chain with individual nucleotide units. The nucleoside unit at the end of the chain which is directly linked to the solid support is termed "the first nucleoside" as used herein. A first nucleoside is bound to a solid support via a linker moiety, *i.e.* a diradical with covalent bonds between either of a CPG, a polymer or other solid support and a nucleoside. The linker stays intact during the synthesis cycles performed to assemble the oligonucleotide chain and is cleaved after the chain assembly to liberate the oligonucleotide from the support.

[00538] Solid supports for solid-phase nucleic acid synthesis include the supports described in, *e.g.*, US patents 4,659,774 , 5,141,813, 4,458,066; Caruthers U.S. Pat. Nos. 4,415,732, 4,458,066, 4,500,707, 4,668,777, 4,973,679, and 5,132,418; Andrus *et al.* U.S. Pat. Nos. 5,047,524, 5,262,530; and Koster U.S. Pat. Nos. 4,725,677 (reissued as Re34,069). In some embodiments, a solid phase is an organic polymer support. In some embodiments, a solid phase

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is an inorganic polymer support. In some embodiments, an organic polymer support is polystyrene, aminomethyl polystyrene, a polyethylene glycol-polystyrene graft copolymer, polyacrylamide, polymethacrylate, polyvinylalcohol, highly cross-linked polymer (HCP), or other synthetic polymers, carbohydrates such as cellulose and starch or other polymeric carbohydrates, or other organic polymers and any copolymers, composite materials or combination of the above inorganic or organic materials. In some embodiments, an inorganic polymer support is silica, alumina, controlled polyglass (CPG), which is a silica-gel support, or aminopropyl CPG. Other useful solid supports include fluorous solid supports (see *e.g.,* WO/2005/070859), long chain alkylamine (LCAA) controlled pore glass (CPG) solid supports (see *e.g.*, S. P. Adams, K. S. Kavka, E. J. Wykes, S. B. Holder and G. R. Galluppi, *J. Am. Chem. Soc.*, **1983**, *105*, 661-663; G. R. Gough, M. J. Bruden and P. T. Gilham, *Tetrahedron Lett.*, **1981**, *22*, 4177-4180). Membrane supports and polymeric membranes (see *e.g.* Innovation and Perspectives in Solid Phase Synthesis, Peptides, Proteins and Nucleic Acids, ch 21 pp 157-162, 1994, Ed. Roger Epton and U.S. Pat. No. 4,923,901) are also useful for the synthesis of nucleic acids. Once formed, a membrane can be chemically functionalized for use in nucleic acid synthesis. In addition to the attachment of a functional group to the membrane, the use of a linker or spacer group attached to the membrane is also used in some embodiments to minimize steric hindrance between the membrane and the synthesized chain.

[00539] Other suitable solid supports include those generally known in the art to be suitable for use in solid phase methodologies, including, for example, glass sold as PrimerTM 200 support, controlled pore glass (CPG), oxalyl-controlled pore glass (see, *e.g.*, Alul, *et al.*, *Nucleic Acids Research*, **1991**, *19*, 1527), TentaGel Support-an aminopolyethyleneglycol derivatized support (see, *e.g.*, Wright, *et al.*, *Tetrahedron Lett.*, **1993**, *34*, 3373), and Poros-a copolymer of polystyrene/divinylbenzene.

[00540] Surface activated polymers have been demonstrated for use in synthesis of natural and modified nucleic acids and proteins on several solid supports mediums. A solid support material can be any polymer suitably uniform in porosity, having sufficient amine content, and sufficient flexibility to undergo any attendant manipulations without losing integrity. Examples of suitable selected materials include nylon, polypropylene, polyester, polytetrafluoroethylene, polystyrene, polycarbonate, and nitrocellulose. Other materials can serve as a solid support, depending on the design of the investigator. In consideration of some designs, for example, a

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coated metal, in particular gold or platinum can be selected (see *e.g.*, US publication No. 20010055761). In one embodiment of oligonucleotide synthesis, for example, a nucleoside is anchored to a solid support which is functionalized with hydroxyl or amino residues. Alternatively, a solid support is derivatized to provide an acid labile trialkoxytrityl group, such as a trimethoxytrityl group (TMT). Without being bound by theory, it is expected that the presence of a trialkoxytrityl protecting group will permit initial detritylation under conditions commonly used on DNA synthesizers. For a faster release of oligonucleotide material in solution with aqueous ammonia, a diglycoate linker is optionally introduced onto the support.

[00541] In some embodiments, a provided oligonucleotide alternatively is synthesized from the 5′ to 3′ direction. In some embodiments, a nucleic acid is attached to a solid support through its 5′ end of the growing nucleic acid, thereby presenting its 3′ group for reaction, i.e. using 5′-nucleoside phosphoramidites or in enzymatic reaction (*e.g.* ligation and polymerization using nucleoside 5′-triphosphates). When considering the 5' to 3' synthesis the iterative steps of the present invention remain unchanged (i.e. capping and modification on the chiral phosphorus).

Linking moiety

[00542] A linking moiety or linker is optionally used to connect a solid support to a compound comprising a free nucleophilic moiety. Suitable linkers are known such as short molecules which serve to connect a solid support to functional groups (*e.g.*, hydroxyl groups) of initial nucleosides molecules in solid phase synthetic techniques. In some embodiments, the linking moiety is a succinamic acid linker, or a succinate linker (-CO-CH_2-CH_2-CO-), or an oxalyl linker (-CO-CO-). In some embodiments, the linking moiety and the nucleoside are bonded together through an ester bond. In some embodiments, a linking moiety and a nucleoside are bonded together through an amide bond. In some embodiments, a linking moiety connects a nucleoside to another nucleotide or nucleic acid. Suitable linkers are disclosed in, for example, *Oligonucleotides And Analogues A Practical Approach*, Ekstein, F. Ed., IRL Press, N.Y., **1991**, Chapter 1 and Solid-Phase Supports for Oligonucleotide Synthesis, Pon, R. T., *Curr. Prot. Nucleic Acid Chem.*, **2000**, 3.1.1-3.1.28.

[00543] A linker moiety is used to connect a compound comprising a free nucleophilic moiety to another nucleoside, nucleotide, or nucleic acid. In some embodiments, a linking moiety is a phosphodiester linkage. In some embodiments, a linking moiety is an *H*-phosphonate

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moiety. In some embodiments, a linking moiety is a modified phosphorus linkage as described herein. In some embodiments, a universal linker (UnyLinker) is used to attached the oligonucleotide to the solid support (Ravikumar et al., *Org. Process Res. Dev.*, **2008**, *12* (3), 399–410). In some embodiments, other universal linkers are used (Pon, R. T., *Curr. Prot. Nucleic Acid Chem.*, **2000**, 3.1.1-3.1.28). In some embodiments, various orthogonal linkers (such as disulfide linkers) are used (Pon, R. T., *Curr. Prot. Nucleic Acid Chem.*, **2000**, 3.1.1-3.1.28).

General conditions - solvents for synthesis

[00544] Syntheses of provided oligonucleotides are generally performed in aprotic organic solvents. In some embodiments, a solvent is a nitrile solvent such as, e.g., acetonitrile. In some embodiments, a solvent is a basic amine solvent such as, e.g., pyridine. In some embodiments, a solvent is an ethereal solvent such as, e.g., tetrahydrofuran. In some embodiments, a solvent is a halogenated hydrocarbon such as, e.g., dichloromethane. In some embodiments, a mixture of solvents is used. In certain embodiments a solvent is a mixture of any one or more of the above-described classes of solvents.

[00545] In some embodiments, when an aprotic organic solvent is not basic, a base is present in the reacting step. In some embodiments where a base is present, the base is an amine base such as, e.g., pyridine, quinoline, or *N*,*N*-dimethylaniline. Exemplary other amine bases include pyrrolidine, piperidine, *N*-methyl pyrrolidine, pyridine, quinoline, *N*,*N*-dimethylaminopyridine (DMAP), or *N*,*N*-dimethylaniline.

[00546] In some embodiments, a base is other than an amine base.

[00547] In some embodiments, an aprotic organic solvent is anhydrous. In some embodiments, an anhydrous aprotic organic solvent is freshly distilled. In some embodiments, a freshly distilled anhydrous aprotic organic solvent is a basic amine solvent such as, e.g., pyridine. In some embodiments, a freshly distilled anhydrous aprotic organic solvent is an ethereal solvent such as, e.g., tetrahydrofuran. In some embodiments, a freshly distilled anhydrous aprotic organic solvent is a nitrile solvent such as, e.g., acetonitrile.

Chiral reagent

[00548] In provided methods, chiral reagents are used to confer stereoselectivity in the production of chirally controlled olignucleotides. Many different chiral reagents, also referred to

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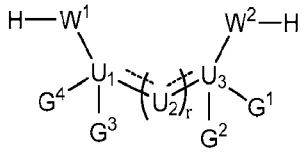
by those of skill in the art and herein as chiral auxiliaries, may be used in accordance with methods of the present invention. Exemplary such chiral reagents are described herein and in Wada I, II and III, referenced above. In certain embodiments, a chiral reagent is as described by Wada I. In some embodiments, a chiral reagent for use in accordance with the methods of the present invention are of Formula **3-I**, below:

[00549]



Formula 3-I

wherein W^1 and W^2 are any of -O-, -S-, or -NG^5-, U_1 and U_3 are carbon atoms which are bonded to U_2 if present, or to each other if r is 0, via a single, double or triple bond. U_2 is -C-, -CG^8-, -CG^8G^8-, -NG^8-, -N-, -O-, or -S- where r is an integer of 0 to 5 and no more than two heteroatoms are adjacent. When any one of U_2 is C, a triple bond must be formed between a second instance of U_2, which is C, or to one of U_1 or U_3. Similarly, when any one of U_2 is CG^8, a double bond is formed between a second instance of U_2 which is -CG^8- or -N-, or to one of U_1 or U_3.

[00550] In some embodiments, -U_1-$(U_2)_r$-U_3- is -CG^3G^4-CG^1G^2-. In some embodiments, -U_1-$(U_2)_r$-U_3- is -CG^3= CG^1-. In some embodiments, -U_1-$(U_2)_r$-U_3- is -C≡C-. In some embodiments, -U_1-$(U_2)_r$-U_3- is -CG^3=CG^8-CG^1G^2-. In some embodiments, -U_1-$(U_2)_r$-U_3- is -CG^3G^4-O-CG^1G^2-. In some embodiments, -U_1-$(U_2)_r$-U_3- is -CG^3G^4-NG^8-CG^1G^2-. In some embodiments, -U_1-$(U_2)_r$-U_3- is -CG^3G^4-N-CG^2-. In some embodiments, -U_1-$(U_2)_r$-U_3- is -CG^3G^4-N=C G^8-CG^1G^2-.

[00551] As defined herein, G^1, G^2, G^3, G^4, G^5, and G^8 are independently hydrogen, or an optionally substituted group selected from alkyl, aralkyl, cycloalkyl, cycloalkylalkyl, heterocyclyl, heteroaryl, and aryl; or two of G^1, G^2, G^3, G^4, and G^5 are G^6 taken together to form an optionally substituted, saturated, partially unsaturated or unsaturated carbocyclic or heteroatom-containing ring of up to about 20 ring atoms which is monocyclic or polycyclic, and is fused or unfused. In some embodiments, a ring so formed is substituted by oxo, thioxo, alkyl, alkenyl, alkynyl, heteroaryl, or aryl moieties. In some embodiments, when a ring formed by taking two G^6 together is substituted, it is substituted by a moiety which is bulky enough to confer stereoselectivity during the reaction.

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[00552] In some embodiments, a ring formed by taking two of G^6 together is optionally substituted cyclopentyl, pyrrolyl, cyclopropyl, cyclohexenyl, cyclopentenyl, tetrahydropyranyl, or piperazinyl. In some embodiments, a ring formed by taking two of G^6 together is optionally substituted cyclopentyl, pyrrolyl, cyclopropyl, cyclohexenyl, cyclopentenyl, tetrahydropyranyl, pyrrolidinyl, or piperazinyl.

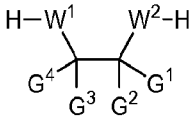
[00553] In some embodiments, G^1 is optionally substituted phenyl. In some embodiments, G^1 is phenyl. In some embodiments, G^2 is methyl or hydrogen. In some embodiments, G^1 is optionally substituted phenyl and G^2 is methyl. In some embodiments, G^1 is phenyl and G^2 is methyl.

[00554] In some embodiments, r is 0.

[00555] In some embodiments, W^1 is $-NG^5-$. In some embodiments, one of G^3 and G^4 is taken together with G^5 to form an optionally substituted pyrrolidinyl ring. In some embodiments, one of G^3 and G^4 is taken together with G^5 to form a pyrrolidinyl ring.

[00556] In some embodiments, W^2 is $-O-$.

[00557] In some embodiments, a chiral reagent is a compound of Formula 3-AA:



Formula 3-AA

wherein each variable is independently as defined above and described herein.

[00558] In some embodiments of Formula 3AA, W^1 and W^2 are independently $-NG^5-$, -O-, or -S-; G^1, G^2, G^3, G^4, and G^5 are independently hydrogen, or an optionally substituted group selected from alkyl, aralkyl, cycloalkyl, cycloalkylalkyl, heterocyclyl, heteroaryl, or aryl; or two of G^1, G^2, G^3, G^4, and G^5 are G^6 taken together to form an optionally substituted saturated, partially unsaturated or unsaturated carbocyclic or heteroatom-containing ring of up to about 20 ring atoms which is monocyclic or polycyclic, fused or unfused, and no more than four of G^1, G^2, G^3, G^4, and G^5 are G^6. Similarly to the compounds of Formula 3-I, any of G^1, G^2, G^3, G^4, or G^5 are optionally substituted by oxo, thioxo, alkyl, alkenyl, alkynyl, heteroaryl, or aryl moieties. In some embodiments, such substitution induces stereoselectivity in chirally controlled oligonucleotide production.

[00559] In some embodiments, a chiral reagent has one of the following formulae:

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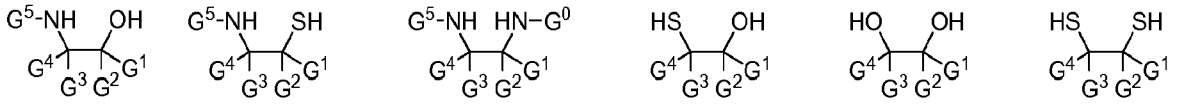
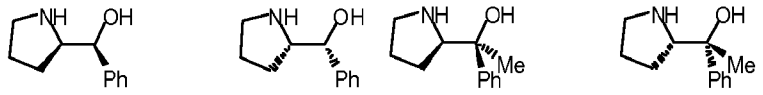


Formulae 3-AB 3-BB 3-CC 3-DD 3-EE 3-FF

[00560] In some embodiments, a chiral reagent is an aminoalcohol. In some embodiments, a chiral reagent is an aminothiol. In some embodiments, a chiral reagent is an aminophenol. In some embodiments, a chiral reagent is (*S*)- and (*R*)-2-methylamino-1-phenylethanol, (1*R*, 2*S*)-ephedrine, or (1*R*, 2*S*)-2-methylamino-1,2-diphenylethanol.

[00561] In some embodiments of the invention, a chiral reagent is a compound of one of the following formulae:



Formula O Formula P Formula Q Formula R

[00562] The choice of chiral reagent, for example, the isomer represented by Formula Q or its stereoisomer, Formula R, permits specific control of chirality at a linkage phosphorus. Thus, either an *R*p or *S*p configuration can be selected in each synthetic cycle, permitting control of the overall three dimensional structure of a chirally controlled oligonucleotide. In some embodiments, a chirally controlled oligonucleotide has all *R*p stereocenters. In some embodiments of the invention, a chirally controlled oligonucleotide has all *S*p stereocenters. In some embodiments of the invention, each linkage phosphorus in the chirally controlled oligonucleotide is independently *R*p or *S*p. In some embodiments of the invention, each linkage phosphorus in the chirally controlled oligonucleotide is independently *R*p or *S*p, and at least one is *R*p and at least one is *S*p. In some embodiments, the selection of *R*p and *S*p centers is made to confer a specific three dimensional superstructure to a chirally controlled oligonucleotide. Exemplary such selections are described in further detail herein.

[00563] In some embodiments, a chiral reagent for use in accordance with the present invention is selected for its ablility to be removed at a particular step in the above-depicted cycle. For example, in some embodiments it is desirable to remove a chiral reagent during the step of modifying the linkage phosphorus. In some embodiments, it is desirable to remove a chiral reagent before the step of modifying the linkage phosphorus. In some embodiments, it is desirable to remove a chiral reagent after the step of modifying the linkage phosphorus. In some embodiments, it is desirable to remove a chiral reagent after a first coupling step has occurred

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but before a second coupling step has occurred, such that a chiral reagent is not present on the growing oligonucleotide during the second coupling (and likewise for additional subsequent coupling steps). In some embodiments, a chiral reagent is removed during the "deblock" reaction that occurs after modification of the linkage phosphorus but before a subsequent cycle begins. Exemplary methods and reagents for removal are described herein.

[00564] In some embodiments, removal of chiral auxiliary is achieved when performing the modification and/or deblocking step, as illustrated in Scheme I. It can be beneficial to combine chiral auxiliary removal together with other transformations, such as modification and deblocking. A person of ordinary skill in the art would appreciate that the saved steps/transformation could improve the overall efficiency of synthesis, for instance, with respect to yield and product purity, especially for longer oligonucleotides. One example wherein the chiral auxiliary is removed during modification and/or deblocking is illustrated in Scheme I.

[00565] In some embodiments, a chiral reagent for use in accordance with methods of the present invention is characterized in that it is removable under certain conditions. For instance, in some embodiments, a chiral reagent is selected for its ability to be removed under acidic conditions. In certain embodiments, a chiral reagent is selected for its ability to be removed under mildly acidic conditions. In certain embodiments, a chiral reagent is selected for its ability to be removed by way of an E1 elimination reaction (e.g., removal occurs due to the formation of a cation intermediate on the chiral reagent under acidic conditons, causing the chiral reagent to cleave from the oligonucleotide). In some embodiments, a chiral reagent is characterized in that it has a structure recognized as being able to accommodate or facilitate an E1 elimination reaction. One of skill in the relevant arts will appreciate which structures would be envisaged as being prone toward undergoing such elimination reactions.

[00566] In some embodiments, a chiral reagent is selected for its ability to be removed with a nucleophile. In some embodiments, a chiral reagent is selected for its ability to be removed with an amine nucleophile. In some embodiments, a chiral reagent is selected for its ability to be removed with a nucleophile other than an amine.

[00567] In some embodiments, a chiral reagent is selected for its ability to be removed with a base. In some embodiments, a chiral reagent is selected for its ability to be removed with an amine. In some embodiments, a chiral reagent is selected for its ability to be removed with a base other than an amine.

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Further Embodiments of Chiral Reagents

[00568] In some embodiments, the present invention is directed to a chiral reagent that is used to synthesize chirally controlled oligonucleotides.

[00569] In some embodiments, the present invention provides chiral reagents that are stable to the coupling, capping, modifying and deblocking steps described above and herein. In some embodiments, the present invention provides chiral reagents that are stable to the modifying and deblocking steps described above and herein. In some embodiments, the present invention provides chiral reagents that are stable to the sulfurization and deblocking steps described above and herein. In some embodiments, the present invention provides chiral reagents that are stable to the oxidation step described above and herein. In some embodiments, such a chiral reagent has a structure of formula Z-I.

[00570] In some embodiments, the present invention provides chiral reagents that are removed by treatment with a base and/or a nucleophile. In some embodiments, the present invention provides chiral reagents that are removed by treatment with a base and/or a nucleophile, and are stable to the coupling, capping, modifying and deblocking steps described above and herein. In some embodiments, the present invention provides chiral reagents that are removed by treatment comprising an amine. In some embodiments, the present invention provides chiral reagents that are removed by treatment comprising an amine, and are stable to the coupling, capping, modifying and deblocking steps described above and herein. In some embodiments, the present invention provides chiral reagents that are removed by the deprotection/cleavage conditions described in this application, and are stable to the coupling, capping, modifying and deblocking steps described above and herein. In some embodiments, such a chiral reagent has a structure of formula Z-I.

[00571] In some embodiments, the chiral reagents that are stable to the coupling, capping, modifying and deblocking steps are used to synthesize chirally controlled oligonucleotides described above and herein. In some embodiments, the chiral reagents that are stable to the coupling, capping, modifying and deblocking steps are used to synthesize chirally controlled oligonucleotides described above and herein, wherein the chirally controlled oligonucleotides comprise one or more phosphate diester or phosphorothioate diester linkages. In some embodiments, the chiral reagents that are stable to the coupling, capping, modifying and

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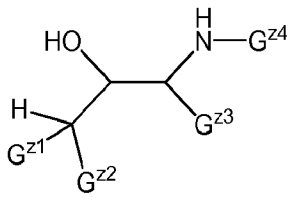
deblocking steps are used to synthesize chirally controlled oligonucleotides comprising one or more phosphate diester or phosphorothioate diester linkages, and are not removed until the desired oligonucleotide lengths have been achieved. In some embodiments, the chiral reagents that are stable to the coupling, capping, modifying and deblocking steps are used to synthesize chirally controlled oligonucleotides comprising one or more phosphate diester or phosphorothioate diester linkages, and are not removed until after cycle exit. In some embodiments, the chiral reagents that are stable to the coupling, capping, modifying and deblocking steps are used to synthesize chirally controlled oligonucleotides comprising one or more phosphate diester or phosphorothioate diester linkages, and are not removed until cleavage from solid support. In some embodiments, the chiral reagents that are stable to the coupling, capping, modifying and deblocking steps are used to synthesize chirally controlled oligonucleotides comprising one or more phosphate diester or phosphorothioate diester linkages, and are not removed until cleavage from solid support, and the removal is performed in the same step as cleavage from solid support. In some embodiments, such a chiral reagent has a structure of formula Z-I.

[00572] In some embodiments, when a chiral reagent that is stable to the coupling, capping, modifying and deblocking steps is used in oligonucleotide synthesis, the oligonucleotide with 5'–OH ready for coupling can be from any synthetic cycle, including those described in Schemes I, I-b, I-c, I-d, Z-1 and Z-2. In some embodiments, the oligonucleotide with 5'–OH for coupling comprises various types of internucleotidic linkages as described above and herein. After coupling, the modifying step as described in this application installs the desired modification to the linkage phosphorus. The product can either go to cycle exit before/after deblocking, or enter the next cycle after deblocking the 5'–OH. It is understood by a person of ordinary skill in the art that the next cycle can be any of the synthetic cycles described in this application, including but not limited to those in Schemes I, I-b, I-c, I-d, Z-1 and Z-2.

[00573] In some embodiments, a chiral reagent or a salt thereof for use in accordance with the present invention is of chemical formula (Z-I).



(Z-I)

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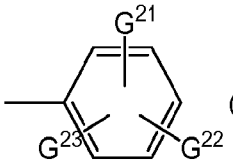
[00574] In the formula (Z-I), G^{z1} and G^{z2} are independently a hydrogen atom, a nitro group (-NO_2), a halogen atom, a cyano group (-CN), a group of formula (Z-II) or (Z-III), or both G^1 and G^2 taken together to form a group of formula (Z-IV).

[00575] In some embodiments, a group of formula (Z-II) is as depicted below:

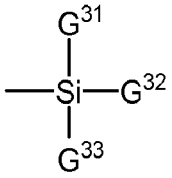


(Z-II)

,

wherein G^{21} to G^{23} are independently a hydrogen atom, a nitro group, a halogen atom, a cyano group or C_{1-3} alkyl group.

[00576] In some embodiments, a group of formula (Z-III) is as depicted below:

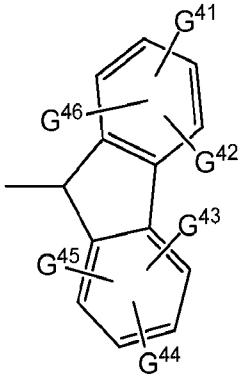


(Z-III)

,

wherein G^{31} to G^{33} are independently C_{1-4} alkyl group, C_{6-14} aryl group C_{1-4} alkoxy group, C_{7-14} aralkyl group, C_{1-4} alkyl C_{6-14} aryl group, C_{1-4} alkoxy C_{6-14} aryl group, or C_{6-14} aryl C_{1-4} alkyl group.

[00577] In some embodiments, a group of formula (Z-IV) is as depicted below:



(Z-IV)

,

wherein G^{41} to G^{46} are independently a hydrogen atom, a nitro group, a halogen atom, a cyano group or C_{1-3} alkyl group.

[00578] G^{z3} and G^{z4} are independently a hydrogen atom, C_{1-3} alkyl group, C_{6-14} aryl group, or both G^{z3} and G^{z4} taken together to form a heteroatom-containing ring that has 3 to 16 carbon atoms, together with the NH moiety in formula (Z-I).

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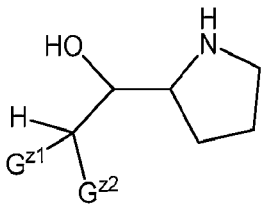
[00579] In some embodiments, a chiral reagent has following chemical formula (Z-I'):



(Z-I')

wherein G^{z1} and G^{z2} are same as above. Namely, G^{z1} and G^{z2} are independently a hydrogen atom, a nitro group, a halogen atom, a cyano group, a group of formula (Z-II) or (Z-III), or both G^{z1} and G^{z2} taken together to form a group of formula (Z-IV).

[00580] In certain embodiments, a chiral reagent has chemical formula (Z-I') and each of G^{z1} and G^{z2} is a group of formula (Z-II), wherein G^{21} to G^{23} are independently a hydrogen atom, a nitro group, a halogen atom, a cyano group or C_{1-3} alkyl group.

[00581] In certain embodiments, a chiral reagent has chemical formula (Z-I') and each of G^{z1} and G^{z2} is a group of formula (Z-II) and each of G^{21} to G^{23} is a hydrogen atom.

[00582] In certain embodiments, a chiral reagent has chemical formula (Z-I') and G^{z1} is a hydrogen atom, G^{z2} is a group of formula (Z-II), and G^{21} to G^{23} are independently a hydrogen atom, a nitro group, a halogen atom, a cyano group or C_{1-3} alkyl group.

[00583] In certain embodiments, a chiral reagent has chemical formula (Z-I') and G^{z1} is a hydrogen atom, G^{z2} is a group of formula (Z-II), each of G^{21} and G^{22} is a hydrogen atom and G^{23} is a nitro group.

[00584] In certain embodiments, a chiral reagent has chemical formula (Z-I') and G^{z1} is a hydrogen atom and G^{z2} is a group of formula (III), and G^{31} to G^{33} are independently C_{1-4} alkyl group, C_{6-14} aryl group, C_{7-14} aralkyl group, C_{1-4} alkyl C_{6-14} aryl group, C_{1-4} alkoxy C_{6-14} aryl group, or C_{6-14} aryl C_{1-4} alkyl group.

[00585] In some embodiments, the chiral reagent has chemical formula (I') and G^1 is a hydrogen atom and G^2 is a group of formula (III), and G^{31} to G^{33} are independently C_{1-4} alkyl group, C_6 aryl group, C_{7-10} aralkyl group, C_{1-4} alkyl C_6 aryl group, C_{1-4} alkoxy C_6 aryl group, or C_6 aryl C_{1-4} alkyl group.

[00586] In certain embodiments, a chiral reagent has chemical formula (Z-I') and G^{z1} is a hydrogen atom, G^{z2} is a group of formula (Z-III), and G^{31} to G^{33} are independently C_{1-4} alkyl group or C_6 aryl group. Examples of C_{1-4} alkyl group are methyl group, ethyl group, *n*-propyl group, *iso*-propyl group, *n*-buthyl group and *tert*-buthyl group.

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[00587] In certain embodiments, a chiral reagent has chemical formula (Z-I') and G^{z1} is a hydrogen atom, G^{z2} is a group of formula (Z-III), and G^{31} to G^{33} are independently C_{1-4} alkyl group.

[00588] In certain embodiments, a chiral reagent has chemical formula (Z-I') and G^{z1} is a hydrogen atom, G^{z2} is a group of formula (Z-III), and G^{31} and G^{33} are C_6 aryl group and G^{32} is C_{1-4} alkyl group.

[00589] In certain embodiments, a chiral reagent has chemical formula (Z-I') and G^{z1} and G^{z2} are taken together to form a group of formula (Z-IV), and G^{41} to G^{46} are independently a hydrogen atom, a nitro group, a halogen atom, a cyano group or C_{1-4} alkyl group.

[00590] In certain embodiments, a chiral reagent has chemical formula (Z-I') and G^{z1} and G^{z2} are taken together to form a group of formula (Z-IV), wherein each of G^{41} to G^{46} is a hydrogen atom.

[00591] In certain embodiments, a chiral reagent is selected from one of chemical formulae 3a, 3b, 5a, Z-5b, 7a, 7b, 9a, 9b, 11a and 11b:

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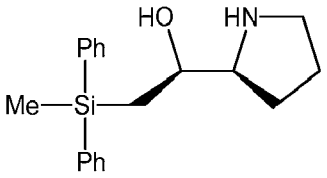
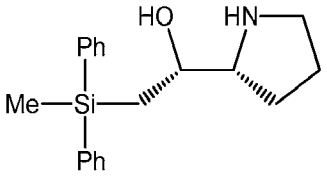
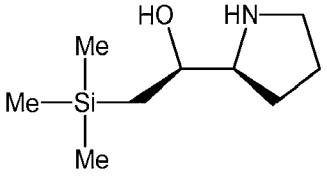
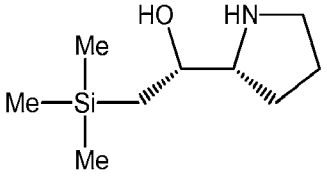
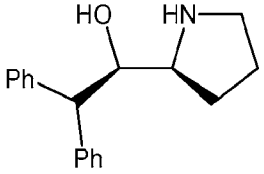
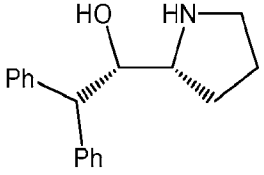

(3a)

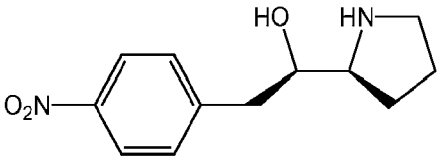

(3b)

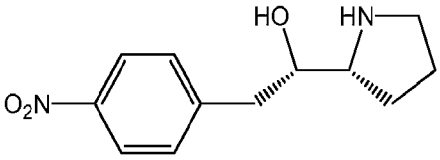

(5a)

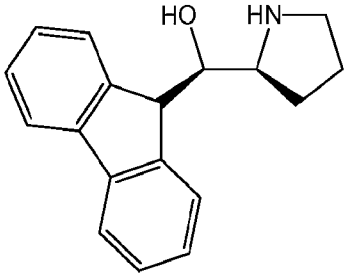

(Z-5b)

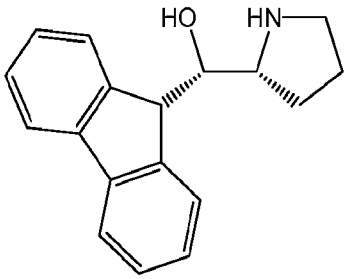

(7a)


(7b)

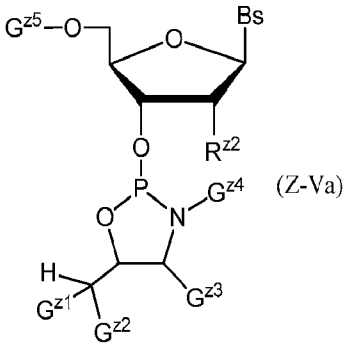

(9a)

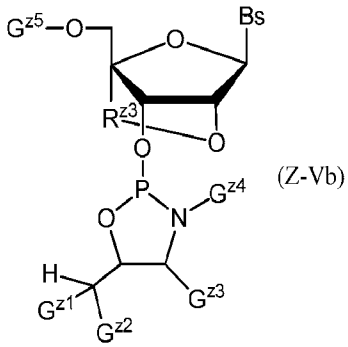

(9b)


(11a)

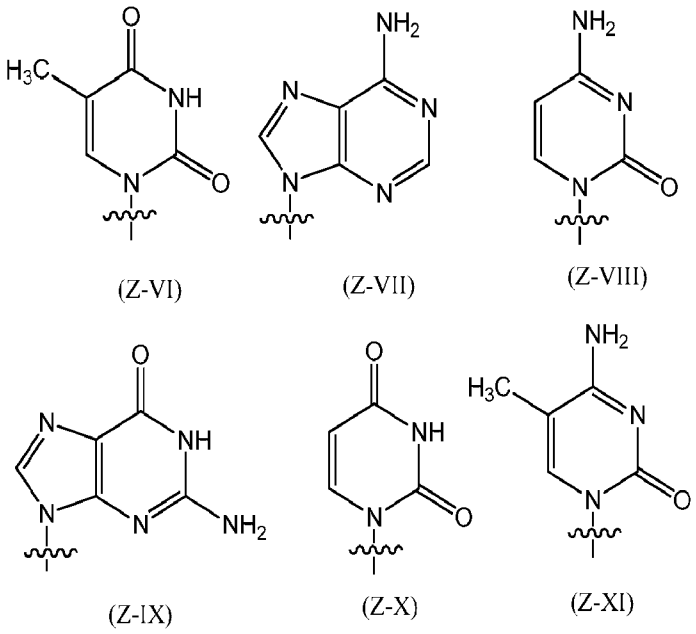

(11b)

[00592] In some embodiments, a nucleoside 3'-phosphoramidite derivative for use in accordance with the present invention is represented by formula (Z-Va) or (Z-Vb):


(Z-Va)


(Z-Vb)

wherein G^{z1} to G^{z4} are the same as above, G^{z5} is a protective group of the hydroxyl group, and Bs is a group selected from the groups represented by following formula (Z-VI) to (Z-XI), or derivatives thereof.



(Z-VI) (Z-VII) (Z-VIII)

(Z-IX) (Z-X) (Z-XI)

Examples of Bs are an adenine, a thymine, a cytosine, a guanine , an uracil, a 5-methylcytosine or derivative thereof;

R^{z2} is independently hydrogen, -OH, -SH, $-NR^dR^d$, $-N_3$, halogen, alkyl, alkenyl, alkynyl, alkyl-Y^1-, alkenyl-Y^1-, alkynyl-Y^1-, aryl-Y^1-, heteroaryl-Y^1-, $-OR^b$, or $-SR^b$, wherein R^b is a blocking moiety;

Y^1 is O, NR^d, S, or Se;

R^d is independently hydrogen, alkyl, alkenyl, alkynyl, aryl, acyl, substituted silyl, carbamate, $-P(O)(R^e)_2$, or $-HP(O)(R^e)$;

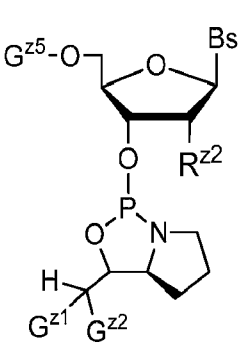
R^e is independently hydrogen, alkyl, aryl, alkenyl, alkynyl, alkyl-Y^2-, alkenyl- Y^2- , alkynyl-Y^2-, aryl-Y^2-, or heteroaryl-Y^2-, or a cation which is Na^+, Li^+, or K^+, or $-O^-$;

Y^2 is O, NR^d, or S;

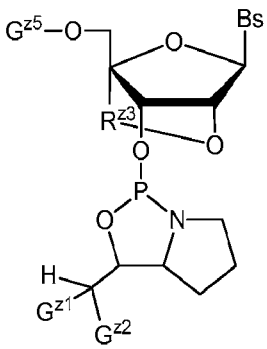
R^{z3} is a group represented by $-CH_2-$, $-(CH_2)_2-$, $-CH_2NH-$, or $-CH_2N(CH_3)-$.

[00593] Examples of G^5 are trityl, 4-monomethoxytrityl, 4,4'-dimethoxytrityl, 4,4',4"-trimethoxytrityl, 9-phenylxanthin-9-yl (Pixyl) and 9-(*p*-methoxyphenyl)xanthin-9-yl (MOX).

[00594] In some embodiments, a nucleoside 3'- phosphoramidite derivative is represented by formula (Z-Va') or (Z-Vb'):



(Z-Va')



(Z-Vb')

wherein each of G^{z1}, G^{z2}, G^{z5}, Bs, R^{z2}, and R^{z3} is independently as defined above and described herein.

[00595] In some embodiments, the invention relates to a method for synthesis of a chirally controlled oligonucleotide.

[00596] In some embodiments, a provided method comprises a first step of reacting a molecule comprising an achiral *H*-phosphonate moiety, the first activating reagent and a chiral reagent or a salt thereof to form a monomer. In some embodiments, a chiral reagent has chemical formula (Z-I) and the monomer may be represented by formula (Z-Va), (Z-Vb), (Z-Va'), or (Z-Vb'). The monomer reacts with the second activating reagent and a nucleoside to form a condensed intermediate. In some embodiments, a subsequent step comprises converting the condensed intermediate to the nucleic acid comprising a chiral X-phosphonate moiety.

[00597] In some embodiments, the present methods provide stable and commercially available materials as starting materials. In some embodiments, the present methods provide a stereocontrolled phosphorous atom-modified oligonucleotide using an achiral starting material.

[00598] As shown in the working examples, in some embodiments methods of the present invention do not cause degradation during deprotection steps. Further the method does not require special capping agents to produce phosphorus atom-modified oligonucleotide derivatives.

[00599] In some embodiments, the present invention provides a method for synthesis of stereocontrolled phosphorus atom-modified oligonucleotide derivatives using a chiral monomer. In some embodiments, the first step is reacting a nucleoside 3'-phosphoramidite derivative which is represented by formula (Z-Va), (Z-Vb), (Z-Va'), or (Z-Vb') with the second activating reagent and a nucleoside to form a condensed intermediate. The second step is converting the condensed intermediate to the nucleic acid comprising a chiral X-phosphonate moiety.

[00600] All publications and patent applications disclosed herein in this specification are herein incorporated by reference in their entirety to the same extent as if each individual

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publication or patent application was specifically and individually indicated to be incorporated by reference.

[00601] As used in this "*Further Embodiments of Chiral Reagents*" section, in a condensation reaction, the term "activating reagent" refers to a reagent that activates a less reactive site and renders it more susceptible to attack by a nucleophile.

[00602] As used in this "*Further Embodiments of Chiral Reagents*" section, an "alkyl" group refers to an aliphatic hydrocarbon group. The alkyl moiety may be a saturated alkyl group (which means that it does not contain any units of unsaturation, e.g. carbon-carbon double bonds or carbon-carbon triple bonds) or the alkyl moiety may be an unsaturated alkyl group (which means that it contains at least one unit of unsaturation). The alkyl moiety, whether saturated or unsaturated, may be branched, straight chain, or include a cyclic portion. The point of attachment of an alkyl is at a carbon atom that is not part of a ring. The "alkyl" moiety may have 1 to 10 carbon atoms (whenever it appears herein, a numerical range such as "1 to 10" refers to each integer in the given range; e.g., "1 to 10 carbon atoms" means that the alkyl group may consist of 1 carbon atom, 2 carbon atoms, 3 carbon atoms, etc., up to and including 10 carbon atoms, although the present definition also covers the occurrence of the term "alkyl" where no numerical range is designated). Alkyl includes both branched and straight chain alkyl groups. The alkyl group of the compounds described herein may be designated as "C_1-C_6 alkyl" or similar designations. By way of example only, "C_1-C_6 alkyl" indicates that there are one, two, three, four, five, or six carbon atoms in the alkyl chain, i.e., the alkyl chain is selected from e.g., methyl, ethyl, propyl, iso-propyl, n-butyl, iso-butyl, sec-butyl, and tert-butyl. Typical alkyl groups include, but are in no way limited to, methyl, ethyl, propyl, isopropyl, butyl, isobutyl, tertiary butyl, pentyl, hexyl, allyl, cyclopropylmethyl, cyclobutylmethyl, cyclopentylmethyl, cyclohexylmethyl, and the like. In one aspect, an alkyl is a C_1-C_6 alkyl. C_{1-3} alkyl group means straight or branched alkyl group that has 1 to 3 carbon atoms. Examples of C_{1-3} alkyl group are methyl, ethyl, propyl and isopropyl. C_{1-4} alkyl group means straight or branched alkyl group that has 1 to 4 carbon atoms. Examples of C_{1-4} alkyl group are methyl, ethyl, propyl, isopropyl, butyl, isobutyl, and tert-butyl.

[00603] As used in this "*Further Embodiments of Chiral Reagents*" section, the term "aryl" refers to an aromatic ring wherein each of the atoms forming the ring is a carbon atom. Aryl rings are formed by five, six, seven, eight, nine, or more than nine carbon atoms. Aryl

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groups are a substituted or unsubstituted. In one aspect, an aryl is a phenyl or a naphthalenyl. Depending on the structure, an aryl group can be a monoradical or a diradical (i.e., an arylene group). In one aspect, an aryl is a C_6-C_{10} aryl. C_{6-14} aryl group means aryl group that has 6 to 14 carbon atoms. The examples of C_{6-14} aryl group are phenyl, biphenyl, naphthyl, anthracyl, indanyl, phthalimidyl, naphthimidyl, phenanthridinyl, and tetrahydronaphthyl.

[00604] The term "aralkyl" refers to an alkyl group substituted with an aryl group. Suitable aralkyl groups include benzyl, picolyl, and the like, all of which may be optionally substituted.

[00605] As used in this "*Further Embodiments of Chiral Reagents*" section, An "acyl moiety" refers to an alkyl(C=O), aryl(C=O), or aralkyl(C=O) group. An acyl moiety can have an intervening moiety (Y) that is oxy, amino, thio, or seleno between the carbonyl and the hydrocarbon group. For example, an acyl group can be alkyl-Y-(C=O), aryl- Y-(C=O) or aralkyl-Y-(C=O).

[00606] As used in this "*Further Embodiments of Chiral Reagents*" section, "alkenyl" groups are straight chain, branch chain, and cyclic hydrocarbon groups containing at least one carbon-carbon double bond. Alkenyl groups can be substituted.

[00607] As used in this "*Further Embodiments of Chiral Reagents*" section, "alkynyl" groups are straight chain, branch chain, and cyclic hydrocarbon groups containing at least one carbon-carbon triple bond. Alkynyl groups can be substituted.

[00608] As used in this "*Further Embodiments of Chiral Reagents*" section, an "alkoxy" group refers to an alklyl group linked to oxygen i.e. (alkyl)-O- group, where alkyl is as defined herein. Examples include methoxy (-OCH3) or ethoxy (-OCH2CH3) groups.

[00609] As used in this "*Further Embodiments of Chiral Reagents*" section, an "alkenyloxy" group refers to an alkenyl group linked to oxygen i.e. (alkenyl)-O- group, where alkenyl is as defined herein.

[00610] As used in this "*Further Embodiments of Chiral Reagents*" section, an "alkynyloxy" group refers to an alkynyl group linked to oxygen i.e. (alkynyl)-O- group, where alkynyl is as defined herein.

[00611] As used in this "*Further Embodiments of Chiral Reagents*" section, an "aryloxy" group refers to an aryl group linked to oxygen i.e. (aryl)-O-group, where the aryl is as defined herein. An example includes phenoxy ($-OC_6H_5$) group.

[00612] As used in this "*Further Embodiments of Chiral Reagents*" section, the term "alkylseleno" refers to an alkyl group having a substituted seleno group attached thereto i.e. (alkyl)-Se- group, wherein alkyl is defined herein.

[00613] As used in this "*Further Embodiments of Chiral Reagents*" section, the term "alkenylseleno" refers to an alkenyl group having a substituted seleno group attached thereto i.e. (alkenyl)-Se- group, wherein alkenyl is defined herein.

[00614] As used in this "*Further Embodiments of Chiral Reagents*" section, the term "alkynylseleno" refers to an alkynyl group having a substituted seleno group attached thereto i.e. (alkynyl)-Se- group, wherein alkenyl is defined herein.

[00615] As used in this "*Further Embodiments of Chiral Reagents*" section, the term "alkylthio" refers to an alkyl group attached to a bridging sulfur atom i.e. (alkyl)-S-group, wherein alkyl is defined herein. For example, an alkylthio is a methylthio and the like.

[00616] As used in this "*Further Embodiments of Chiral Reagents*" section, the term "alkenylthio" refers to an alkenyl group attached to a bridging sulfur atom i.e. (alkenyl)- S-group, wherein alkenyl is defined herein.

[00617] As used in this "*Further Embodiments of Chiral Reagents*" section, the term "alkynylthio" refers to an alkynyl group attached to a bridging sulfur atom i.e. (alkynyl)- S-group, wherein alkenyl is defined herein.

[00618] As used in this "*Further Embodiments of Chiral Reagents*" section, the term "alkylamino" refers to an amino group substituted with at least one alkyl group i.e. –NH(alkyl) or –N(alkyl)2, wherein alkyl is defined herein.

[00619] As used in this "*Further Embodiments of Chiral Reagents*" section, the term "alkenylamino" refers to an amino group substituted with at least one alkenyl group Le -NH(alkenyl) or -N(alkenyl)2, wherein alkenyl is defined herein.

[00620] As used in this "*Further Embodiments of Chiral Reagents*" section, the term "alkynylamino" refers to an amino group substituted with at least one alkynyl group i.e. -NH(alkynyl) or -N(alkynyl)$_2$, wherein alkynyl is defined herein.

[00621] As used in this "*Further Embodiments of Chiral Reagents*" section, the term "halogen" is intended to include fluorine, chlorine, bromine and iodine.

[00622] As used in this "*Further Embodiments of Chiral Reagents*" section, a "fluorescent group" refers to a molecule that, when excited with light having a selected wavelength, emits

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light of a different wavelength. Fluorescent groups include, but are not limited to, indole groups, fluorescein, tetramethylrhodamine, Texas Red, BODIPY, 5-[(2-aminoethyl)amino]napthalene-l-sulfonic acid (EDANS), coumarin and Lucifer yellow.

[00623] As used in this "*Further Embodiments of Chiral Reagents*" section, an "ammonium ion" is a positively charged polyatomic cation of the chemical formula NH_4^+.

[00624] As used in this "*Further Embodiments of Chiral Reagents*" section, an "alkylammonium ion" is an ammonium ion that has at least one of its hydrogen atoms replaced by an alkyl group, wherein alkyl is defined herein. Examples include triethylammonium ion, *N, N*-diisopropylethylammonium ion.

[00625] As used in this "*Further Embodiments of Chiral Reagents*" section, an "iminium ion" has the general structure $(R^x)_2C=N(R^x)_2^+$ The R^x groups refer to alkyl, alkenyl, alkynyl, aryl groups as defined herein. A "heteroaromatic iminium ion" refers to an imminium ion where the nitrogen and its attached R^x groups form a heteroaromatic ring. A "heterocyclic iminium ion" refers to an imminium ion where the nitrogen and its attached R^x groups form a heterocyclic ring.

[00626] As used in this "*Further Embodiments of Chiral Reagents*" section, the terms "amino" or "amine" refers to a $-N(R^h)_2$ radical group, where each R^h is independently hydrogen, alkyl, fluoroalkyl, carbocyclyl, carbocyclylalkyl, aryl, aralkyl, heterocyclyl, heterocyclylalkyl, heteroaryl or heteroarylalkyl, unless stated otherwise specifically in the specification. When a $-N(R^h)_2$ group has two R^h other than hydrogen they can be combined with the nitrogen atom to form a 4-, 5-, 6-, or 7-membered ring. For example, $-N(R^h)_2$ is meant to include, but not be limited to, 1-pyrrolidinyl and 4-morpholinyl. Any one or more of the hydrogen, alkyl, fluoroalkyl, carbocyclyl, carbocyclylalkyl, aryl, aralkyl, heterocyclyl, heterocyclylalkyl, heteroaryl or heteroarylalkyl are optionally substituted by one or more substituents which independently are alkyl, heteroalkyl, alkenyl, alkynyl, cycloalkyl, heterocycloalkyl, aryl, arylalkyl, heteroaryl, heteroarylalkyl, hydroxy, halo, cyano, trifluoromethyl, trifluoromethoxy, nitro, trimethylsilyl, $-OR^i$, $-SR^i$, $-OC(O)R^i$, $-N(R^i)_2$, $-C(O)R^i$, $-C(O)OR^i$, $-OC(O)N(R^i)_2$, $-C(O)N(R^i)_2$, $-N(R^i)C(O)OR$, $-N(R^i)C(O)R^i$, $-N(R^i)C(O)N(R^i)_2$, $N(R^i)C(NR^i)N(R^i)_2$, $-N(R^i)S(O)_tR^i$ (where t is 1 or 2), $-S(O)$, or $-S(O)_tN(R^i)_2$ (where t is 1 or 2), where each R^i is independently hydrogen, alkyl, fluoroalkyl, carbocyclyl, carbocyclylalkyl, aryl, aralkyl, heterocyclyl, heterocyclylalkyl, heteroaryl or heteroarylalkyl.

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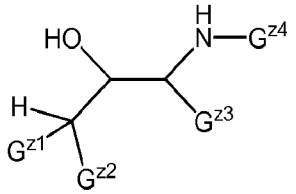
[00627] As used in this "*Further Embodiments of Chiral Reagents*" section, "carbamate" as used herein, refers to a moiety attached to an amino group which has the formula -C(O)OR where R is alkyl, fluoroalkyl, carbocyclyl, carbocyclylalkyl, aryl, aralkyl, heterocyclyl, heterocyclylalkyl, heteroaryl or heteroarylalkyl. Examples include but are not limited to Boc (*tert*-butyl-OC(O)-), CBz (benzyl-OC(O)-), Teoc ($Me_3SiCH_2CH_2OC(O)$-), alloc (allyl-OC(O)-), or Fmoc (9-fluorenylmethyl-OC(O)-) group

[00628] As used in this "*Further Embodiments of Chiral Reagents*" section, "substituted silyl" as used herein, refers to a moiety which has the formula R^x_3Si-. Examples include, but are not limited to, TBDMS (tert-butyldimethylsilyl), TBDPS (tert-butyldiphenylsilyl) or TMS (trimethylsilyl).

[00629] As used in this "*Further Embodiments of Chiral Reagents*" section, the term "thiol" refers to -SH groups, and include substituted thiol groups i.e. -SRJ groups, wherein RJ are each independently a substituted or unsubstituted alkyl, cycloalkyl, alkenyl, alkynyl, aryl aralkyl, heterocyclyl or heterocyclylalkyl group as defined herein.

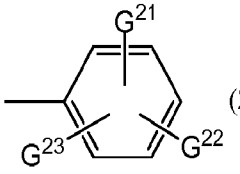
[00630] In some embodiments, the present invention provides a chiral reagent or a salt thereof. In some embodiments, a chiral reagent is of the following chemical formula (Z-I):



(Z-I)

wherein G^{z1} and G^{z2} are independently a hydrogen atom, a nitro group, a halogen atom, a cyano group (-CN), a group of formula (Z-II) or (Z-III), or both G^{z1} and G^{z2} taken together to form a group of formula (Z-IV). In some embodiments, the term "chiral reagent" is a chemical composition which is used to produce stereocontrolled phosphorous atom-modified nucleotide or oligonucleotide derivatives. A chiral reagent reacts with a nucleoside to form a chiral intermediate.

[00631] In some embodiments, a group of formula (Z-II) is of the following formula:

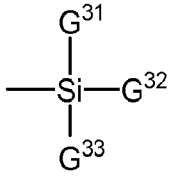


(Z-II)

,

wherein G^{21} to G^{23} are independently a hydrogen atom, a nitro group, a halogen atom, a cyano group or C_{1-3} alkyl group. In some embodiments, examples of G21 to G23 are a hydrogen atom.

[00632] In some embodiments, a group of formula (Z-III) is of the following formula:

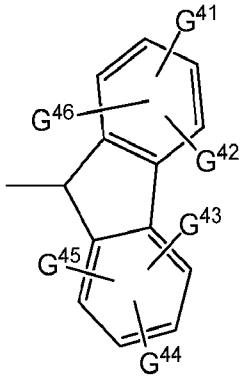


(Z-III)

,

wherein G^{31} to G^{33} are independently C_{1-4} alkyl group, C_{6-14} aryl group, C_{1-4} alkoxy group, C_{7-14} aralkyl group, C_{1-4} alkyl C_{6-14} aryl group, C_{1-4} alkoxy C_{6-14} aryl group, or C_{6-14} aryl C_{1-4} alkyl group. Examples of C_{1-4} alkyl C_{6-14} aryl group are methylphenyl group, and ethylphenyl group. Examples of C_{1-4} alkoxy C_{6-14} aryl group are methoxyphenyl group and ethoxyphenyl group. Examples of C_{6-14} aryl C_{1-4} alkyl groups are benzyl group and phenylethyl group. In some embodiments, examples of G^{31} to G^{33} are independently a methyl group and a phenyl group.

[00633] In some embodiments, a group of formula (Z-IV) is of the following formula:



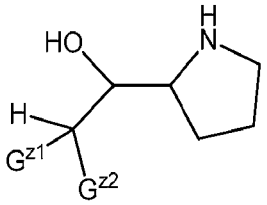
(Z-IV)

,

wherein G^{41} to G^{46} are independently a hydrogen atom, a nitro group, a halogen atom, a cyano group or C_{1-3} alkyl group. In some embodiments, examples of G41 to G46 are a hydrogen atom.

[00634] G^{z3} and G^{z4} are independently a hydrogen atom, C_{1-3} alkyl group, C_{6-14} aryl group, or both G^{z3} and G^{z4} taken together to form a heteroatom-containing ring that has 3 to 16 carbon atoms. In some embodiments, examples of G^3 and G^4 are that taken together to form a heteroatom-containing ring that has 3 to 16 carbon atoms with NH moiety in the formula (I).

[00635] In certain embodiments, a chiral reagent has following chemical formula (Z-I').



(Z-I')

[00636] In the formula (Z-I'), G^{z1} and G^{z2} are same as above and G^{z1} and G^{z2} are independently a hydrogen atom, a nitro group, a halogen atom, a cyano group, a group of formula (Z-II) or (Z-III), or both G^{z1} and G^{z2} taken together to form a group of formula (Z-IV)..

[00637] In certain embodiments, a chiral reagent has chemical formula (Z-I') and each of G^{z1} and G^{z2} is a group of formula (Z-II), wherein G^{21} to G^{23} are independently a hydrogen atom, a nitro group, a halogen atom, a cyano group or C_{1-3} alkyl group.

[00638] In certain embodiments, a chiral reagent has chemical formula (Z-I') and each of G^{z1} and G^{z2} is a group of formula (Z-II) and each of G^{21} to G^{23} is a hydrogen atom.

[00639] In certain embodiments, a chiral reagent has chemical formula (Z-I') and G^{z1} is a hydrogen atom, G^{z2} is a group of formula (Z-II), and G^{21} to G^{23} are independently a hydrogen atom, a nitro group, a halogen atom, a cyano group or C_{1-3} alkyl group.

[00640] In certain embodiments, a chiral reagent has chemical formula (Z-I') and G^{z1} is a hydrogen atom, G^{z2} is a group of formula (Z-II), each of G^{21} and G^{22} is a hydrogen atom and G^{23} is a nitro group ($-NO_2$).

[00641] In certain embodiments, a chiral reagent has chemical formula (Z-I') and G^{z1} is a hydrogen atom and G^{z2} is a group of formula (Z-III), and G^{31} to G^{33} are independently C_{1-4} alkyl group, C_{6-14} aryl group, C_{7-14} aralkyl group, C_{1-4} alkyl C_{6-14} aryl group, C_{1-4} alkoxy C_{6-14} aryl group, or C_{6-14} aryl C_{1-4} alkyl group.

[00642] In some embodiments, a chiral reagent has chemical formula (Z-I') and G^{z1} is a hydrogen atom, G^{z2} is a group of formula (Z-III), and G^{31} to G^{33} are independently C_{1-4} alkyl group or C_6 aryl group (a phenyl group). Examples of C_{1-4} alkyl group are methyl group, ethyl group, *n*-propyl group, *iso*-propyl group, *n*-butyl group and *tert*-butyl group.

[00643] In certain embodiments, a chiral reagent has chemical formula (Z-I') and G^{z1} is a hydrogen atom, G^{z2} is a group of formula (Z-III), and G^{31} to G^{33} are independently C_{1-2} alkyl group (a methyl group or an ethyl group) or C_6 aryl group (a phenyl group).

[00644] In certain embodiments, a chiral reagent has chemical formula (Z-I') and G^{z1} is a hydrogen atom, G^{z2} is a group of formula (Z-III), and G^{31} to G^{33} are independently C_{1-4} alkyl group.

[00645] In certain embodiments, a chiral reagent has chemical formula (Z-I') and G^{z1} is a hydrogen atom, G^{z2} is a group of formula (Z-III), and G^{31} and G^{33} are C_6 aryl group (a phenyl group) and G^{32} is C_{1-2} alkyl group.

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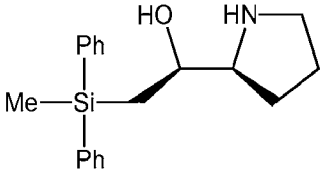
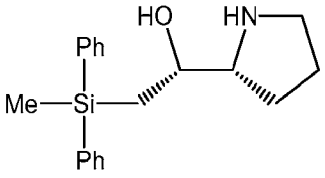
[00646] In certain embodiments, a chiral reagent has chemical formula (Z-I') and G^{z1} and G^{z2} are taken together to form a group of formula (Z-IV), and G^{41} to G^{46} are independently a hydrogen atom, a nitro group, a halogen atom, a cyano group or C_{1-3} alkyl group.

[00647] In certain embodiments, a chiral reagent has chemical formula (Z-I') and G^{z1} and G^{z2} are taken together to form a group of formula (Z-IV), wherein each of G^{41} to G^{46} is a hydrogen atom.

[00648] In certain embodiments, a chiral reagent is selected from one of chemical formulae 3a, 3b, 5a, Z-5b, 7a, 7b, 9a, 9b, 11a and 11b:


(3a)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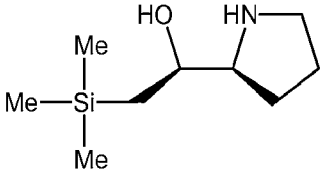
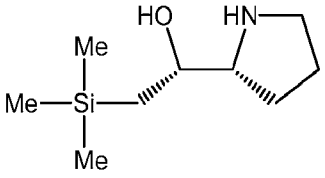
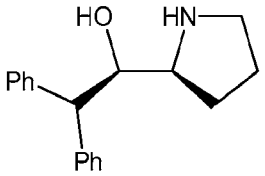
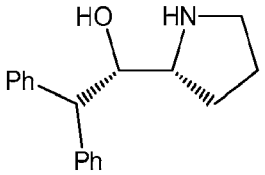
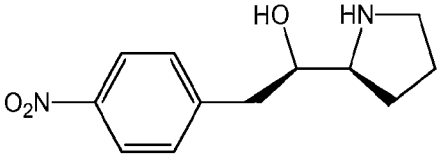
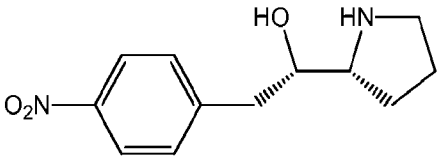

(5a)

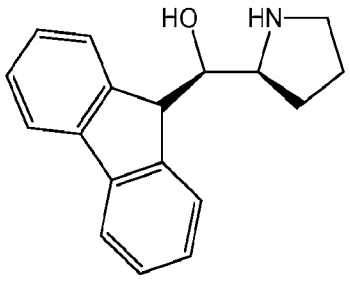

(Z-5b)

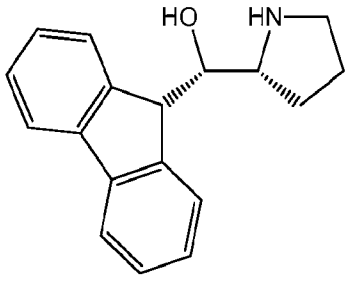

(7a)


(7b)


(9a)


(9b)


(11a)


(11b)

Namely, in some embodiments, a chiral reagent is selected from:

(S)-2-(Methyldiphenylsilyl)-1-((S)-1-pyrrolidin-2-yl)ethanol (**3a**),

(R)-2-(Methyldiphenylsilyl)-1-((R)-1-pyrrolidin-2-yl)ethanol (**3b**),

(S)-2-(Trimethylsilyl)-1-((S)-1-pyrrolidin-2-yl)ethanol (**5a**),

(R)-2-(Trimethylsilyl)-1-((R)-1-pyrrolidin-2-yl)ethanol (**Z-5b**),

(R)-2,2-Diphenyl-1-((S)-pyrrolidin-2-yl)ethanol (**7a**),

(S)-2,2-Diphenyl-1-((R)-pyrrolidin-2-yl)ethanol (**7b**),

(R)-2-(4-Nitrophenyl)-1-((S)-pyrrolidin-2-yl)ethanol (**9a**),

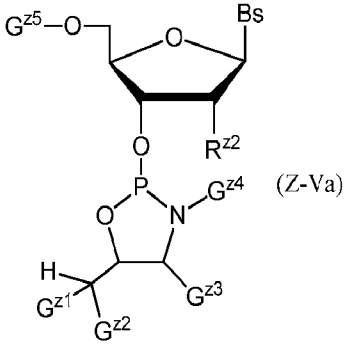
(S)-2-(4-Nitrophenyl)-1-((R)-pyrrolidin-2-yl)ethanol (**9b**),

(R)-(9*H*-Fluororen-9-yl)((S)-pyrrolidin-2-yl)methanol (**11a**), or

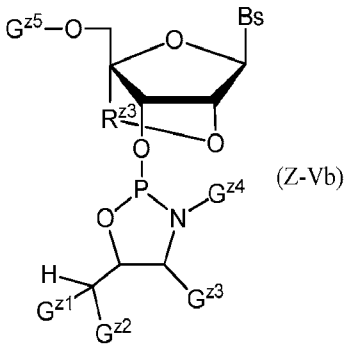
(S)-(9*H*-Fluororen-9-yl)((R)-pyrrolidin-2-yl)methanol (**11b**).

[00649] The chiral reagent reacts with a nucleic acid or modified nucleic acid to be an asymmetric auxiliary group. A nucleoside 3'- phosphoramidite derivative, which is an intermediate of manufacturing a stereocontrolled phosphorous atom-modified oligonucleotide derivative, is obtained by chiral reagent reacting with a nucleic acid or modified nucleic acid.

[00650] In some embodiments, the invention provides a nucleoside 3'- phosphoramidite derivative which is represented by formula (Z-Va) or (Z-Vb). The compounds of formula (Z-Va) and (Z-Vb) are known as monomers that are used in synthesizing oligonucleotide derivatives. These compounds are also known as oxazaphospholidine monomers. The sugar moieties of the compounds represented by formula (Z-Vb) are known as BNA and LNA (when R^{z3} is a methylene group).

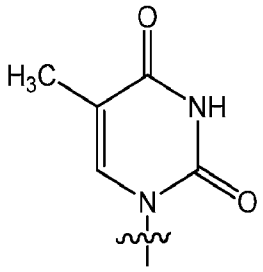


(Z-Va)

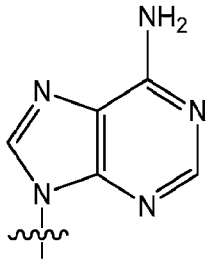


(Z-Vb)

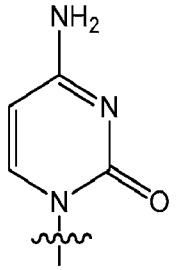
[00651] In the formula (Z-Va) and (Z-Va), G^{z1} to G^{z4} are same as above, G^{z5} is a protective group of the hydroxyl group, and Bs is a group selected from the groups represented by formula (Z-VI) to (Z-XI) or derivatives thereof.



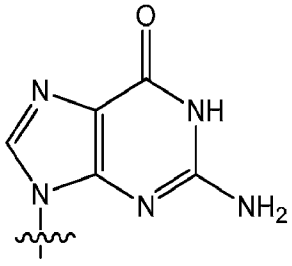
(Z-VI)



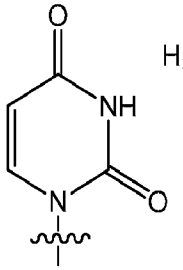
(Z-VII)



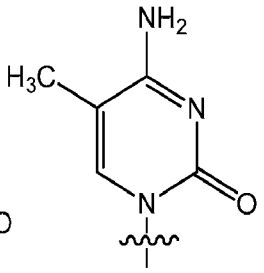
(Z-VIII)



(Z-IX)



(Z-X)



(Z-XI)

Examples of Bs are an adenine, a thymine, a cytosine, a guanine , an uracil, a 5-methylcytosine or derivative thereof;

R^{z2} is independently hydrogen, -OH, -SH, $-NR^dR^d$, $-N_3$, halogen, alkyl, alkenyl, alkynyl, alkyl-Y^1-, alkenyl-Y^1-, alkynyl-Y^1-, aryl-Y^1-, heteroaryl-Y^1-, $-OR^b$, or $-SR^b$, wherein R^b is a blocking moiety;

Y^1 is O, NR^d, S, or Se;

R^d is independently hydrogen, alkyl, alkenyl, alkynyl, aryl, acyl, substituted silyl, carbamate, $-P(O)(R^e)_2$, or $-HP(O)(R^e)$;

R^e is independently hydrogen, alkyl, aryl, alkenyl, alkynyl, alkyl-Y^2-, alkenyl- Y^2- , alkynyl-Y^2-, aryl-Y^2-, or heteroaryl-Y^2-, or a cation which is Na^+, Li^+, or K^+, or $-O^-$;

Y^2 is O, NR^d, or S;

R^{z3} is a group represented by $-CH_2-$, $-(CH_2)_2-$, $-CH_2NH-$, or $-CH_2N(CH_3)-$.

[00652] Examples of G^{z5} is trityl, 4-monomethoxytrityl, 4,4'-dimethoxytrityl, 4,4',4"-trimethoxytrityl, 9-phenylxanthin-9-yl (Pixyl) and 9-(*p*-methoxyphenyl)xanthin-9-yl (MOX).

[00653] In some embodiments, Bs is an adenine, a thymine, a cytosine, a guanine, or derivative thereof. In some embodiments, Bs is a nucleobase or a modified nucleobase.

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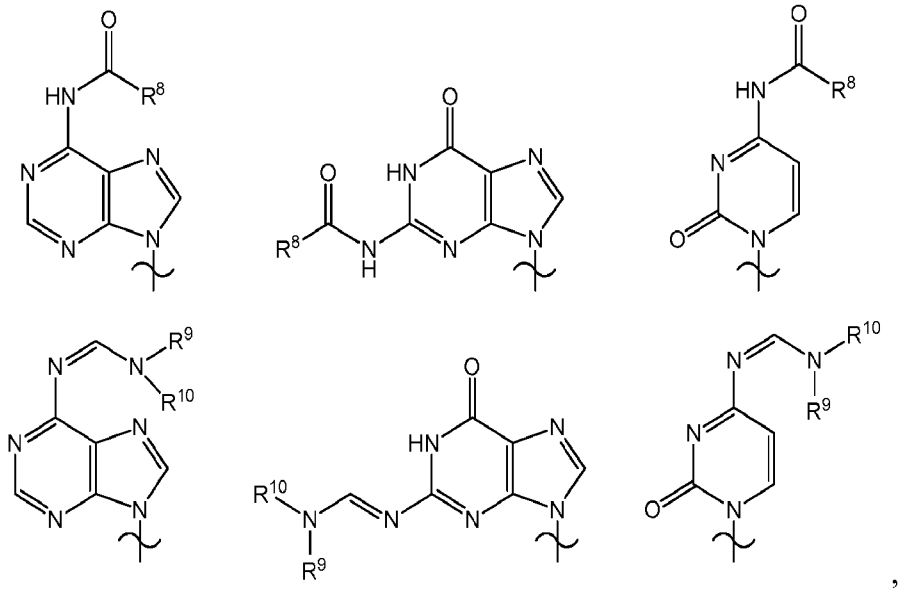
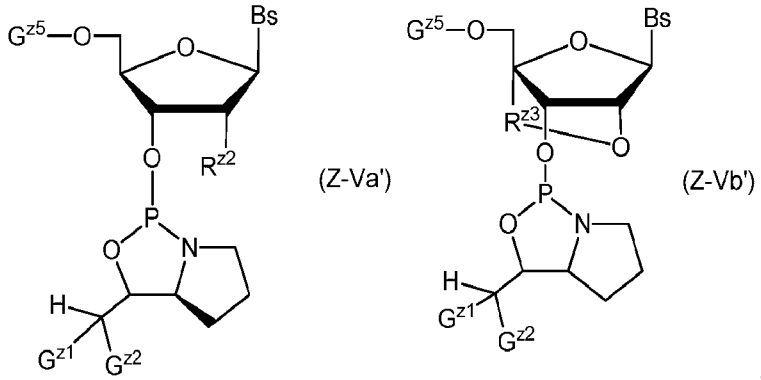
Exemplary derivatives are, for instance, those disclosed in JP 2005-89441 A, and are represented as follows:



wherein, in the above formula, each of R^8 to R^{10} is independently C_{1-10} alkyl, C_6-C_{10} aryl, C_6-C_{10} aralkyl, or C_6-C_{10} aryloxyalkyl. In some embodiments, R^8 is methyl, isopropyl, phenyl, benzyl, and phenoxymethyl. In some embodiments, R^9 and $R^{10}$are C_{1-4} alkyl group.

[00654] In some embodiments, a nucleoside 3'- phosphoramidite derivative is represented by formula (Z-Va') or (Z-Vb'):



(Z-Va') (Z-Vb')

wherein, in the formula (Z-Va') and (Z-Vb'), each of G^{z1}, G^{z2}, G^{z5}, Bs, R^{z2} and R^{z3} are the same as above. In certain embodiments, a nucleoside 3'- phosphoramidite derivative is a chiral monomer which is used to produce stereocontrolled phosphorous atom-modified nucleotide and oligonucleotide. Examples of the nucleoside 3'-phosphoramidite derivatives are represented by the following formulae: **12a**, **12b**, **13a**, **13b**, **14a**, **14b**, **15a**, **15b**, **16a**, **16b**, **17a**, **17b**, **18a**, **18b**, **19a**, **19b**, **20a**, **20b**, **21a**, **21b**, **22a**, **22b**, **23a**, **23b**, **24a**, **24b**, **25a**, **25b**, **26a**, **26b**, **27a**, **27b**, **28a**, **28b**, **29a**, **29b**, **30a**, **30b**, **31a**, **31b**, **32a**, **32b**, **33a**, **33b**, **34a**, **34b** and **35a**.

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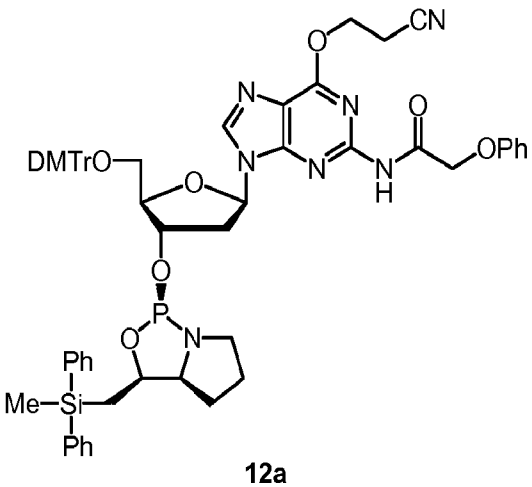
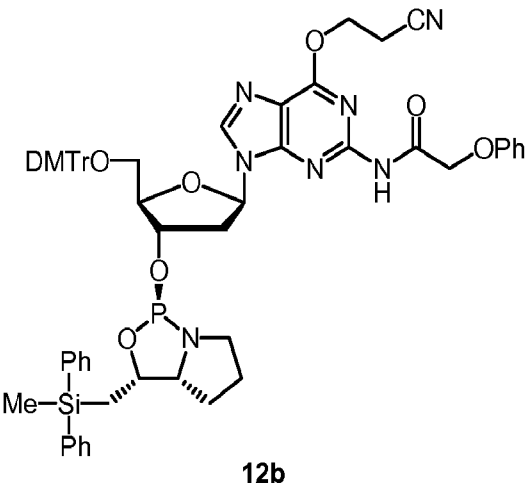


12a



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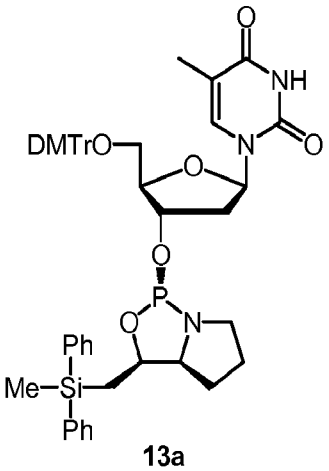
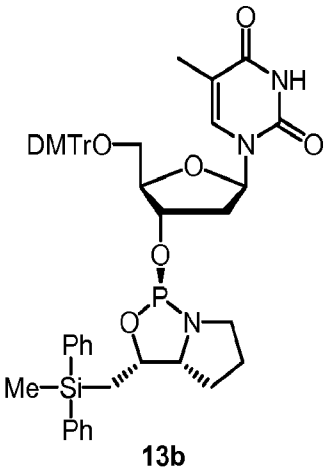


13a



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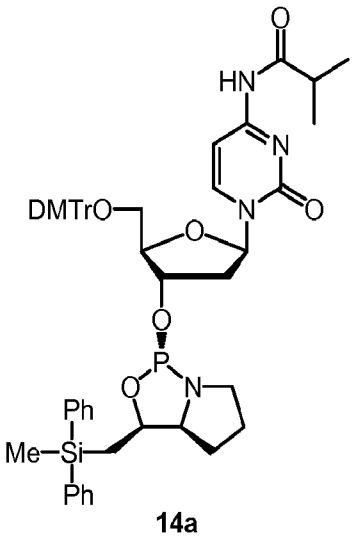
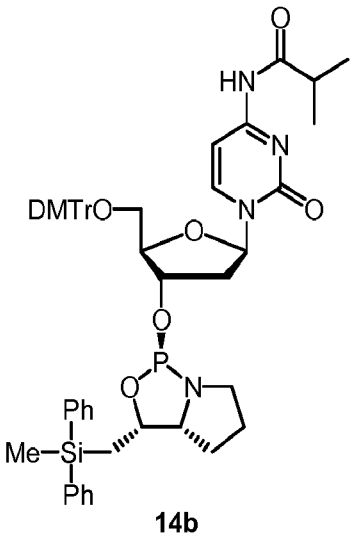


14a



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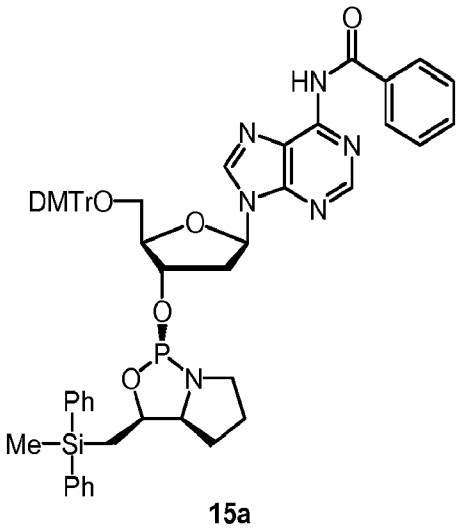

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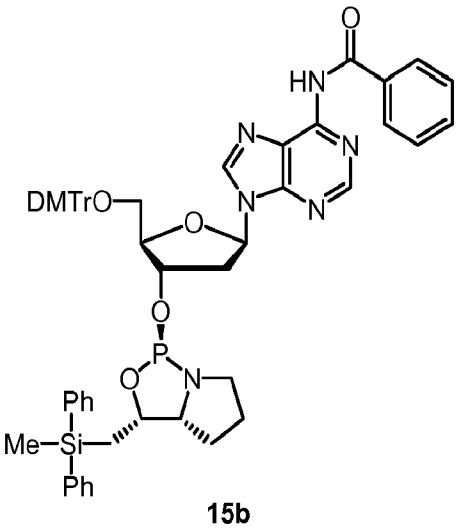

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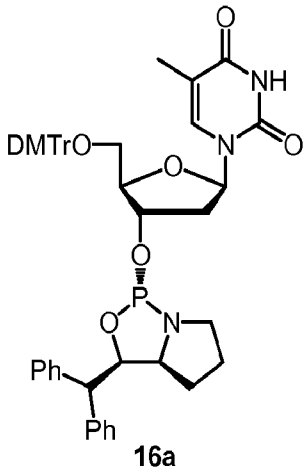

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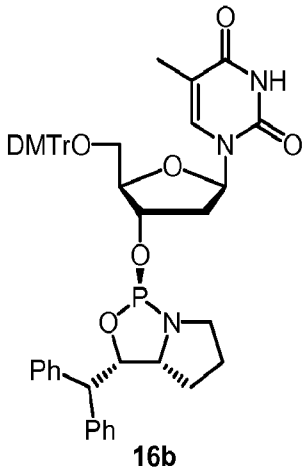

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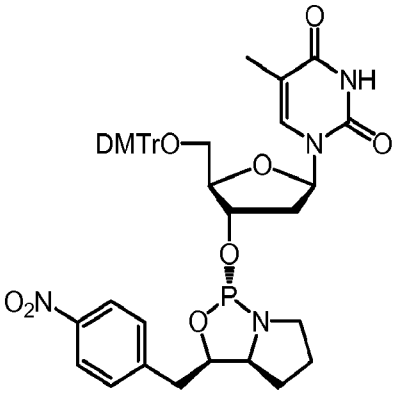
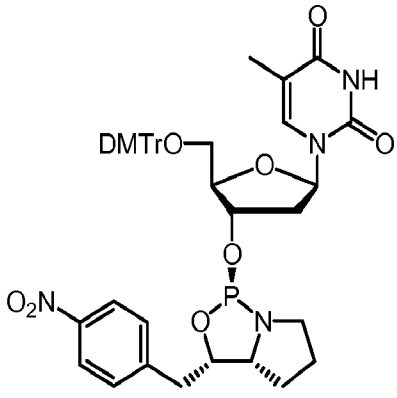
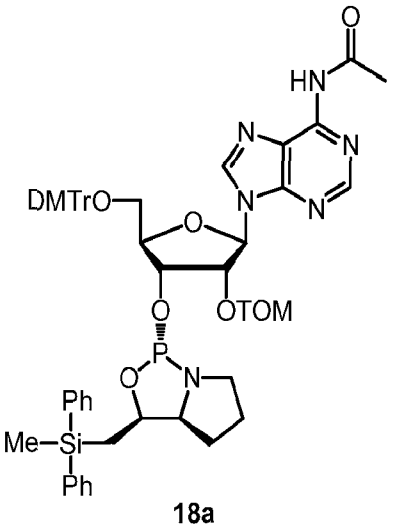


17a



17b



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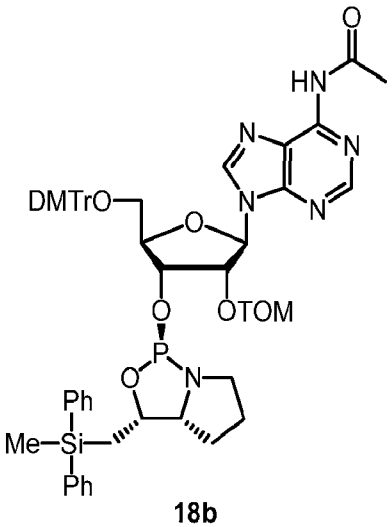

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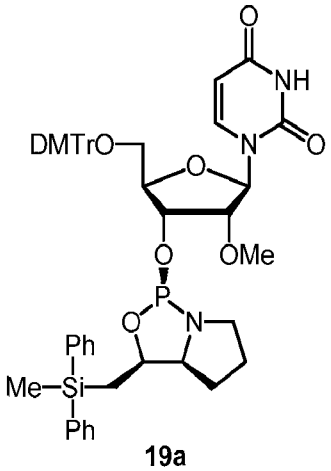
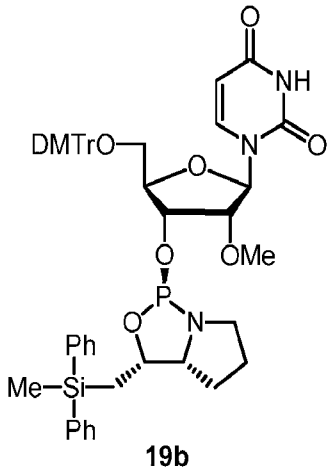


19a



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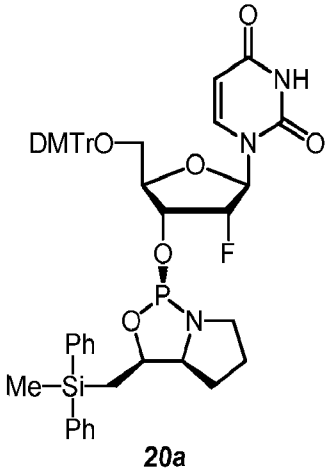

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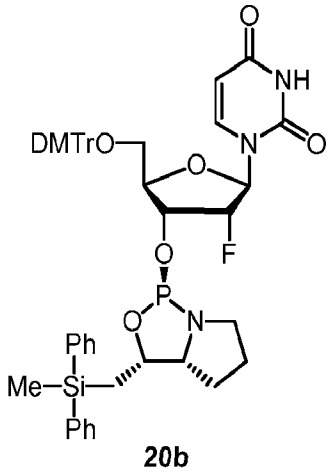

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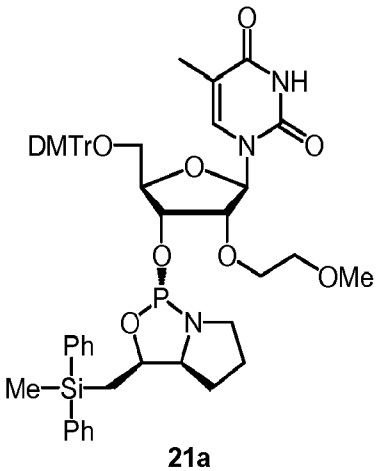

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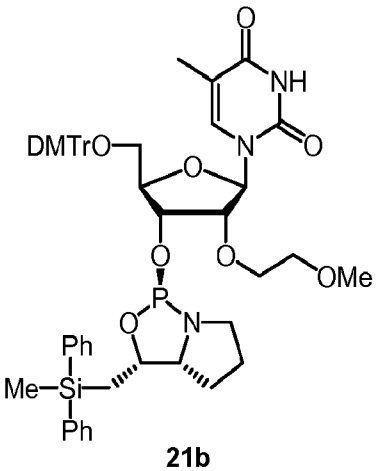

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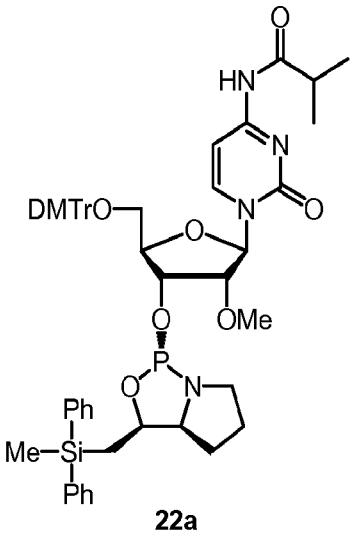

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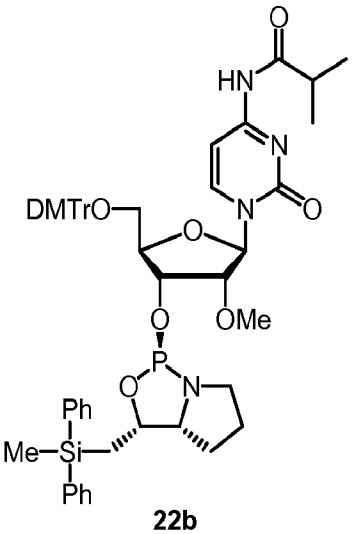

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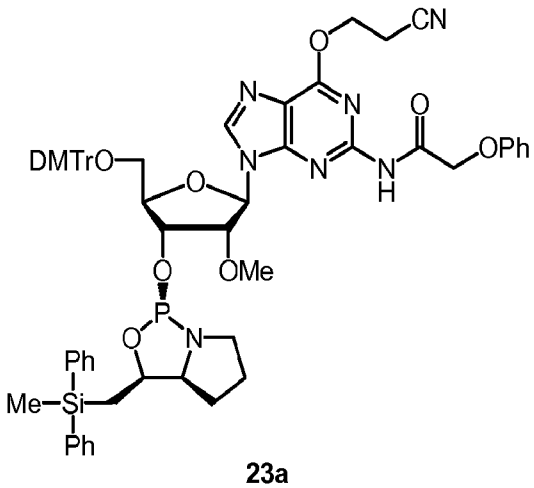

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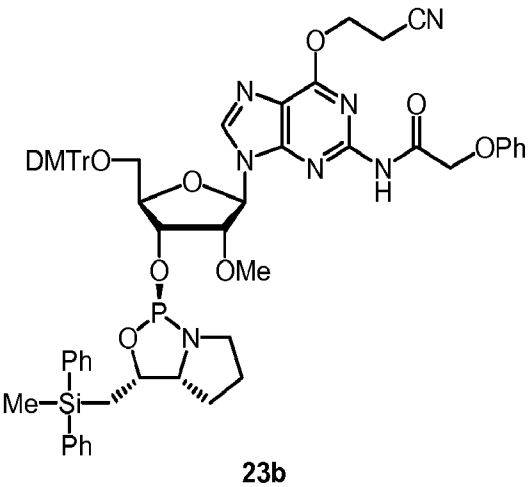

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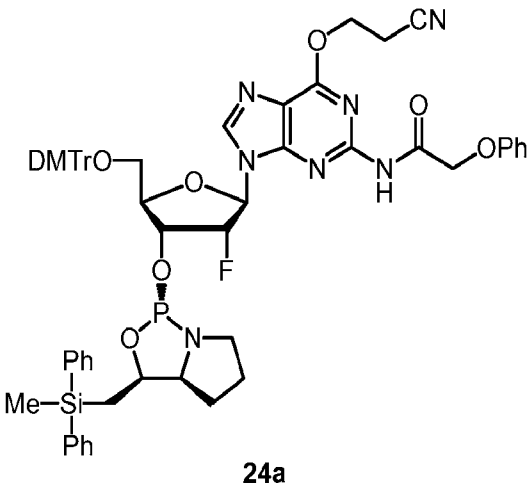

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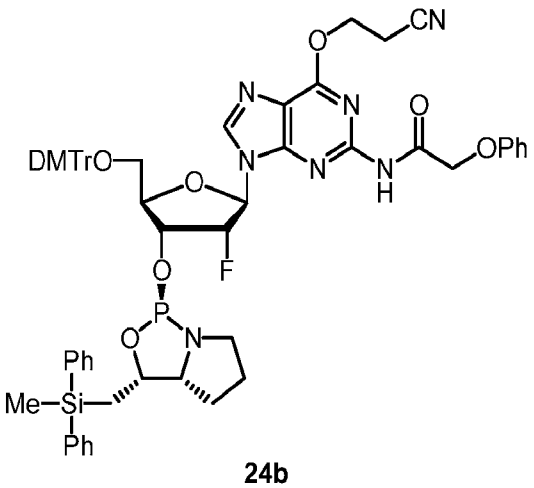

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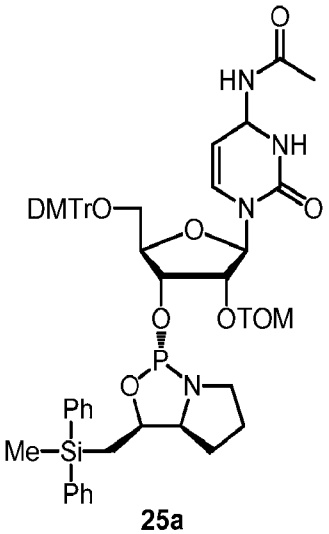

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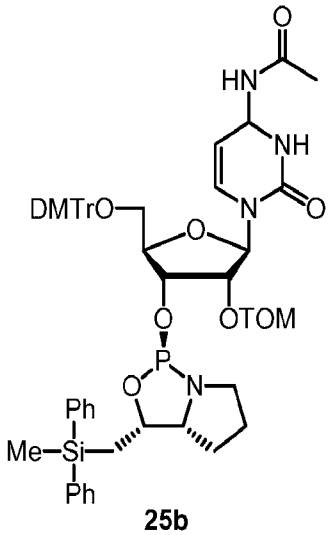

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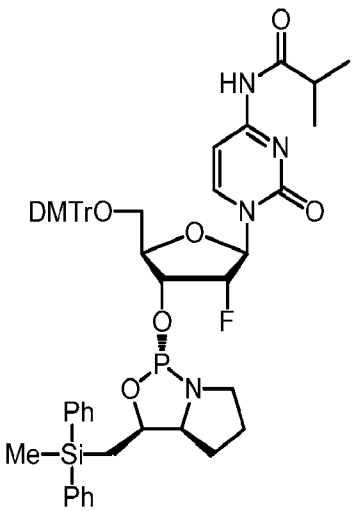

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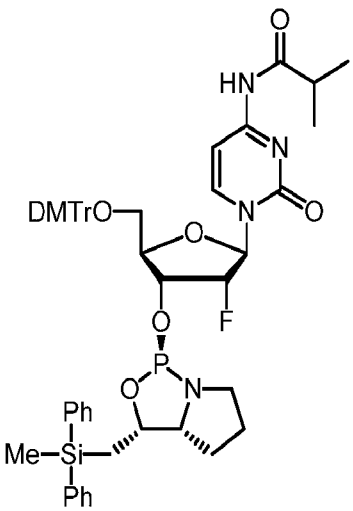

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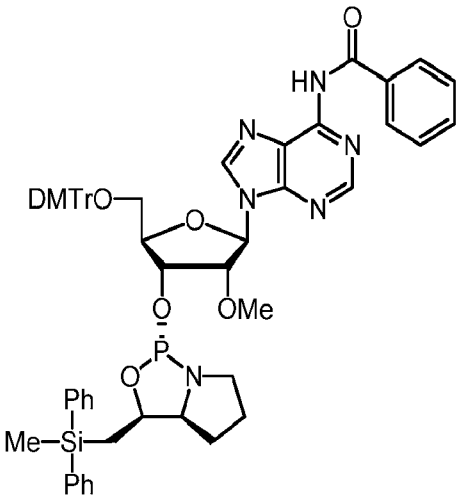

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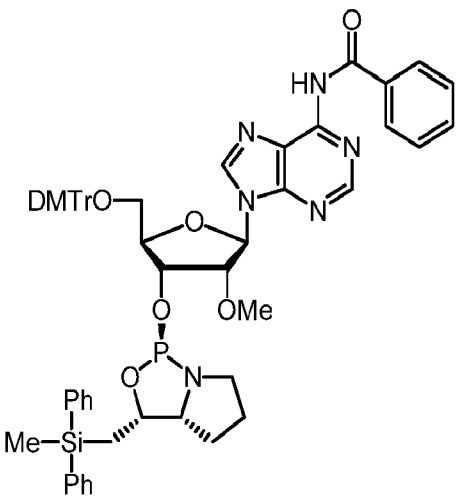

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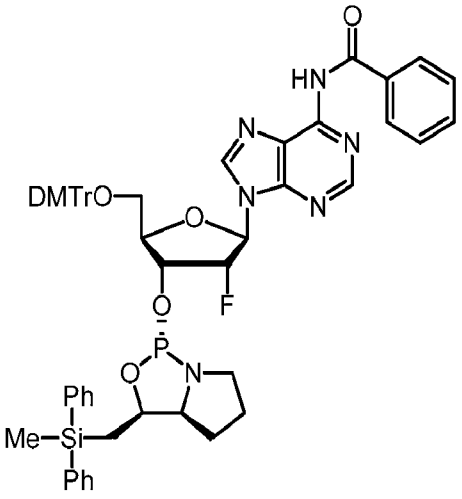

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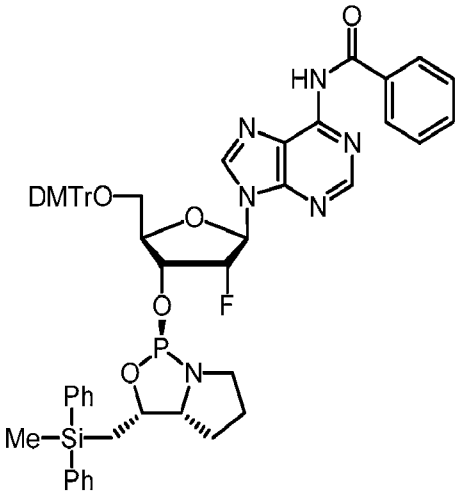

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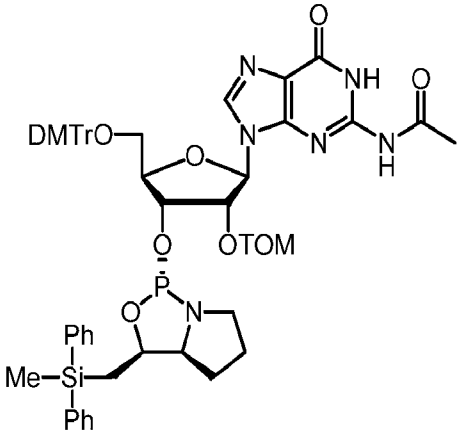

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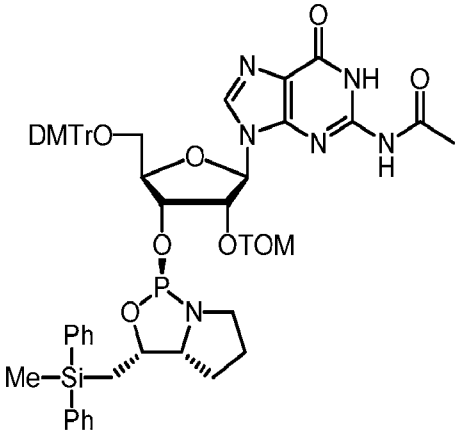

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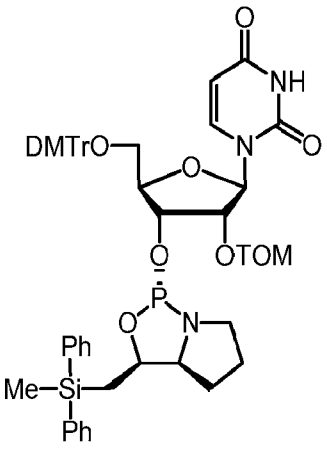

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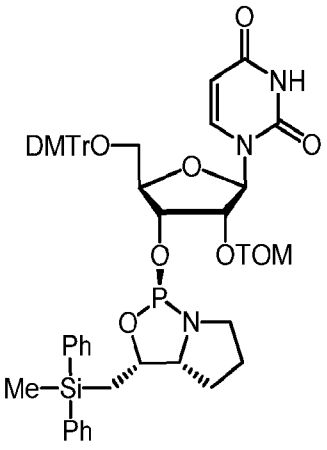

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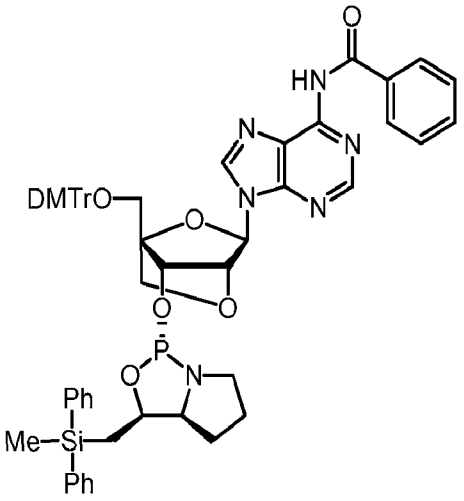

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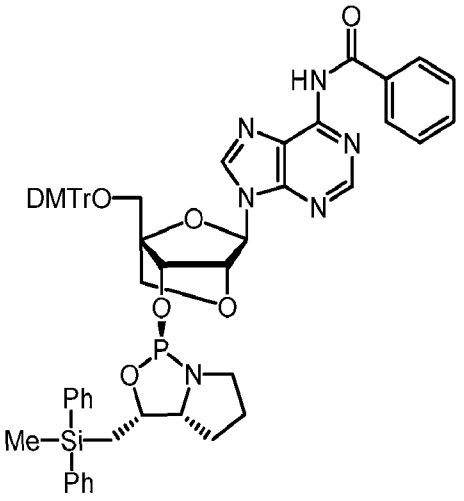

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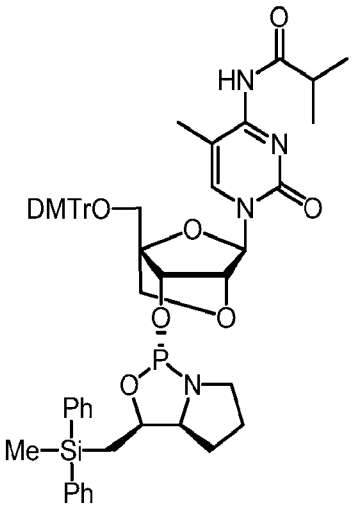

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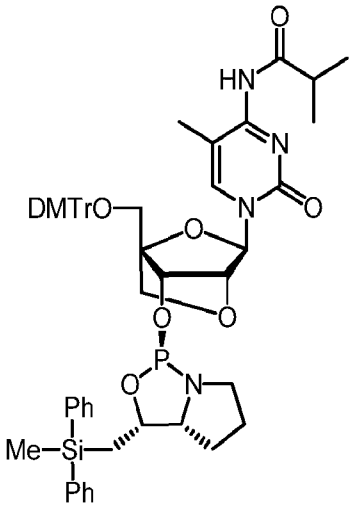

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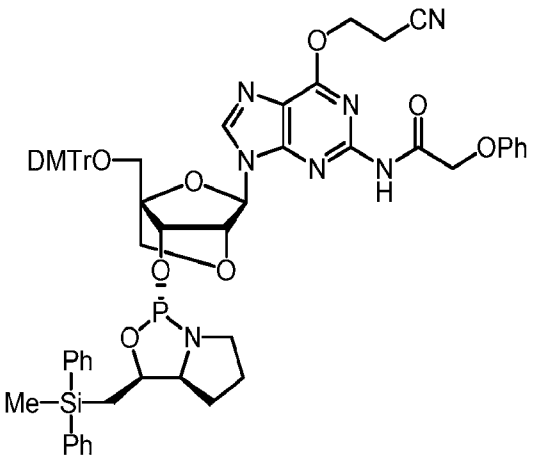

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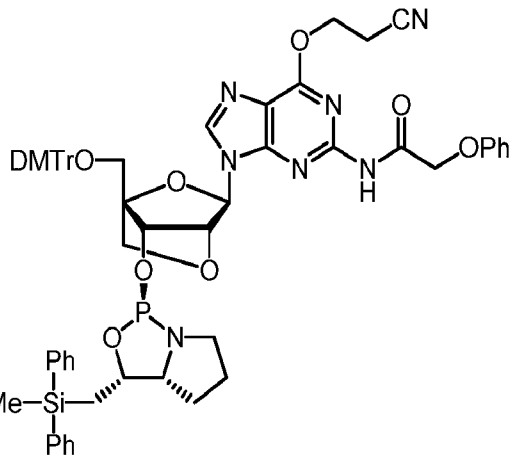

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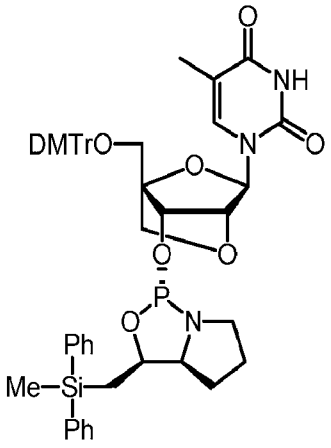

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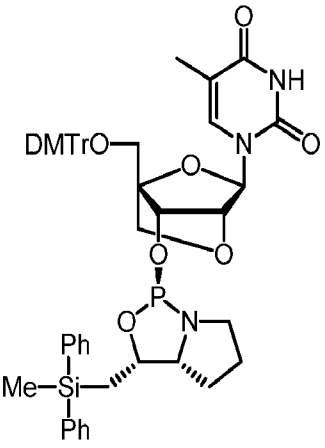

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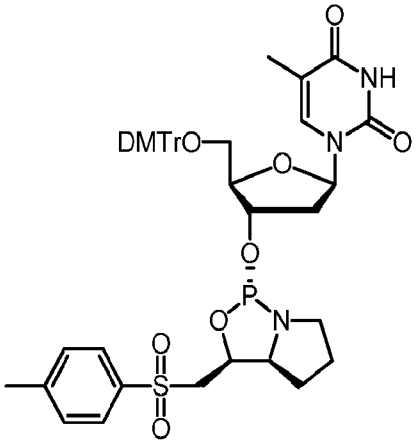

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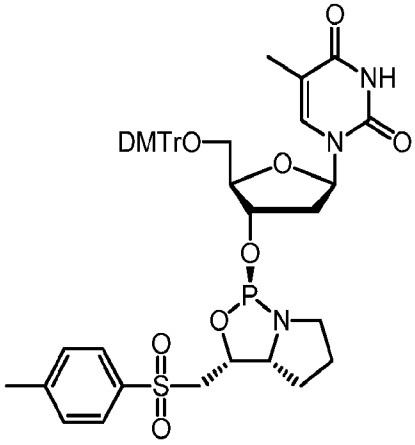


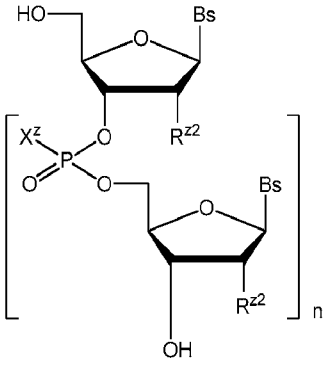
35a

, and

DMTr represents a 4,4'-dimethoxytrityl group and TOM represents a triisopropylsiloxymethyl group.

[00655] Examples using a nucleoside 3'- phosphoramidite derivative are disclosed in, e.g, JP 2005-89441 A. By repeating steps of condensation and de-protection, methods of the present invention facilitate lengthening the chain of oligonucleotide, as disclosed therein.

[00656] In some embodiments, an oligonucleotide is as shown in formula (Z-X):



(Z-X)

,

wherein, in the formula (Z-X), X^z represents sulfide (=S), C_{1-3} alkyl, C_{1-3} alkoxy, C_{1-3} alkylthio, C_6-C_{10} aryl, C_6-C_{10} aralkyl, or C_6-C_{10} aryloxialkyl. In some embodiments, X^z represents sulfide (=S). n^z is an integer that represents 1 to 150, 1 to 100, 1 to 50, or 1 to 30. In some embodiments, n^z is preferably 2 to 100, preferably 10 to 100, preferably 10 to 50, and more preferably 15 to 30.

[00657] In some embodiments, the present invention provides methods for synthesis of a stereocontrolled phosphorus atom-modified oligonucleotide derivative. In some embodiments, , the first step is a step of reacting a molecule comprising an achiral *H*-phosphonate moiety, the first activating reagent and a chiral reagent or a salt thereof to form a monomer. In some embodiments, the chiral reagent has chemical formula (Z-I) or (Z-I') and the monomer may be represented by fomura (Z-Va), (Z-Vb), (Z-Va'), or (Z-Vb'). The monomer reacts with the second activating reagent and a nucleoside to form a condensed intermediate. Next step is a step of converting the condensed intermediate to the nucleic acid comprising a chiral X-phosphonate moiety. In some embodiments, the methods are as described in WO 2010/064146. In some embodiments, the steps are as described in route A and route B of WO 2010/064146.

[00658] In some embodiments, the present invention provides a method of synthesizing chirally controlled oligonucleotide as illustrated in Scheme Z-1 below.

Scheme Z-1

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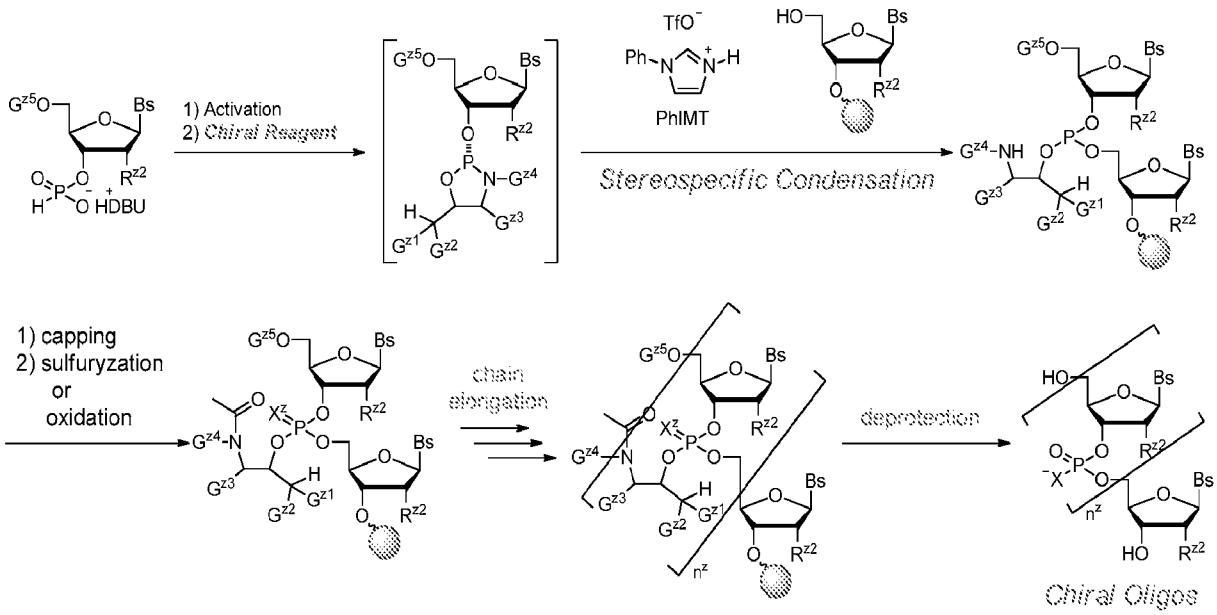

Activation

[00659] An achiral *H*-phosphonate moiety is treated with the first activating reagent to form the first intermediate. In one embodiment, the first activating reagent is added to the reaction mixture during the condensation step. Use of the first activating reagent is dependent on reaction conditions such as solvents that are used for the reaction. Examples of the first activating reagent are phosgene, trichloromethyl chloroformate, bis(trichloromethyl)carbonate (BTC), oxalyl chloride, Ph_3PCl_2, $(PhO)_3PCl_2$, *N,N'*-bis(2-oxo-3-oxazolidinyl)phosphinic chloride (BopCl), 1,3-dimethyl-2-(3-nitro-1,2,4-triazol-1-yl)-2-pyrrolidin-1-yl-1,3,2-diazaphospholidinium hexafluorophosphate (MNTP), or 3-nitro-1,2,4-triazol-1-yl-tris(pyrrolidin-1-yl)phosphonium hexafluorophosphate (PyNTP).

[00660] The example of achiral *H*-phosphonate moiety is a compound shown in the above Scheme. DBU represents 1,8-diazabicyclo[5.4.0]undec-7-ene. H^+DBU may be, for example, ammonium ion, alkylammonium ion, heteroaromatic iminium ion, or heterocyclic iminium ion, any of which is primary, secondary, tertiary or quaternary, or a monovalent metal ion.

Reacting with Chiral Reagent

[00661] After the first activation step, the activated achiral *H*-phosphonate moiety reacts with a chiral reagent, which is represented by formula (Z-I) or (Z-I'), to form a chiral intermediate of formula (Z-Va), (Z-Vb), (Z-Va'), or (Z-Vb').

Stereospecific Condensation Step

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[00662] A chiral intermediate of Formula Z-Va ((Z-Vb), (Z-Va'), or (Z-Vb')) is treated with the second activating reagent and a nucleoside to form a condensed intermediate. The nucleoside may be on solid support. Examples of the second activating reagent are 4,5-dicyanoimidazole (DCI), 4,5-dichloroimidazole, 1-phenylimidazolium triflate (PhIMT), benzimidazolium triflate (BIT), benztriazole, 3-nitro-l,2,4-triazole (NT), tetrazole, 5-ethylthiotetrazole (ETT), 5-benzylthiotetrazole (BTT), 5-(4-nitrophenyl)tetrazole, *N*-cyanomethylpyrrolidinium triflate (CMPT), *N*-cyanomethylpiperidinium triflate, *N*-cyanomethyldimethylammonium triflate. A chiral intermediate of Formula Z-Va ((Z-Vb), (Z-Va'), or (Z-Vb')) may be isolated as a monomer. Usually, the chiral intermediate of Z-Va ((Z-Vb), (Z-Va'), or (Z-Vb')) is not isolated and undergoes a reaction in the same pot with a nucleoside or modified nucleoside to provide a chiral phosphite compound, a condensed intermediate. In other embodiments, when the method is performed via solid phase synthesis, the solid support comprising the compound is filtered away from side products, impurities, and/or reagents.

Capping Step

[00663] If the final nucleic acid is larger than a dimer, the unreacted -OH moiety is capped with a blocking group and the chiral auxiliary in the compound may also be capped with a blocking group to form a capped condensed intermediate. If the final nucleic acid is a dimer, then the capping step is not necessary.

Modifying Step

[00664] The compound is modified by reaction with an electrophile. The capped condensed intermediate may be executed modifying step. In some embodiments, the modifying step is performed using a sulfur electrophile, a selenium electrophile or a boronating agent. Examples of modifying steps are step of oxidation and sulfurization.

[00665] In some embodiments of the method, the sulfur electrophile is a compound having one of the following formulas:

$$S_8 \text{ (Formula Z-B)}, Z^{z1}\text{-S-S-}Z^{z2}, \text{ or } Z^{z1}\text{-S-}V^{z}\text{-}Z^{z2};$$

wherein Z^{z1} and Z^{z2} are independently alkyl, aminoalkyl, cycloalkyl, heterocyclic, cycloalkylalkyl, heterocycloalkyl, aryl, heteroaryl, alkyloxy, aryloxy, heteroaryloxy, acyl, amide, imide, or thiocarbonyl, or Z^{z1} and Z^{z2} are taken together to form a 3 to 8 membered alicyclic or

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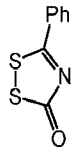
heterocyclic ring, which may be substituted or unsubstituted; V^z is SO_2, O, or NR^f; and R^f is hydrogen, alkyl, alkenyl, alkynyl, or aryl.

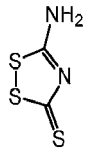
[00666] In some embodiments of the method, the sulfur electrophile is a compound of following Formulae Z-A, Z-B, Z-C, Z-D, Z-E, or Z-F:



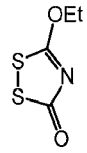
Formula Z-A



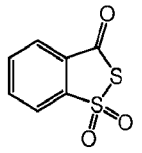
Formula Z-B



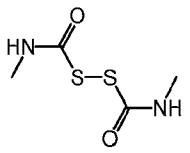
Formula Z-C



Formula Z-D



Formula Z-E



Formula Z-F

[00667] In some embodiments, the selenium electrophile is a compound having one of the following formulae:

Se (Formula Z-G), Z^{z3}-Se-Se-Z^{z4}, or Z^{z3}-Se-V^z-Z^{z4};

wherein Z^{z3} and Z^{z4} are independently alkyl, aminoalkyl, cycloalkyl, heterocyclic, cycloalkylalkyl, heterocycloalkyl, aryl, heteroaryl, alkyloxy, aryloxy, heteroaryloxy, acyl, amide, imide, or thiocarbonyl, or Z^{z3} and Z^{z4} are taken together to form a 3 to 8 membered alicyclic or heterocyclic ring, which may be substituted or unsubstituted; V^z is SO_2, S, O, or NR^f; and R^f is hydrogen, alkyl, alkenyl, alkynyl, or aryl.

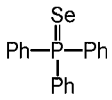
[00668] In some embodiments, the selenium electrophile is a compound of Formula Z-G, Z-H, Z-I, Z-J, Z-K, or Z-L.



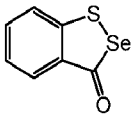
Formula Z-G



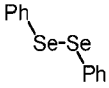
Formula Z-H



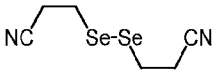
Formula Z-I



Formula Z-J



Formula Z-K



Formula Z-L

[00669] In some embodiments, the boronating agent is borane-*N*,*N*-diisopropylethylamine (BH_3 DIPEA), borane-pyridine (BH_3 Py), borane-2-chloropyridine (BH_3 CPy), borane-aniline (BH_3 An), borane-tetrahydrofiirane (BH_3 THF), or borane-dimethylsulfide (BH_3 Me_2S).

[00670] In some embodiments of the method, the modifying step is an oxidation step. In some embodiments of the method, the modifying step is an oxidation step using similar conditions as described above in this application. In some embodiments, an oxidation step is as disclosed in, *e.g.*, JP 2010-265304 A and WO2010/064146.

Chain Elongation Cycle and De-protection Step

[00671] The capped condensed intermediate is deblocked to remove the blocking group at the 5'-end of the growing nucleic acid chain to provide a compound. The compound is

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optionally allowed to re-enter the chain elongation cycle to form a condensed intermediate, a capped condensed intermediate, a modified capped condensed intermediate, and a 5'-deprotected modified capped intermediate. Following at least one round of chain elongation cycle, the 5'-deprotected modified capped intermediate is further deblocked by removal of the chiral auxiliary ligand and other protecting groups for, *e.g.*, nucleobase, modified nucleobase, sugar and modified sugar protecting groups, to provide a nucleic acid. In other embodiments, the nucleoside comprising a 5'-OH moiety is an intermediate from a previous chain elongation cycle as described herein. In yet other embodiments, the nucleoside comprising a 5'-OH moiety is an intermediate obtained from another known nucleic acid synthetic method. In embodiments where a solid support is used, the phosphorus-atom modified nucleic acid is then cleaved from the solid support. In certain embodiments, the nucleic acids is left attached on the solid support for purification purposes and then cleaved from the solid support following purification.

[00672] In yet other embodiments, the nucleoside comprising a 5'-OH moiety is an intermediate obtained from another known nucleic acid synthetic method. In yet other embodiments, the nucleoside comprising a 5'-OH moiety is an intermediate obtained from another known nucleic acid synthetic method as described in this application. In yet other embodiments, the nucleoside comprising a 5'-OH moiety is an intermediate obtained from another known nucleic acid synthetic method comprising one or more cycles illustrated in Scheme I. In yet other embodiments, the nucleoside comprising a 5'-OH moiety is an intermediate obtained from another known nucleic acid synthetic method comprising one or more cycles illustrated in Scheme I-b, I-c or I-d.

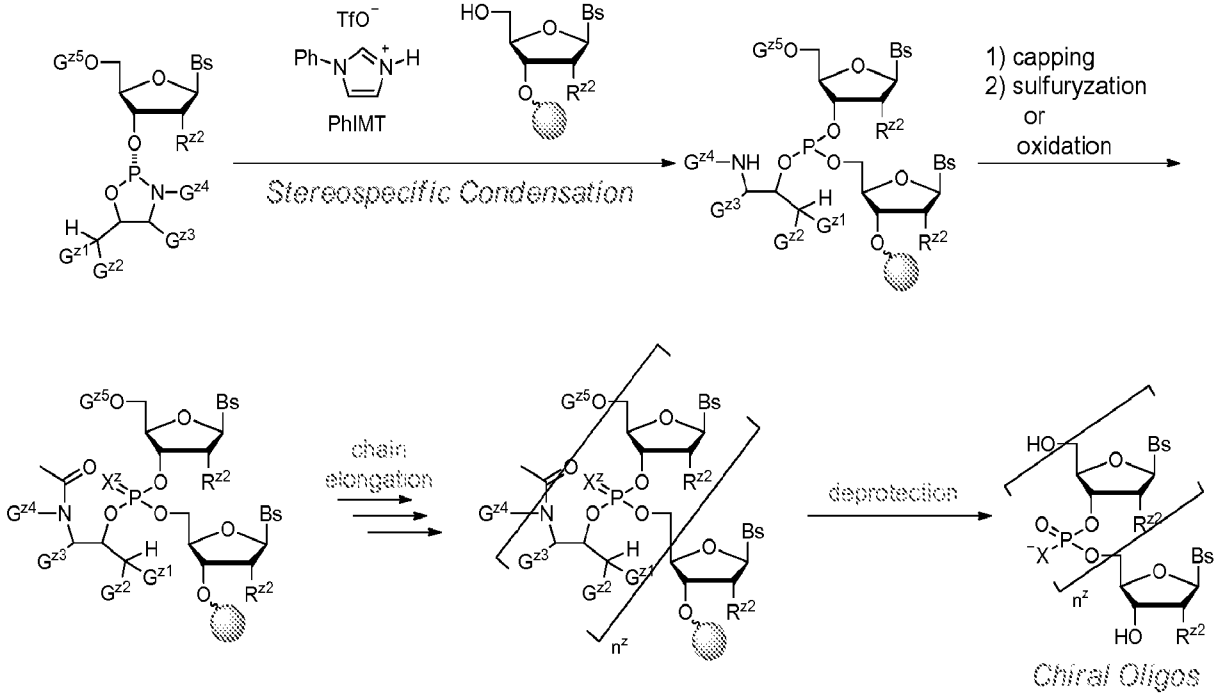
[00673] In some embodiments, the present invention provides oligonucleotide synthesis methods that use stable and commercially available materials as starting materials. In some embodiments, the present invention provides oligonucleotide synthesis methods to produce stereocontrolled phosphorus atom-modified oligonucleotide derivatives using an achiral starting material.

[00674] In some embodiments, the method of the present invention does not cause degradations under the de-protection steps. Further the method does not require special capping agents to produce phosphorus atom-modified oligonucleotide derivatives.

[00675] In some embodiments, the present invention provides methods for the synthesis of stereocontrolled phosphorus atom-modified oligonucleotide derivatives using a chiral monomer.

In some embodiments, the first step is reacting a nucleoside 3'-phosphoramidite derivative which is represented by formula (Z-Va), (Z-Vb), (Z-Va'), or (Z-Vb') with the second activating reagent and a nucleoside to form a condensed intermediate. The second step is converting the condensed intermediate to the nucleic acid comprising a chiral X-phosphonate moiety. An exemplary method is illustrated Scheme Z-2 below.

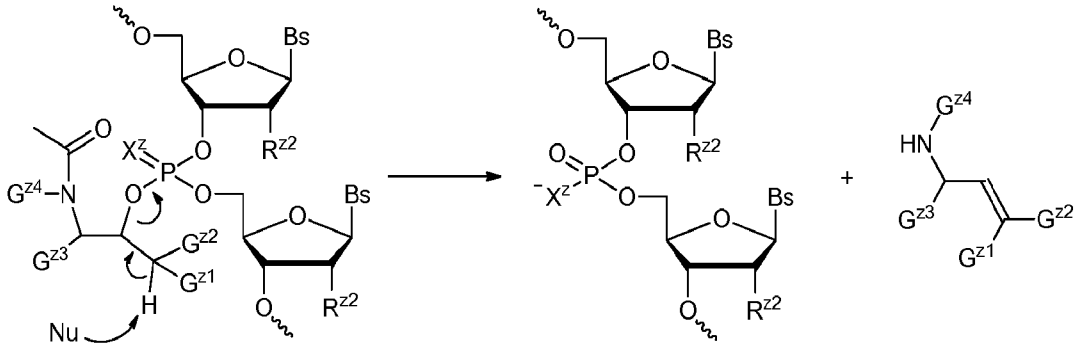
Scheme Z-2



[00676] The detailed conditions of the Scheme Z-2 are similar to that of Scheme Z-1. The starting material of formula Z-Va (Z-Vb), especially of formula Z-Va' (or Z-Vb'), is chemically stable. As shown in a working example, the method of the present invention does not cause degradations under the de-protection steps. Further the method does not require special capping agents to produce phosphorus atom-modified oligonucleotide derivatives.

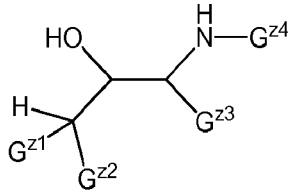
[00677] In some embodiments, mechanism for the removal of auxiliaries is shown as illustrated in Scheme Z-3, below.

Scheme Z-3.



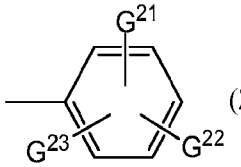
In Scheme Z-3, Nu stands for is a nucleophile. In some embodiments, the mechanism in Scheme Z-3 is thought to be different from the previous mechanism for the removal of auxiliaries.

[00678] In some embodiments, the present invention provides a chiral reagent or a salt thereof, the chiral reagent having following chemical formula (Z-I):



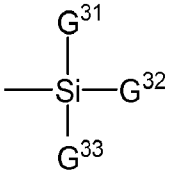
(Z-I)

wherein G^{z1} and G^{z2} are independently a hydrogen atom, a nitro group, a halogen atom, a cyano group, a group of formula (Z-II) or (Z-III), or both G^{z1} and G^{z2} taken together to form a group of formula (Z-IV),



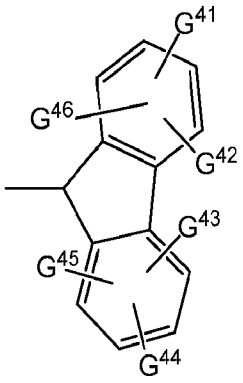
(Z-II)

wherein G^{21} to G^{23} are independently a hydrogen atom, a nitro group, a halogen atom, a cyano group or C_{1-3} alkyl group,



(Z-III)

wherein G^{31} to G^{33} are independently C_{1-4} alkyl group, C_{1-4} alkoxy group, C_{6-14} aryl group, C_{7-14} aralkyl group, C_{1-4} alkyl C_{6-14} aryl group, C_{1-4} alkoxy C_{6-14} aryl group, or C_{6-14} aryl C_{1-4} alkyl group,

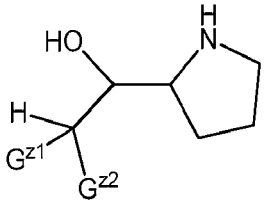


(Z-IV)

wherein G^{41} to G^{46} are independently a hydrogen atom, a nitro group, a halogen atom, a cyano group or C_{1-3} alkyl group,

G^{z3} and G^{z4} are independently a hydrogen atom, C_{1-3} alkyl group, C_{6-14} aryl group, or both G^{z3} and G^{z4} taken together to form a heteroatom-containing ring that has 3 to 16 carbon atoms.

[00679] In some embodiments, the present invention provides a chiral reagent, or a salt thereof, of formula Z-1 has following chemical formula (Z-I')



(Z-I')

wherein each variable is independently as defined above and described herein. In some embodiments, the present invention provides a chiral reagent, or a salt thereof, of formula (Z-1'), wherein each of G^{z1} and G^{z2} is a group of formula (Z-II), wherein G^{21} to G^{23} are independently a hydrogen atom, a nitro group, a halogen atom, a cyano group or C_{1-3} alkyl group. In some embodiments, the present invention provides a chiral reagent, or a salt thereof, of formula (Z-1'), wherein each of G^{z1} and G^{z2} is a group of formula (Z-II), wherein each of G^{21} to G^{23} is a hydrogen atom. In some embodiments, the present invention provides a chiral reagent, or a salt thereof, of formula (Z-1'), wherein G^{z1} is a hydrogen atom, and G^{z2} is a group of formula (Z-II), wherein G^{21} to G^{23} are independently a hydrogen atom, a nitro group, a halogen atom, a cyano group or C_{1-3} alkyl group. In some embodiments, the present invention provides a chiral reagent, or a salt thereof, of formula (Z-1'), wherein G^{z1} is a hydrogen atom, and G^{z2} is a group of formula (Z-II), wherein each of G^{21} and G^{22} is a hydrogen atom and G^{23} is a nitro group. In some embodiments, the present invention provides a chiral reagent, or a salt thereof, of formula (Z-1'), wherein G^{z1} is a hydrogen atom, and G^{z2} is a group of formula (Z-III), wherein G^{31} to G^{33} are independently C_{1-4} alkyl group, C_{6-14} aryl group, C_{7-14} aralkyl group, C_{1-4} alkyl C_{6-14} aryl

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group, C_{1-4} alkoxy C_{6-14} aryl group, or C_{6-14} aryl C_{1-4} alkyl group. In some embodiments, the present invention provides a chiral reagent, or a salt thereof, of formula (Z-1'), wherein G^{z1} is a hydrogen atom, and G^{z2} is a group of formula (Z-III), wherein G^{31} to G^{33} are independently C_{1-4} alkyl group, C_6 aryl group, C_{7-10} aralkyl group, C_{1-4} alkyl C_6 aryl group, C_{1-4} alkoxy C_6 aryl group, or C_6 aryl C_{1-4} alkyl group. In some embodiments, the present invention provides a chiral reagent, or a salt thereof, of formula (Z-1'), wherein G^{z1} is a hydrogen atom, and G^{z2} is a group of formula (Z-III), wherein G^{31} to G^{33} are independently C_{1-4} alkyl group, or C_6 aryl group. In some embodiments, the present invention provides a chiral reagent, or a salt thereof, of formula (Z-1'), wherein G^{z1} is a hydrogen atom, and G^{z2} is a group of formula (Z-III), wherein G^{31} and G^{33} are C_6 aryl group and G^{32} is C_{1-2} alkyl group. In some embodiments, the present invention provides a chiral reagent, or a salt thereof, of formula (Z-1'), wherein G^{z1} is a hydrogen atom, and G^{z2} is a group of formula (Z-III), wherein G^{31} to G^{33} are independently C_{1-4} alkyl group. In some embodiments, the present invention provides a chiral reagent, or a salt thereof, of formula (Z-1'), wherein G^{z1} is a hydrogen atom, and G^{z2} is a group of formula (Z-III), wherein G^{31} and G^{33} are C_6 aryl group and G^{32} is C_{1-4} alkyl group. In some embodiments, the present invention provides a chiral reagent, or a salt thereof, of formula (Z-1'), wherein G^{z1} is a hydrogen atom, and G^{z2} is a group of formula (Z-IV), wherein G^{41} to G^{46} are independently a hydrogen atom, a nitro group, a halogen atom, a cyano group or C_{1-3} alkyl group. In some embodiments, the present invention provides a chiral reagent, or a salt thereof, of formula (Z-1'), wherein G^{z1} and G^{z2} are taken together to form a group of formula (Z-IV), wherein G^{41} to G^{46} are independently a hydrogen atom, a nitro group, a halogen atom, a cyano group or C_{1-3} alkyl group. In some embodiments, the present invention provides a chiral reagent, or a salt thereof, of formula (Z-1'), wherein G^{z1} and G^{z2} are taken together to form a group of formula (Z-IV), wherein each of G^{41} to G^{46} is a hydrogen atom.

[00680] In some embodiments, a chiral reagent or a salt thereof is selected from formulae **3a**, **3b**, **5a**, Z-**5b**, **7a**, **7b**, **9a**, **9b**, **11a** and **11b**.

[00681] In some embodiments, the present invention provides a nucleoside 3'-phosphoramidite derivative which is represented by formula Z-Va or Z-Vb:

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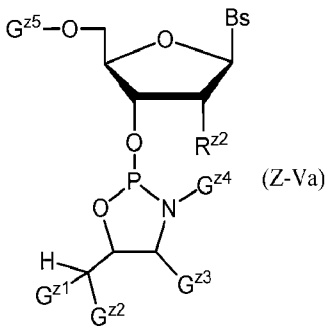
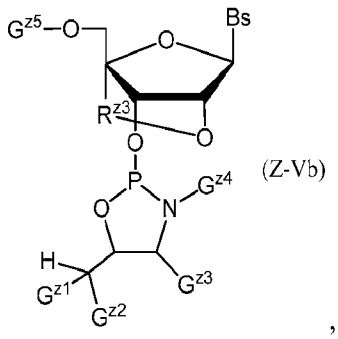
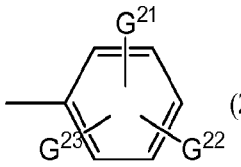

(Z-Va)

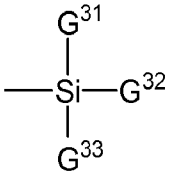

(Z-Vb)

,

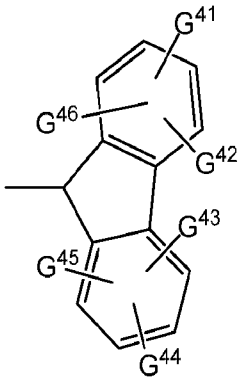
wherein G^{z1} and G^{z2} are independently a hydrogen atom, a nitro group, a halogen atom, a cyano group, a group of formula (Z-II) or (Z-III), or both G^{z1} and G^{z2} taken together to form a group of formula (Z-IV),


(Z-II)

wherein G^{21} to G^{23} are independently a hydrogen atom, a nitro group, a halogen atom, a cyano group or C_{1-3} alkyl group,


(Z-III)

wherein G^{31} to G^{33} are independently C_{1-4} alkyl group, C_{1-4} alkoxy group, C_{6-14} aryl group, C_{7-14} aralkyl group, C_{1-4} alkyl C_{6-14} aryl group, C_{1-4} alkoxy C_{6-14} aryl group, or C_{6-14} aryl C_{1-4} alkyl group,


(Z-IV)

wherein G^{41} to G^{46} are independently a hydrogen atom, a nitro group, a halogen atom, a cyano group or C_{1-3} alkyl group;

G^{z3} and G^{z4} are independently a hydrogen atom, C_{1-3} alkyl group, C_{6-14} aryl group, or both G^{z3} and G^{z4} taken together to form a heteroatom-containing ring that has 3 to 16 carbon atoms;

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G^{z5} is a protective group of a hydroxyl group;

R^{z2} is independently hydrogen, -OH, -SH, $-NR^dR^d$, $-N_3$, halogen, alkyl, alkenyl, alkynyl, alkyl-Y^1-, alkenyl-Y^1-, alkynyl-Y^1-, aryl-Y^1-, heteroaryl-Y^1-, $-OR^b$, or $-SR^b$, wherein R^b is a blocking moiety;

Y^1 is O, NR^d, S, or Se;

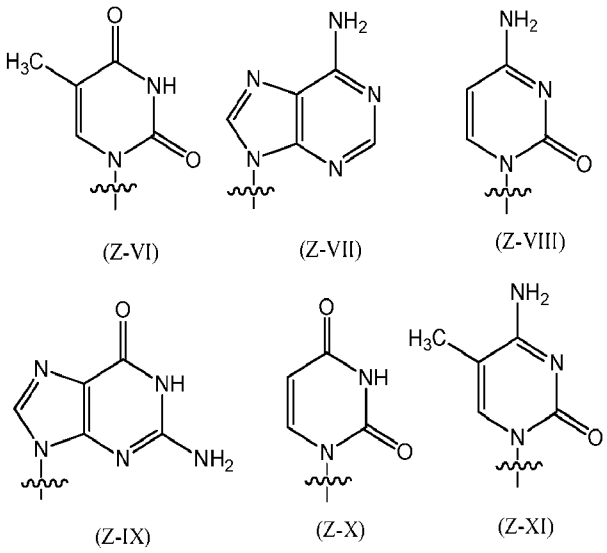
R^d is independently hydrogen, alkyl, alkenyl, alkynyl, aryl, acyl, substituted silyl, carbamate, $-P(O)(R^e)_2$, or $-HP(O)(R^e)$;

R^e is independently hydrogen, alkyl, aryl, alkenyl, alkynyl, alkyl-Y^2-, alkenyl- Y^2- , alkynyl-Y^2-, aryl-Y^2-, or heteroaryl-Y^2-, or a cation which is Na^+, Li^+, or K^+, or $-O^-$;

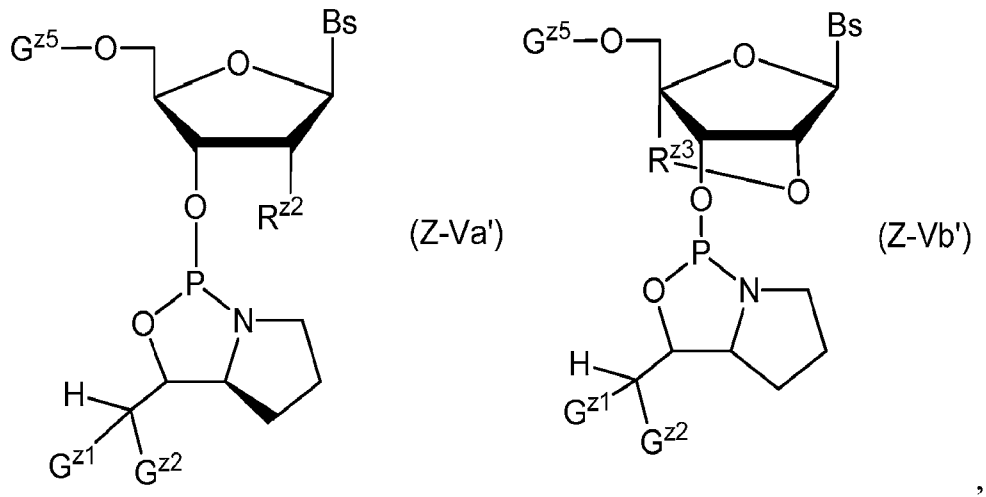
Y^2 is O, NR^d, or S;

R^{z3} is a group represented by $-CH_2-$, $-(CH_2)_2-$, $-CH_2NH-$, or $-CH_2N(CH_3)-$; and

Bs is a group selected from the groups represented by following formula (Z-VI) to (Z-XI) or derivatives thereof.



[00682] In some embodiments, the present invention provides a nucleoside 3'-phosphoramidite derivative of formula Z-Va or Z-Vb, having the structure of (Z-Va') or (Z-Vb'):



,

wherein each variable is independently as defined above and described herein.

[00683] In some embodiments, the present invention provides a nucleoside 3'-phosphoramidite derivative selected from formulae **12a**, **12b**, **13a**, **13b**, **14a**, **14b**, **15a**, **15b**, **16a**, **16b**, **17a**, **17b**, **18a**, **18b**, **19a**, **19b**, **20a**, **20b**, **21a**, **21b**, **22a**, **22b**, **23a**, **23b**, **24a**, **24b**, **25a**, **25b**, **26a**, **26b**, **27a**, **27b**, **28a**, **28b**, **29a**, **29b**, **30a**, **30b**, **31a**, **31b**, **32a**, **32b**, **33a**, **33b**, **34a**, **34b** and **35a**. In some embodiments, the present invention provides a nucleoside 3'-phosphoramidite derivative selected from formulae **12a**, **12b**, **13a**, **13b**, **14a**, **14b**, **15a**, **15b**, **16a**, **16b**, **17a**, **17b**, **18a**, **18b**, **19a**, **19b**, **20a**, **20b**, **21a**, **21b**, **22a**, **22b**, **23a**, **23b**, **24a**, **24b**, **25a**, **25b**, **26a**, or **26b**.

[00684] In some embodiments, the present invention provides a method for synthesis of stereocontrolled phosphorus atom-modified oligonucleotide derivatives comprising steps of:

reacting a molecule comprising an achiral *H*-phosphonate moiety, a chiral reagent or a salt thereof to form a monomer of a nucleoside 3'-phosphoramidite derivative;

reacting the monomer and a nucleoside to form a condensed intermediate; and

converting the condensed intermediate to the nucleic acid comprising a chiral X-phosphonate moiety;

wherein the chiral reagent has following chemical formula (Z-I).

[00685] In some embodiments, the present invention provides a method for synthesis of stereocontrolled phosphorus atom-modified oligonucleotide derivatives comprising steps of:

reacting a molecule comprising an achiral *H*-phosphonate moiety, a chiral reagent or a salt thereof to form a monomer of a nucleoside 3'-phosphoramidite derivative;

reacting the monomer and a nucleoside to form a condensed intermediate; and

converting the condensed intermediate to the nucleic acid comprising a chiral X-phosphonate moiety;

wherein the chiral reagent has following chemical formula (Z-I').

[00686] In some embodiments, the present invention provides a method for synthesis of stereocontrolled phosphorus atom-modified oligonucleotide derivatives comprising steps of:

reacting a molecule comprising an achiral *H*-phosphonate moiety, a chiral reagent or a salt thereof to form a monomer of a nucleoside 3'-phosphoramidite derivative;

reacting the monomer and a nucleoside to form a condensed intermediate; and

converting the condensed intermediate to the nucleic acid comprising a chiral X-phosphonate moiety;

wherein the chiral reagent is selected from formulae **3a**, **3b**, **5a**, **Z-5b**, **7a**, **7b**, **9a**, **9b**, **11a** and **11b**.

[00687] In some embodiments, the present invention provides a method for synthesis of stereocontrolled phosphorus atom-modified oligonucleotide derivatives comprising steps of:

reacting a nucleoside 3'-phosphoramidite derivative which is represented by formula (Z-Va) or (Z-Vb), with an activating reagent and a nucleoside to form a condensed intermediate; and converting the condensed intermediate to the nucleic acid comprising a chiral X-phosphonate moiety.

[00688] In some embodiments, the present invention provides a method for synthesis of stereocontrolled phosphorus atom-modified oligonucleotide derivatives comprising steps of:

reacting a nucleoside 3'-phosphoramidite derivative represented by formula (Z-Va) or (Z-Vb), with an activating reagent and a nucleoside to form a condensed intermediate; and converting the condensed intermediate to the nucleic acid comprising a chiral X-phosphonate moiety; and wherein the nucleoside 3'-phosphoramidite derivative represented by formula (Z-Va) or (Z-Vb) is selected from formulae **12a**, **12b**, **13a**, **13b**, **14a**, **14b**, **15a**, **15b**, **16a**, **16b**, **17a**, **17b**, **18a**, **18b**, **19a**, **19b**, **20a**, **20b**, **21a**, **21b**, **22a**, **22b**, **23a**, **23b**, **24a**, **24b**, **25a**, **25b**, **26a**, **26b**, **27a**, **27b**, **28a**, **28b**, **29a**, **29b**, **30a**, **30b**, **31a**, **31b**, **32a**, **32b**, **33a**, **33b**, **34a**, **34b** and **35a**. In some embodiments, the present invention provides a method for synthesis of stereocontrolled phosphorus atom-modified oligonucleotide derivatives comprising steps of:

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reacting a nucleoside 3'-phosphoramidite derivative represented by formula (Z-Va) or (Z-Vb), with an activating reagent and a nucleoside to form a condensed intermediate; and converting the condensed intermediate to the nucleic acid comprising a chiral X-phosphonate moiety; and wherein the nucleoside 3'-phosphoramidite derivative represented by formula (Z-Va) or (Z-Vb) is selected from formulae **12a**, **12b**, **13a**, **13b**, **14a**, **14b**, **15a**, **15b**, **16a**, **16b**, **17a**, **17b**, **18a**, **18b**, **19a**, **19b**, **20a**, **20b**, **21a**, **21b**, **22a**, **22b**, **23a**, **23b**, **24a**, **24b**, **25a**, **25b**, **26a**, and **26b**.

Preparation and Use of Certain Chiral Auxiliaries of Formula Z-I

Abbreviation

ac: acetyl

bz: benzoyl

CSO: (1*S*)-(+)-(10-camphorsulfonyl)oxaziridine

DBU: 1,8-diazabicyclo[5.4.0]undec-7-ene

DCA: dichloroacetic acid

DCM: dichloromethane, CH_2Cl_2

Tr: trityl, triphenylmethyl

MeIm: *N*-methylimidazole

NIS: *N*-iodosuccinimide

pac: phenoxyacetyl

Ph: phenyl

PhIMT: *N*-phenylimidazolium triflate

POS: 3-phenyl-1,2,4-dithiazoline-5-one

TBS: *tert*-butyldimethylsilyl

TBDPS: *tert*-butyldiphenylsilyl

TOM: triisopropylsiloxymethyl

TFA: trifluoroacetic acid

General procedure for the synthesis of chirally controlled oligonucleotides -1.

[00689] The automated solid-phase synthesis of chirally controlled oligonucleotides was performed according to the cycles shown in Table Z-1.

Table Z-1. Synthesis procedure.

step	operation	reagents and solvent	volume	waiting time
1	detritylation	3% DCA/DCM	1.6 mL	20 s
2	coupling	0.1M monomer/MeCN + 1M PhIMT	0.5 mL	5 min
3	capping	Ac_2O/THF-pyridine + 16% MeIm/THF	0.5 mL	30 s
4	oxidation / sulfurization	0.5M CSO/MeCN or 0.1M POS/MeCN	0.5 mL	90 s

General procedure for the synthesis of chirally controlled oligonucleotides -2.

[00690] The automated solid-phase synthesis of chirally controlled oligonucleotides was performed according to the cycles shown in Table Z-2.

Table Z-2.

step	operation	reagents and solvent	volume	waiting time
1	detritylation	3% DCA/DCM	1.6 mL	20 s
2	coupling	pre-activated monomer* + 1M PhIMT	0.5 mL	5 min
3	capping	Ac_2O/THF-pyridine + 16% MeIm/THF	0.5 mL	30 s
4	oxidation / sulfurization	0.5M CSO/MeCN or 0.1M POS/MeCN	0.5 mL	90 s

* *preparation of pre-activated monomer in Step 2 of TableZ- 2:*

[00691] Nucleoside-3′-*H*-phosphonate monoester is dried by repeated coevaporations with dry toluene and then dissolved in dry MeCN. Ph_3PCl_2 is added to the solution, and the mixture is stirred for 5 min. To the mixture, a solution of chiral reagent, which is repeated coevaportions with dry toluene and dissolved in dry MeCN, is added dropwise via syringe, and the mixture is stirred for 5 min under argon.

[00692] After the synthesis, the resin was treated with a 25% NH_3 aqueous solution (1 mL) for 12 h at 55 °C. The mixture was cooled to room temperature and the resin was removed by membrane filtration. The filtrate was concentrated to dryness under reduced pressure. The residue was dissolved in H_2O (3 mL) and analyzed by RP-UPLC-MS with a linear gradient of acetonitrile (0–50%/30 min) in 0.1 M triethylammonium acetate buffer (pH 7.0) at 50 °C at a rate of 0.3 mL/min.

[00693] Example Z-1.

[00694] **(*S*)-1-Tritylpyrrolidin-2-carbaldehyde (1a).**

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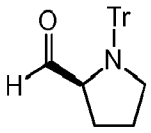
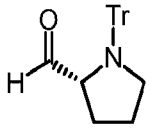


1a

[00695] Compound **1a** was synthesized from L-proline according to the procedure described in the literature (Guga, P. *Curr. Top. Med. Chem.* **2007**, *7*, 695-713.).

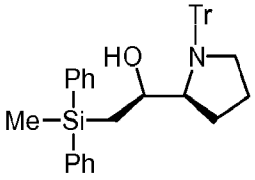
[00696] (*R*)-1-Tritylpyrrolidin-2-carbaldehyde (1b).



1b

[00697] Compound **1b** was synthesized from D-proline in a similar manner to compound **1a**.

[00698] (*S*)-2-(Methyldiphenylsilyl)-1-((*S*)-1-tritylpyrrolidin-2-yl)ethanol (2a).



2a

[00699] To a solution of methyldiphenylsilylmethyl magnesium chloride in THF prepared from chloromethyldiphenylmethylsilane (4.02 g, 16.3 mmol) and magnesium (402 mg, 16.3 mmol) in THF (14 mL) was added **1a** (2.79 g, 8.14 mmol) in THF (30 mL) solution with ice cooling. After stirring for 1.5 h with ice cooling, the mixture warmed to room temperature and continued stirring for 30 min. Saturated aqueous NH_4Cl (100 mL) was added to the reaction mixture at 0°C, and extraction was performed with diethylether (100 mL) for three times. The combined extract was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was chromatographed on silica gel afforded **2a** as a colorless foam (3.91 g, 87%). ^1H NMR (300 MHz, $CDCl_3$) δ 7.48-7.08 (25H, m), 4.33-4.23 (1H, m), 3.16-2.89 (3H, m), 2.84 (1H, brs), 1.70-1.54 (1H, m), 1.35 (1H, dd, *J* = 14.7, 6.3Hz), 1.10 (1H, dd, *J* = 14.7, 8.1Hz), 1.18-1.05 (1H, m), 1.04-0.90 (1H, m), 0.34 (3H, s), -0.17- -0.36 (1H, m).

[00700] (*S*)-2-(Methyldiphenylsilyl)-1-((*S*)-1-pyrrolidin-2-yl)ethanol (3a).

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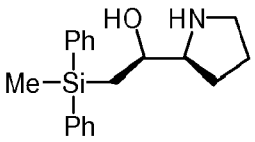
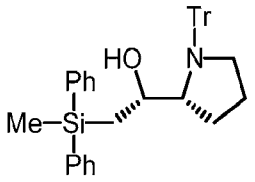


3a

[00701] **2a** (3.91 g, 7.06 mmol) was dissolved in 3% DCA in DCM (70 mL), and stirred for 10 min at room temperature. To the mixture, 1M NaOH (200 mL) was added, and extraction was performed with DCM (100 mL) for three times. The combined extract was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was chromatographed on silica gel afforded **3a** as a light yellow oil (1.99 g, 90%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.57-7.52 (5H, m), 7.38-7.33 (5H, m), 3.77 (1H, ddd, *J* = 8.9, 5.4, 3.5Hz), 3.01 (1H, dt, *J* = 7.4, 3.6Hz), 2.97-2.79 (2H, m), 2.27 (2H, brs), 1.76-1.53 (4H, m), 1.38 (1H, dd, *J* = 15.0, 9.0Hz), 1.24 (1H, dd, *J* = 15.0, 5.4Hz), 0.65 (3H, s); ^{13}C NMR (100.4 MHz, $CDCl_3$) δ 137.4, 137.1, 134.6, 134.5, 129.1, 127.8, 69.5, 64.1, 47.0, 25.8, 24.0, 19.6, -3.4. MALDI TOF-MS *m/z* Calcd for $C_{19}H_{26}NOSi$ $[M+H]^+$ 312.18, found 312.06.

[00702] Example Z-2.

[00703] (*R*)-2-(Methyldiphenylsilyl)-1-((*R*)-1-tritylpyrrolidin-2-yl)ethanol (2b).



2b

[00704] Compound **2b** was obtained by using **1b** instead of **1a** in a similar manner to compound **2a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.48-7.12 (25H, m), 4.33-4.24 (1H, m), 3.16-2.89 (3H, m), 2.86 (1H, brs), 1.69-1.52 (1H, m), 1.35 (1H, dd, *J* = 14.4, 6.0Hz), 1.10 (1H, dd, *J* = 14.4, 8.4Hz), 1.18-1.05 (1H, m), 1.03-0.89 (1H, m), 0.33 (3H, s), -0.19- -0.39 (1H, m); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 144.5, 137.5, 136.8, 134.6, 134.3, 129.8, 129.0, 127.8, 127.7, 127.4, 126.1, 77.9, 71.7, 65.1, 53.5, 25.0, 24.8, 19.6, -4.0. MALDI TOF-MS *m/z* Calcd for $C_{38}H_{40}NOSi$ $[M+H]^+$ 554.29, found 554.09.

[00705] (*R*)-2-(Methyldiphenylsilyl)-1-((*R*)-1-pyrrolidin-2-yl)ethanol (3b).

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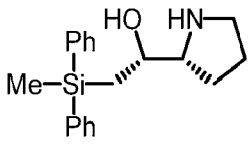


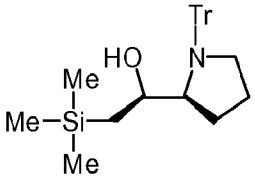
3b

[00706] Compound **3b** was obtained by using **2b** instead of **2a** in a similar manner to compound **3a**.

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.58-7.52 (5H, m), 7.38-7.33 (5H, m), 3.78 (1H, ddd, *J* = 9.0, 5.1, 3.6Hz), 3.00 (1H, dt, *J* = 7.4, 3.3Hz), 2.97-2.78 (2H, m), 2.19 (2H, brs), 1.76-1.53 (4H, m), 1.38 (1H, dd, *J* = 14.6, 9.0Hz), 1.24 (1H, dd, *J* = 14.6, 5.1Hz), 0.66 (3H, s); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 137.5, 137.1, 134.5, 134.4, 129.0, 127.7, 69.2, 64.2, 46.9, 25.8, 24.0, 19.7, -3.4. MALDI TOF-MS *m/z* Calcd for $C_{19}H_{26}NOSi$ $[M+H]^+$ 312.18, found 312.09.

[00707] Example Z-3.

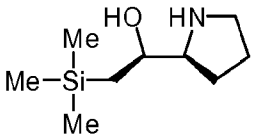
[00708] (*S*)-2-(Trimethylsilyl)-1-((*S*)-1-tritylpyrrolidin-2-yl)ethanol (4a).



4a

[00709] Compound **4a** was obtained by using "chloromethyltrimethylsilane" instead of "chloromethyldiphenylmethylsilane" in a similar manner to compound **2a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.58-7.51 (5H, m), 7.31-7.14 (10H, m), 4.13 (1H, dt, *J* = 7.5, 3.0Hz), 3.39-3.31 (1H, m), 3.20-2.99 (2H, m), 2.84 (1H, s), 1.74-1.57 (1H, m), 1.29-1.10 (2H, m), 0.74 (1H, dd, *J* = 14.4, 7.2Hz), 0.46 (1H, dd, *J* = 14.4, 7.2Hz), -0.15 (9H, s). MALDI TOF-MS *m/z* Calcd for $C_{28}H_{36}NOSi$ $[M+H]^+$ 430.26, found 430.09.

[00710] (*S*)-2-(Trimethylsilyl)-1-((*S*)-1-pyrrolidin-2-yl)ethanol (5a).



5a

[00711] Compound **5a** was obtained by using **4a** instead of **2a** in a similar manner to compound **3a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.76 (1H, ddd, *J* = 8.8, 5.7, 3.3Hz), 3.08 (1H, dt, *J*

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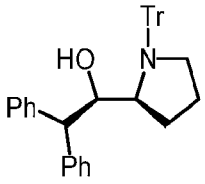
= 7.8, 3.3Hz), 3.02-2.87 (2H, m), 2.48 (2H, brs), 1.81-1.58 (4H, m), 0.83 (1H, dd, J = 14.7, 8.7Hz), 0.68 (1H, dd, J = 14.7, 6.0Hz), 0.05 (9H, s); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 69.6, 64.3, 46.9, 25.8, 23.9, 22.0, -0.8. MALDI TOF-MS *m/z* Calcd for $C_9H_{22}NOSi$ $[M+H]^+$ 188.15, found 188.00.

[00712] Example Z-5.

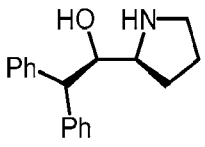
[00713] (*R*)-2,2-Diphenyl-1-((*S*)-1-tritylpyrrolidin-2-yl)ethanol (6a).



6a

[00714] To a solution of diphenylmethane (6.7 mL, 40mmol) in anhydrous THF (36 mL), *n*-BuLi (1.67M solution of Hexane, 24mL, 40mmol) was added dropwise at room temperature and stirred for 1 h. To the mixture, **1a** (3.41g, 10mmol), which was dried by repeated coevaporations with toluene, in anhydrous THF (40mL) was slowly added at 0 °C, and continued stirring for 45 min. A saturated NH_4Cl aqueous solution (100mL) and Et_2O (100mL) were then added, and the organic layer was separated and the aqueous layer was extracted with Et_2O (2 × 100mL). The organic layer were combined, dried over Na_2SO_4, filterd and concentrated under reduced pressure. The residue was purified by chromatography on silica gel to afford **6a** (1.41g, 28%) as white foam.

[00715] (*R*)-2,2-Diphenyl-1-((*S*)-pyrrolidin-2-yl)ethanol (7a).



7a

[00716] **6a**（650mg, 1.27mmol）was dissolved in 3% DCA in DCM (13 mL) , and stirred for 10 min at room temperature. To the mixture, 1M NaOH (40 mL) was added, and extraction was performed with DCM (30 mL) for three times. The combined extract was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was chromatographed on silica gel afforded **7a** as a light yellow oil (316 mg, 93%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.44-7.38 (2H, m), 7.33-7.14 (8H, m), 4.46 (1H, dd, J = 9.9, 3.3Hz), 3.91 (1H, d,

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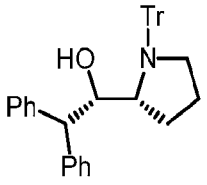
J = 9.9Hz), 3.02-2.88 (2H, m), 2.81-2.69 (1H, m), 2.52 (2H, brs), 1.88-1.56 (4H, m); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 142.3, 142.0, 128.6, 128.5, 128.4, 128.2, 126.5, 126.4, 73.5, 60.1, 55.8, 46.6, 25.8, 23.4. MALDI TOF-MS *m/z* Calcd for $C_{18}H_{22}NO$ $[M+H]^+$ 268.17, found 268.06.

[00717] Example Z-6.

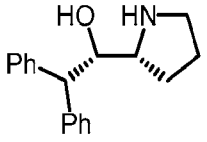
[00718] (*S*)-2,2-Diphenyl-1-((*R*)-1-tritylpyrrolidin-2-yl)ethanol (6b).



6b

[00719] Compound **6b** was obtained by using **1b** instead of **1a** in a similar manner to compound **6a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.44-7.37 (6H, m), 7.30-7.01 (17H, m), 6.66-6.61 (2H, m), 4.80 (1H, d, *J* = 10.8Hz), 3.63 (1H, d, *J* = 10.8Hz), 3.36-3.28 (1H, m), 3.22-3.09 (1H, m), 3.01-2.89 (1H, m), 2.66 (1H, s), 1.90-1.75 (1H, m), 1.29-1.04 (2H, m), 0.00--0.19 (1H, m); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 144.2, 142.9, 141.6, 130.0, 128.5, 128.4, 127.9, 127.8, 127.4, 126.4, 126.2, 77.9, 75.9, 61.9, 55.4, 53.4, 24.7, 24.5. MALDI TOF-MS *m/z* Calcd for $C_{37}H_{36}NO$ $[M+H]^+$ 510.28, found 510.11.

[00720] (*S*)-2,2-Diphenyl-1-((*R*)-pyrrolidin-2-yl)ethanol (7b).

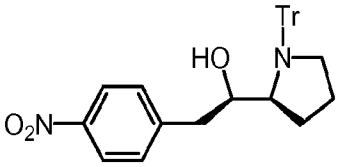


7b

[00721] Compound **7b** was obtained by using **6b** instead of **6a** in a similar manner to compound **7a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.45-7.14 (10H, m), 4.45 (1H, dd, *J* = 9.9, 3.3Hz), 3.91 (1H, d, *J* = 9.9Hz), 3.00-2.89 (2H, m), 2.82-2.71 (1H, m), 2.40 (2H, brs), 1.87-1.55 (4H, m); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 142.3, 142.0, 128.5, 128.3, 128.1, 126.3, 126.2, 73.4, 60.1, 55.9, 46.5, 25.8, 23.5. MALDI TOF-MS *m/z* Calcd for $C_{18}H_{22}NO$ $[M+H]^+$ 268.17, found 268.03.

[00722] Example Z-7.

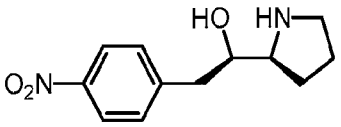
[00723] (*R*)-2-(4-Nitrophenyl)-1-((*S*)-1-tritylpyrrolidin-2-yl)ethanol (8a).



8a

[00724] Compound **8a** was obtained by using "4-nitrobenzylchloride" instead of "diphenylmethane" in a similar manner to compound **6a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.09-8.03 (2H, m), 7.49-7.43 (6H, m), 7.28-7.09 (11H, m), 4.23 (1H, ddd, *J* = 8.3, 5.6, 3.0Hz), 3.43-3.33 (1H, m), 3.23-3.11 (1H, m), 3.07-2.96 (1H, m), 2.83 (1H, brs), 2.74 (1H, dd, *J* = 13.8, 8.4Hz), 2.49 (1H, dd, *J* = 13.8, 5.1Hz), 1.83-1.67 (1H, m), 1.41-1.17 (2H, m), 0.27-0.08 (1H, m); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 147.3, 146.3, 144.3, 129.8, 129.6, 127.5, 126.3, 123.4, 77.9, 74.8, 63.5, 53.2, 39.5, 25.0, 24.9. MALDI TOF-MS *m/z* Calcd for $C_{31}H_{31}N_2O_3$ $[M+H]^-$ 479.23, found 479.08.

[00725] (*R*)-2-(4-Nitrophenyl)-1-((*S*)-pyrrolidin-2-yl)ethanol (9a).

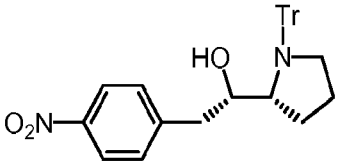


9a

[00726] Compound **9a** was obtained by using **8a** instead of **6a** in a similar manner to compound **7a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.15 (2H, d, *J* = 8.7Hz), 7.42 (2H, d, *J* = 8.7Hz), 3.86-3.79 (1H, m), 3.16-3.07 (1H, m), 2.99-2.68 (6H, m), 1.84-1.68 (4H, m); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 147.4, 146.2, 129.9, 123.2, 72.4, 62.0, 46.6, 40.4, 25.7, 24.4. MALDI TOF-MS *m/z* Calcd for $C_{12}H_{17}N_2O_3$ $[M+H]^+$ 237.12, found 237.01.

[00727] Example Z-8.

[00728] (*S*)-2-(4-Nitrophenyl)-1-((*R*)-1-tritylpyrrolidin-2-yl)ethanol (8b).



8b

[00729] Compound **8b** was obtained by using **1b** instead of **1a** in a similar manner to compound **8a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.09-8.04 (2H, m), 7.49-7.43 (6H, m), 7.28-7.09 (11H, m), 4.22 (1H, ddd, *J* = 8.4, 5.6, 3.0Hz), 3.43-3.33 (1H, m), 3.24-3.10 (1H, m), 3.08-2.94

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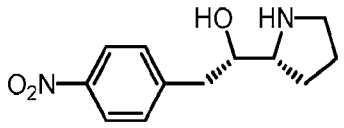
(1H, m), 2.81 (1H, brs), 2.75 (1H, dd, J = 14.0, 8.1Hz), 2.49 (1H, dd, J = 14.0, 5.1Hz), 1.81-1.67 (1H, m), 1.40-1.16 (2H, m), 0.26-0.09 (1H, m); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 147.3, 144.3, 129.8, 129.6, 129.4, 126.3, 123.5, 77.9, 74.8, 63.5, 53.2, 39.5, 25.0, 24.9. MALDI TOF-MS *m/z* Calcd for $C_{31}H_{31}N_2O_3$ $[M+H]^+$ 479.23, found 479.08.

[00730] (*S*)-2-(4-Nitrophenyl)-1-((*R*)-pyrrolidin-2-yl)ethanol (9b).

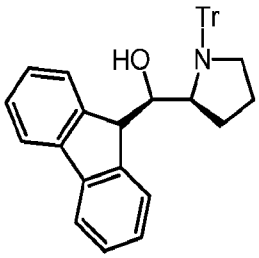


9b

[00731] Compound **9b** was obtained by using **8b** instead of **8a** in a similar manner to compound **9a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.19-8.13 (2H, m), 7.45-7.39 (2H, m), 3.83 (1H, ddd, J = 7.7, 5.4, 3.9Hz), 3.14 (1H, dt, J = 7.7, 3.9Hz), 3.01-2.87 (2H, m), 2.83 (1H, d, J = 3.3Hz), 2.81 (1H, s), 2.62 (2H, brs), 1.79-1.72 (4H, m); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 147.3, 146.5, 130.0, 123.5, 72.7, 61.7, 46.7, 40.1, 25.8, 24.2. MALDI TOF-MS *m/z* Calcd for $C_{12}H_{17}N_2O_3$ $[M+H]^+$ 237.12, found 237.02.

[00732] Example Z-9.

[00733] (*R*)-(*9H*-Fluoren-9-yl)((*S*)-1-tritylpyrrolidin-2-yl)methanol (10a).



10a

[00734] Compound **10a** was obtained by using "fluorene" instead of "diphenylmethane" in a similar manner to compound **6a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.70 (1H, d, J = 7.5Hz), 7.66 (1H, d, J = 7.8Hz), 7.55 (2H, d, J = 7.5Hz), 7.44-7.09 (18H, m), 6.87-6.62 (1H, m), 4.55-4.48 (1H, m), 4.06 (1H, d, J = 7.5Hz), 3.43-3.34 (1H, m), 3.18-3.06 (1H, m), 2.98-2.88 (1H, m), 2.85 (1H, brs), 1.42-1.24 (1H, m), 1.18-1.04 (1H, m), 0.53-0.39 (1H, m), -0.02- -0.20 (1H, m); MALDI TOF-MS *m/z* Calcd for $C_{37}H_{34}NO$ $[M+H]^+$ 508.26, found 508.12.

[00735] (*R*)-(*9H*-Fluororen-9-yl)((*S*)-pyrrolidin-2-yl)methanol (11a).

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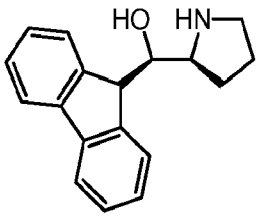
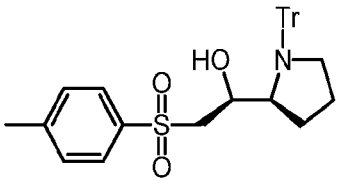


11a

[00736] Compound **11a** was obtained by using **10a** instead of **6a** in a similar manner to compound **7a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.76 (2H, d, *J* = 7.5Hz), 7.68 (2H, t, *J* = 8.0Hz), 7.43-7.35 (2H, m), 7.34-7.25 (2H, m), 4.28 (1H, d, *J* = 6.3Hz), 4.03 (1H, dd, *J* = 6.5, 4.2Hz), 3.19-3.11 (1H, m), 2.97-2.88 (1H, m), 2.86-2.76 (1H, m), 2.02 (2H, brs), 1.77-1.53 (3H, m), 1.38-1.23 (1H, m); MALDI TOF-MS *m/z* Calcd for $C_{18}H_{20}NO$ $[M+H]^+$ 266.15, found 266.04.

[00737] (*S*)-2-Tosyl-1-((*S*)-1-tritylpyrrolidin-2-yl)ethanol (12a').

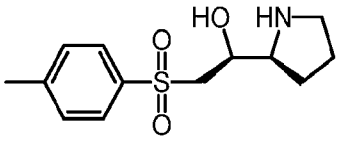


12a'

[00738] Compound **12a'** was obtained by using "chloromethyl *p*-tolyl sulfone" instead of "chloromethyldiphenylmethylsilane" in a similar manner to compound **2a**.

[00739] ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.66 (2H, d, *J* = 8.4Hz), 7.48-7.44 (6H, m), 7.35 (2H, d, *J* = 7.2Hz), 7.21-7.13 (9H, m), 4.39-4.36 (1H, m), 3.33 (1H, s), 3.24-3.20 (1H, m), 3.19-3.10 (2H, m), 2.98-2.92 (2H, m), 2.49 (3H, s), 1.55-1.49 (1H, m), 1.33-1.26 (1H, m), 1.12-1.04 (1H, m), 0.22-0.14 (1H, m); ^{13}C NMR (150.9 MHz, $CDCl_3$) δ 144.6, 144.5, 136.3, 129.9, 129.5, 128.1, 127.5, 126.2, 78.0, 69.1, 63.9, 60.2, 52.6, 25.5, 24.7, 21.7.

[00740] (*S*)-2-Tosyl-1-((*S*)-1-tritylpyrrolidin-2-yl)ethanol (13a').



13a'

[00741] Compound **13a'** was obtained by using **12a'** instead of **2a** in a similar manner to compound **3a**.

[00742] ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.82 (2H, d, *J* = 8.4Hz), 7.37 (2H, d, *J* = 8.4Hz), 4.01 (1H, ddd, *J* = 12.0, 5.1, 3.0Hz), 3.32 (1H, dd, *J* = 14.4, 3.0Hz), 3.25 (1H, dd, *J* = 14.4,

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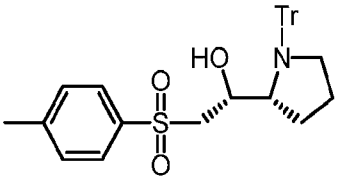
9.0Hz), 3.16 (1H, dt, *J* = 7.8, 5.1Hz), 2.90-2.82 (2H, m), 2.46 (3H, s), 2.04 (2H, brs), 1.78-1.63 (3H, m), 1.62-1.55 (1H, m); ^{13}C NMR (150.9 MHz, $CDCl_3$) δ 144.5, 136.7, 129.7, 127.7, 67.4, 61.8, 60.1, 46.7, 25.7, 21.4. MALDI TOF-MS *m/z* Calcd for $C_{13}H_{20}NO_3S$ $[M+H]^+$ 270.12, found 270.04.

[00743] (*R*)-2-Tosyl-1-((*R*)-1-tritylpyrrolidin-2-yl)ethanol (12b').

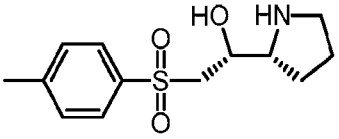


12b'

[00744] Compound **12b'** was obtained by using **1b** instead of **1a** in a similar manner to compound **12a'**.

[00745] ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.66 (2H, d, *J* = 8.4Hz), 7.47-7.44 (6H, m), 7.35 (2H, d, *J* = 7.8Hz), 7.21-7.13 (9H, m), 4.37 (1H, dt, *J* = 8.6, 2.4Hz), 3.33 (1H, s), 3.23-3.20 (1H, m), 3.19-3.12 (2H, m), 2.98-2.92 (2H, m), 2.49 (3H, s), 1.56-1.49 (1H, m), 1.32-1.26 (1H, m), 1.11-1.03 (1H, m), 0.23-0.15 (1H, m); ^{13}C NMR (150.9 MHz, $CDCl_3$) δ 144.6, 144.5, 136.3, 129.9, 129.6, 128.1, 127.6, 126.2, 78.0, 69.1, 63.9, 60.2, 52.6, 25.5, 24.7, 21.7.

[00746] (*R*)-2-Tosyl-1-((*R*)-1-tritylpyrrolidin-2-yl)ethanol (13b').



13b'

[00747] Compound **13b'** was obtained by using **12b'** instead of **12a'** in a similar manner to compound **13a'**.

[00748] ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.82 (2H, d, *J* = 8.4Hz), 7.37 (2H, d, *J* = 8.4Hz), 4.01 (1H, ddd, *J* = 9.0, 5.1, 3.0Hz), 3.32 (1H, dd, *J* = 14.4, 3.0Hz), 3.25 (1H, dd, *J* = 14.4, 9.0Hz), 3.17 (1H, dt, *J* = 7.2, 5.1Hz), 2.89-2.83 (2H, m), 2.46 (3H, s), 2.04 (2H, brs), 1.79-1.64 (3H, m), 1.62-1.55 (1H, m); ^{13}C NMR (150.9 MHz, $CDCl_3$) δ 144.8, 136.6, 129.8, 127.9, 67.7, 61.8, 60.1, 46.8, 25.9, 25.8, 21.6. MALDI TOF-MS *m/z* Calcd for $C_{13}H_{20}NO_3S$ $[M+H]^-$ 270.12, found 270.05.

[00749] Example Z-10.

[00750] Oxazaphospholidine monomer 12a.

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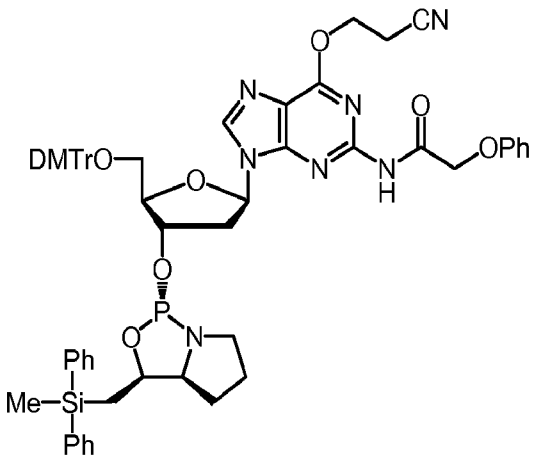

12a

[00751] **3a** (560 mg, 1.80 mmol) were dried by repeated coevaporations with dry toluene and dissolved in dry diethylether (0.90 mL) under argon. *N*-Methylmorpholine (400 micro L, 3.60 mmol) was added to the solution, and the resultant solution was added dropwise to a solution of PCl_3 (160 micro L, 1.80 mmol) in dry diethylether (0.90 mL) at 0 °C under argon with stirring. The mixture was then allowed to warm to room temperature and stirred for 30 min. The resultant *N*-methylmorpholine hydrochloride was removed by filtration under nitrogen, and the filtrate was concentrated to dryness under reduced pressure to afford crude 2-chloro-1,3,2-oxazaphospholidine derivative. The crude materials were dissolved in freshly distilled THF (3.6 mL) to make 0.5 M solutions, which were used to synthesize the nucleoside 3′-*O*-oxazaphospholidines without further purification.

[00752] 5′-*O*-(DMTr)-2-*N*-(phenoxyacetyl)-6-*O*-(cyanoethyl)guanosine (636 mg, 0.84 mmol) was dried by repeated coevaporations with dry toluene, and dissolved in freshly distilled THF (2.5 mL) under argon. Et_3N (0.58 mL, 4.2 mmol) was added, and the mixture was cooled to –78 °C. A 0.5 M solution of the corresponding crude 2-chloro-1,3,2-oxazaphospholidine derivative in freshly distilled THF (3.6 mL, 1.80 mmol) was added dropwise via a syringe, and the mixture was stirred for 15 min at room temperature. A saturated $NaHCO_3$ aqueous solution (70 mL) and $CHCl_3$ (70 mL) were then added, and the organic layer was separated and washed with saturated $NaHCO_3$ aqueous solutions (2 × 70 mL). The combined aqueous layers were back-extracted with $CHCl_3$ (70 mL). The organic layers were combined, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by chromatography on silica gel to afford **12a** (829 mg, 90%) as a white foam. ^1H NMR (300 MHz, $CDCl_3$) δ 8.77 (1H, brs), 7.99 (1H, s), 7.54-6.98 (24H, m), 6.81-6.73 (4H, m), 6.35 (1H, dd, *J* = 8.0, 6.3Hz),

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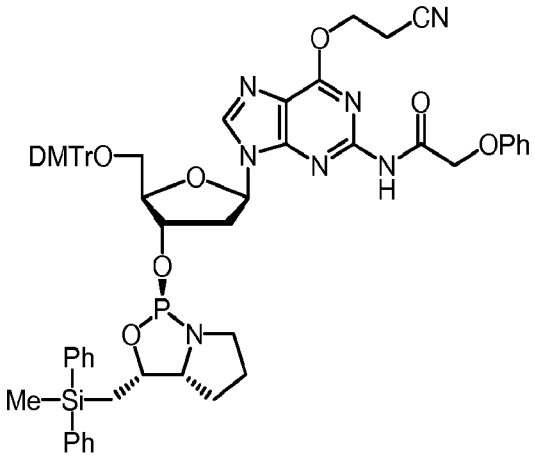
4.89-4.73 (4H, m), 4.68 (2H, brs), 4.05-3.98 (1H, m), 3.75 (6H, s), 3.62-3.46 (1H, m), 3.41-3.20 (3H, m), 3.18-3.04 (1H, m), 3.08 (2H, t, J = 6.6Hz), 2.58-2.36 (2H, m), 1.94-1.59 (2H, m), 1.56 (1H, dd, J = 15.0, 8.7Hz), 1.43 (1H, dd, J = 15.0, 5.7Hz), 1.33-1.16 (2H, m), 0.62 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 153.5 (1P, s).

[00753] Example Z-11.

[00754] Oxazaphospholidine monomer 12b.

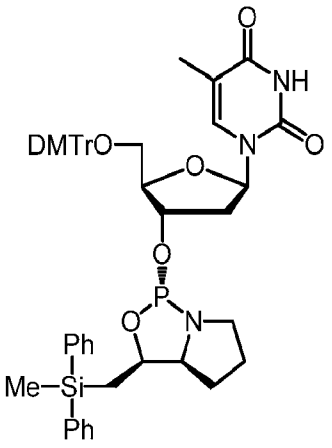


12b

[00755] Compound **12b** was obtained by using **3b** instead of **3a** in a similar manner to compound **12a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.80 (1H, brs), 7.96 (1H, s), 7.54-6.96 (24H, m), 6.79-6.71 (4H, m), 6.19 (1H, t, J = 6.6Hz), 4.90-4.73 (4H, m), 4.66 (2H, brs), 4.16-4.08 (1H, m), 3.76 (6H, s), 3.60-3.36 (2H, m), 3.29 (1H, d, J = 3.9Hz), 3.27-3.12 (2H, m), 3.09 (2H, t, J = 6.6Hz), 2.59-2.46 (1H, m), 2.07-1.97 (1H, m), 1.94-1.41 (5H, m), 1.36-1.18 (1H, m), 0.65 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 157.1 (1P, s).

[00756] Example Z-12.

[00757] Oxazaphospholidine monomer 13a.

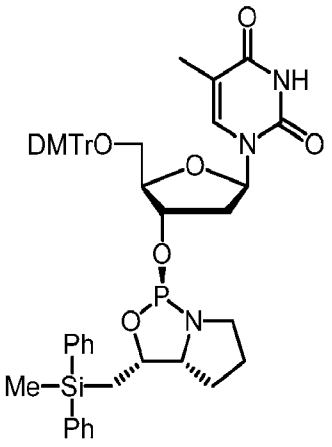


13a

[00758] Compound **13a** was obtained by using "5′-*O*-(DMTr)thymidine" instead of "5′-*O*-(DMTr)-2-*N*-(phenoxyacetyl)-6-*O*-(cyanoethyl)guanosine" in a similar manner to compound **12a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.58-7.23 (21H, m), 6.86-6.79 (4H, m), 6.35 (1H, dd, *J* = 8.1, 5.7Hz), 4.79-4.67 (2H, m), 3.83-3.78 (1H, m), 3.78 (6H, s), 3.59-3.43 (1H, m), 3.34 (1H, dd, *J* = 10.5, 2.4Hz), 3.35-3.24 (1H, m), 3.20 (1H, dd, *J* = 10.5, 2.4Hz), 3.16-3.02 (1H, m), 2.36-2.26 (1H, m), 2.15-2.02 (1H, m), 1.92-1.77 (1H, m), 1.74-1.59 (1H, m), 1.52 (1H, dd, *J* = 14.7, 9.0Hz), 1.40 (3H, s), 1.45-1.15 (3H, m), 0.60 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 153.7 (1P, s).

[00759] Example Z-13.

[00760] Oxazaphospholidine monomer 13b.



13b

[00761] Compound **13b** was obtained by using **3b** instead of **3a** in a similar manner to compound **13a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.46 (1H, brs), 7.59-7.20 (20H, m), 6.86-6.79 (4H, m), 6.26 (1H, t, *J* = 6.8Hz), 4.78-4.65 (2H, m), 4.01-3.95 (1H, m), 3.78 (6H, s), 3.55-3.40 (1H, m), 3.42 (1H, dd, *J* = 10.5, 2.7Hz), 3.40-3.28 (1H, m), 3.22 (1H, dd, *J* = 10.5, 3.0Hz), 3.19-

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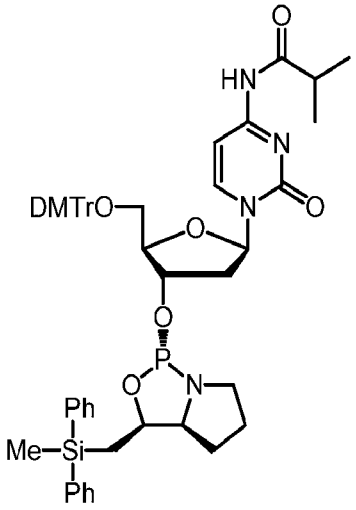
3.06 (1H, m), 2.16-1.95 (2H, m), 1.90-1.54 (3H, m), 1.49-1.35 (1H, m), 1.43 (3H, s), 1.34-1.17 (2H, m), 0.67 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 156.2 (1P, s). Oligos were synthesized using the above compound 13b by the general method disclosed above.

[00762] Example Z-14.

[00763] Oxazaphospholidine monomer 14a.

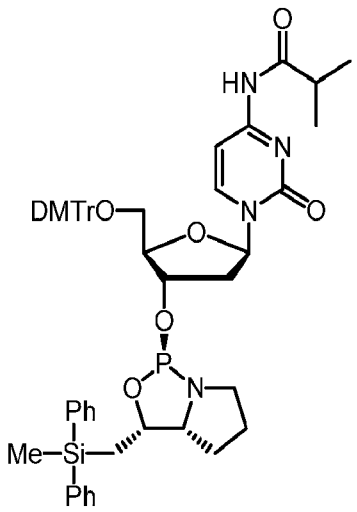


14a

[00764] Compound **14a** was obtained by using "5′-*O*-(DMTr)-4-*N*-(isobutyryl)cytidine" instead of "5′-*O*-(DMTr)-2-*N*-(phenoxyacetyl)-6-*O*-(cyanoethyl)guanosine" in a similar manner to compound **12a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.33 (1H, brs), 8.17 (1H, d, *J* = 7.5Hz), 7.52-7.22 (19H, m), 7.07 (1H, d, *J* = 7.5Hz), 6.88-6.81 (4H, m), 6.20 (1H, t, *J* = 6.2Hz), 4.81-4.64 (2H, m), 3.93-3.87 (1H, m), 3.79 (6H, s), 3.59-3.43 (1H, m), 3.39-3.29 (3H, m), 3.16-3.02 (1H, m), 2.69-2.52 (2H, m), 2.12-2.00 (1H, m), 1.91-1.50 (3H, m), 1.47-1.32 (2H, m), 1.27-1.16 (7H, m), 0.60 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 154.8 (1P, s).

[00765] Example Z-16.

[00766] Oxazaphospholidine monomer 14b.

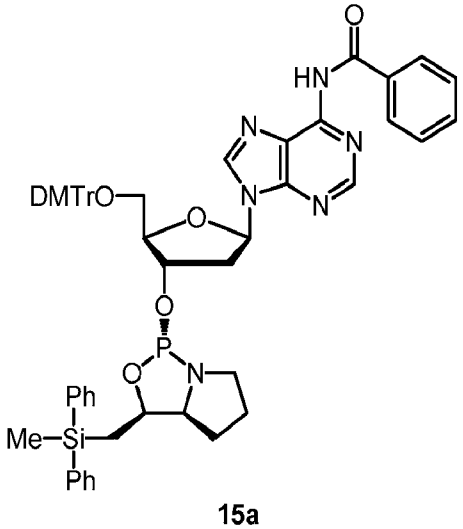


14b

[00767] Compound **14b** was obtained by using **3b** instead of **3a** in a similar manner to compound **14a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.33 (1H, d, *J* = 7.5Hz), 8.23 (1H, brs), 7.57-7.22 (19H, m), 7.12 (1H, d, *J* = 7.5Hz), 6.88-6.81 (4H, m), 6.15 (1H, dd, *J* = 6.6, 4.2Hz), 4.82-4.63 (2H, m), 4.03-3.97 (1H, m), 3.80 (6H, s), 3.55-3.26 (4H, m), 3.19-3.05 (1H, m), 2.59 (1H, quintet, *J* = 6.9Hz), 2.39-2.27 (1H, m), 2.21-2.10 (1H, m), 1.90-1.56 (3H, m), 1.50-1.32 (2H, m), 1.26-1.17 (7H, m), 0.66 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 157.2 (1P, s).

[00768] Example Z-17.

[00769] Oxazaphospholidine monomer 15a.



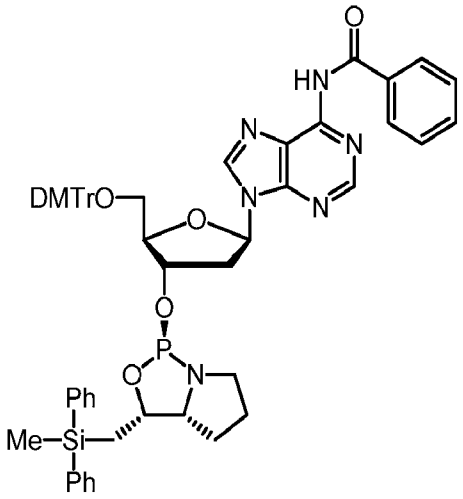
15a

[00770] Compound **15a** was obtained by using "5′-*O*-(DMTr)-6-*N*-(benzoyl)adenosine" instead of "5′-*O*-(DMTr)-2-*N*-(phenoxyacetyl)-6-*O*-(cyanoethyl)guanosine" in a similar manner to compound **12a**.

[00771] ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.71 (1H, s), 8.12 (1H, s), 8.04 (2H, d, *J* = 7.8Hz), 7.62-7.15 (23H, m), 6.80-6.75 (4H, m), 6.37 (1H, dd, *J* = 7.8, 6.0Hz), 4.94-4.88 (1H, m), 4.80 (1H, ddd, *J* = 12.0, 6.0, 5.4Hz), 4.07-4.04 (1H, m), 3.76 (6H, s), 3.58-3.49 (1H, m), 3.41-3.34 (1H, m), 3.33 (1H, dd, *J* = 10.8, 4.8Hz), 3.25 (1H, dd, *J* = 10.8, 4.8Hz), 3.13-3.06 (1H, m), 2.66-2.58 (1H, m), 2.40-2.35 (1H, m), 1.91-1.84 (1H, m), 1.73-1.66 (1H, m), 1.56 (1H, dd, *J* = 15.0, 9.0Hz), 1.44 (1H, dd, *J* = 15.0, 5.4Hz), 1.47-1.41 (1H, m), 1.30-1.23 (1H, m), 0.63 (3H, s); ^{31}P NMR (243.0 MHz, $CDCl_3$) δ 151.8 (1P, s).

[00772] Example Z-18.

[00773] Oxazaphospholidine monomer 15b.



15b

[00774] Compound **15b** was obtained by using **3b** instead of **3a** in a similar manner to compound **15a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.06 (1H, brs), 8.76 (1H, s), 8.12 (1H, s), 8.07-7.99 (2H, m), 7.64-7.14 (22H, m), 6.83-6.75 (4H, m), 6.25 (1H, t, *J* = 6.6Hz), 4.86-4.75 (2H, m), 4.20-4.15 (1H, m), 3.77 (6H, s), 3.61-3.38 (2H, m), 3.36 (1H, dd, *J* = 10.2, 4.2Hz), 3.27 (1H, dd, *J* = 10.2, 4.2Hz), 3.27-3.13 (1H, m), 2.71-2.59 (1H, m), 2.12-2.01 (1H, m), 1.94-1.42 (5H, m), 1.36-1.20 (1H, m), 0.67 (3H, s)); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 157.3 (1P, s)..

[00775] Example Z-19.

[00776] Oxazaphospholidine monomer 16a.

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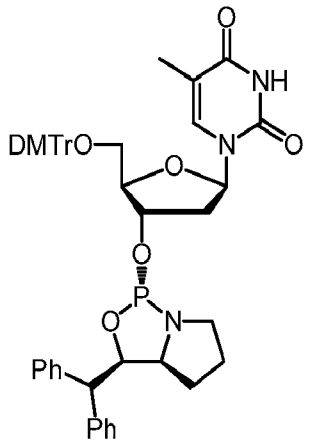
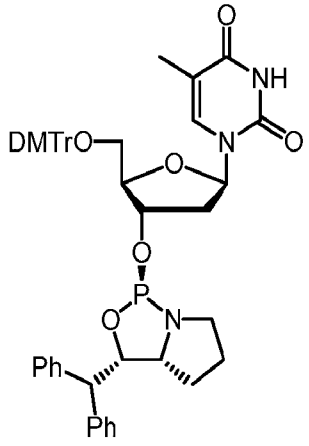


16a

[00777] Compound **16a** was obtained by using **7a** instead of **3a** in a similar manner to compound **13a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.57 (1H, d, *J* = 0.9Hz), 7.37-6.94 (20H, m), 6.87-6.78 (4H, m), 6.48 (1H, dd, *J* = 8.6, 5.7Hz), 5.42 (1H, dd, *J* = 11.0, 5.1Hz), 4.81-4.71 (1H, m), 4.02 (1H, d, *J* = 11.0Hz), 3.83 (1H, d, *J* = 2.1Hz), 3.79 (6H, s), 3.61-3.41 (2H, m), 3.24-3.09 (1H, m), 3.16 (1H, dd, *J* = 10.8, 2.4Hz), 3.02 (1H, dd, *J* = 10.8, 2.4Hz), 2.54-2.44 (1H, m), 2.34-2.22 (1H, m), 1.94-1.79 (1H, m), 1.74-1.56 (1H, m), 1.38 (3H, s), 1.38-1.28 (2H, m); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 160.9 (1P, s).

[00778] Example Z-20.

[00779] Oxazaphospholidine monomer 16b.



16b

[00780] Compound **16b** was obtained by using **3b** instead of **3a** in a similar manner to compound **16a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.57 (1H, d, *J* = 1.5Hz), 7.43-7.11 (20H, m), 6.85-6.78 (4H, m), 6.48 (1H, dd, *J* = 7.5, 5.7Hz), 5.58 (1H, dd, *J* = 11.4, 5.1Hz), 4.82-4.73 (1H, m), 4.17-4.02 (2H, m), 3.78 (6H, s), 3.56-3.40 (3H, m), 3.32 (1H, dd, *J* = 10.7, 2.4Hz), 3.22-3.07

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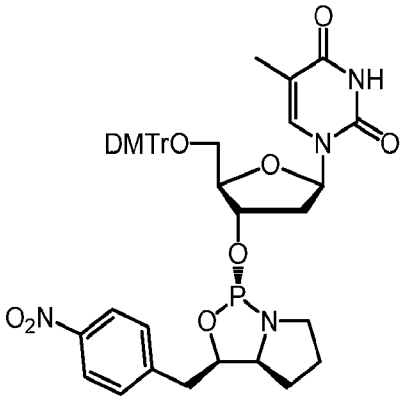
(1H, m), 2.26-2.04 (2H, m), 1.95-1.81 (1H, m), 1.74-1.56 (1H, m), 1.40 (3H, d, J = 1.5Hz), 1.44-1.34 (2H, m); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 162.2 (1P, s).

[00781] Example Z-21.

[00782] Oxazaphospholidine monomer 17a.

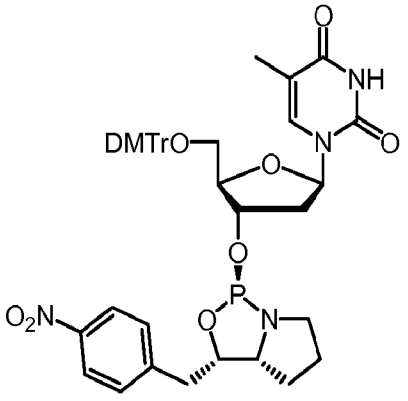


17a

[00783] Compound **17a** was obtained by using **9a** instead of **3a** in a similar manner to compound **13a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.22 (1H, brs), 8.05-7.99 (2H, m), 7.52 (1H, d, J = 1.2Hz), 7.41-7.19 (11H, m), 6.87-6.79 (4H, m), 6.37 (1H, dd, J = 8.4, 5.7Hz), 4.88-4.75 (2H, m), 3.86-3.80 (1H, m), 3.79 (6H, d, J = 1.2Hz), 3.64-3.49 (2H, m), 3.27-3.12 (3H, m), 2.97 (2H, d, J = 6.6Hz), 2.51-2.41 (1H, m), 2.33-2.20 (1H, m), 2.03-1.75 (2H, m), 1.72-1.59 (1H, m), 1.46-1.36 (1H, m), 1.40 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 157.5 (1P, s).

[00784] Example Z-22.

[00785] Oxazaphospholidine monomer 17b.



17b

[00786] Compound **17b** was obtained by using **9b** instead of **9a** in a similar manner to compound **17a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.67 (1H, brs), 8.18-8.11 (2H, m), 7.57 (1H, d, J

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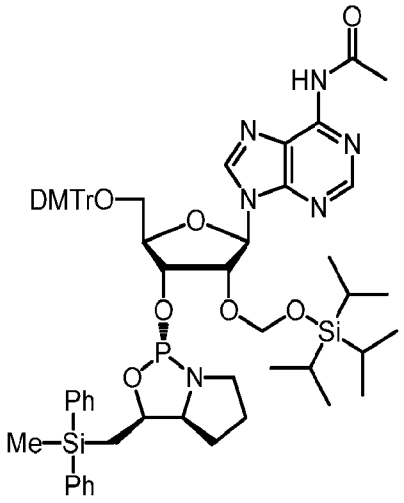
= 1.2Hz), 7.47-7.22 (11H, m), 6.86-6.79 (4H, m), 6.29 (1H, t, *J* = 6.6Hz), 4.87 (1H, dt, *J* = 7.5, 5.7Hz), 4.80-4.72 (1H, m), 4.11-4.05 (1H, m), 3.79 (6H, s), 3.67-3.47 (2H, m), 3.43 (1H, dd, *J* = 10.8, 2.7Hz), 3.27 (1H, dd, *J* = 10.8, 2.4Hz), 3.25-3.13 (1H, m), 3.07-2.99 (2H, m), 2.19-2.12 (2H, m), 2.03-1.62 (3H, m), 1.46-1.30 (1H, m), 1.41 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 158.1 (1P, s).

[00787] Example Z-23.

[00788] Oxazaphospholidine monomer 18a.

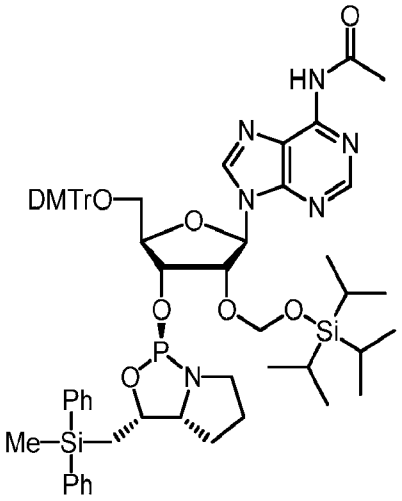


18a

[00789] Compound **18a** was obtained by using "5′-*O*-(DMTr)-2′-*O*-TOM-6-*N*-(acetyl)adenosine" instead of **"**5′-*O*-(DMTr)-2-*N*-(phenoxyacetyl)-6-*O*-(cyanoethyl)guanosine**"** in a similar manner to compound **12a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.82 (1H, brs), 8.49 (1H, s), 8.10 (1H, s), 7.58-7.17 (19H, m), 6.83-6.73 (4H, m), 6.11 (1H, d, *J* = 6.6Hz), 5.15 (1H, dd, *J* = 6.6, 5.4Hz), 4.98-4.77 (4H, m), 4.18-4.11 (1H, m), 3.76 (6H, s), 3.59-3.25 (4H, m), 3.16-3.02 (1H, m), 2.62 (3H, s), 1.91-1.53 (3H, m), 1.49-1.18 (3H, m), 0.96-0.80 (3H, m), 0.90 (18H, s), 0.62 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 156.7 (1P, s).

[00790] Example Z-24.

[00791] Oxazaphospholidine monomer 18b.

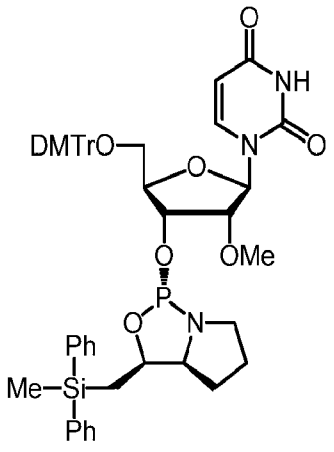


18b

[00792] Compound **18b** was obtained by using **3b** instead of **3a** in a similar manner to compound **18a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.56 (1H, brs), 8.55 (1H, s), 8.13 (1H, s), 7.57-7.17 (19H, m), 6.82-6.73 (4H, m), 6.16 (1H, d, *J* = 5.7Hz), 5.06 (1H, t, *J* = 5.6Hz), 4.93 (1H, d, *J* = 5.1Hz), 4.83 (1H, d, *J* = 5.1Hz), 4.81-4.69 (2H, m), 4.27-4.19 (1H, m), 3.76 (6H, s), 3.55-3.40 (2H, m), 3.33-3.16 (2H, m), 3.12-2.97 (1H, m), 2.63 (3H, s), 1.88-1.52 (3H, m), 1.45-1.16 (3H, m), 0.91-0.79 (3H, m), 0.86 (18H, s), 0.64 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 154.8 (1P, s).

[00793] Example Z-25.

[00794] Oxazaphospholidine monomer 19a.



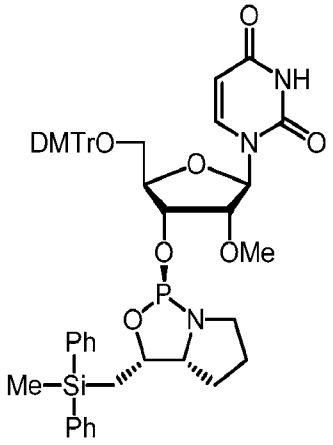
19a

[00795] Compound **19a** was obtained by using "5′-*O*-(DMTr)-2′-*O*-(methyl)uridine" instead of "5′-*O*-(DMTr)-2-*N*-(phenoxyacetyl)-6-*O*-(cyanoethyl)guanosine" in a similar manner to compound **12a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.91 (1H, d, *J* = 7.8Hz), 7.58-7.20 (19H, m), 6.88-6.80 (4H, m), 5.96 (1H, d, *J* = 3.3Hz), 5.19 (1H, d, *J* = 7.8Hz), 4.88-4.78 (1H, m), 4.66-4.57

(1H, m), 4.03-3.95 (1H, m), 3.90-3.74 (1H, m), 3.78 (6H, s), 3.77-3.71 (1H, m), 3.58-3.29 (2H, m), 3.45 (3H, s), 3.13-2.82 (2H, m), 1.88-1.53 (3H, m), 1.49-1.16 (3H, m), 0.60 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 155.3 (1P, s).

[00796] Example Z-26.

[00797] Oxazaphospholidine monomer 19b.

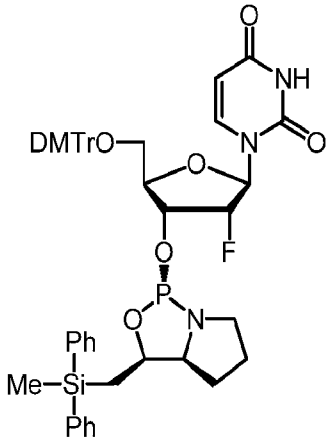


19b

[00798] Compound **19b** was obtained by using **3b** instead of **3a** in a similar manner to compound **19a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.10 (1H, d, J = 8.4Hz), 7.58-7.20 (19H, m), 6.87-6.79 (4H, m), 5.89 (1H, d, J = 1.5Hz), 5.21 (1H, d, J = 8.4Hz), 4.92-4.82 (1H, m), 4.73-4.63 (1H, m), 4.15-4.08 (1H, m), 3.89-3.73 (1H, m), 3.78 (6H, s), 3.66-3.62 (1H, m), 3.57-3.27 (2H, m), 3.30 (3H, s), 3.17-2.82 (2H, m), 1.89-1.55 (3H, m), 1.55-1.40 (1H, m), 1.35-1.15 (2H, m), 0.66 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 157.5 (1P, s).

[00799] Example Z-27.

[00800] Oxazaphospholidine monomer 20a.

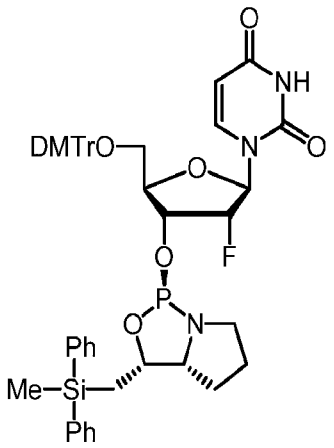


20a

[00801] Compound **20a** was obtained by using "5′-*O*-(DMTr)-2′-deoxy-2′-fluorouridine" instead of "5′-*O*-(DMTr)-2-*N*-(phenoxyacetyl)-6-*O*-(cyanoethyl)guanosine" in a similar manner to compound **12a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.85 (1H, d, *J* = 8.1Hz), 7.58-7.20 (19H, m), 6.87-6.79 (4H, m), 5.98 (1H, d, *J* = 16.5Hz), 5.23 (1H, d, *J* = 8.1Hz), 4.86-4.61 (3H, m), 3.99 (1H, d, *J* = 6.9Hz), 3.76 (6H, d, *J* = 3.0Hz), 3.56-3.34 (4H, m), 3.10-2.96 (1H, m), 1.88-1.74 (1H, m), 1.72-1.52 (2H, m), 1.48-1.16 (3H, m), 0.61 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 154.3 (1P, d, *J* = 8.9Hz).

[00802] Example Z-28.

[00803] Oxazaphospholidine monomer 20b.



20b

[00804] Compound **20b** was obtained by using **3b** instead of **3a** in a similar manner to compound **20a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.01 (1H, d, *J* = 8.4Hz), 7.58-7.20 (19H, m), 6.87-6.79 (4H, m), 6.03 (1H, d, *J* = 16.2Hz), 5.29 (1H, d, *J* = 8.4Hz), 4.96 (1H, dd, *J* = 13.1, 7.5Hz), 4.80-4.54 (2H, m), 4.15 (1H, d, *J* = 9.0Hz), 3.78 (6H, s), 3.61-3.39 (3H, m), 3.37-3.25 (1H, m), 3.23-3.09 (1H, m), 1.91-1.56 (3H, m), 1.51-1.13 (3H, m), 0.66 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 158.9 (1P, d, *J* = 4.4Hz).

[00805] Example Z-29.

[00806] Oxazaphospholidine monomer 21a.

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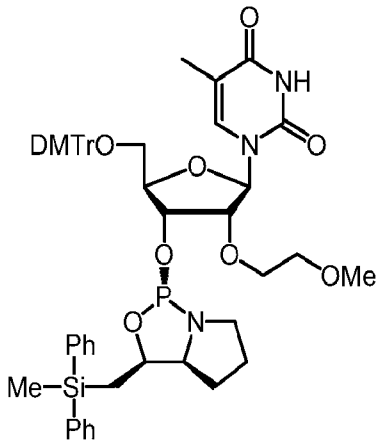
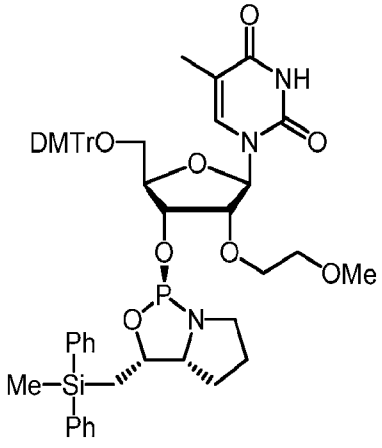


21a

[00807] Compound **21a** was obtained by using "5′-*O*-(DMTr)-2′-*O*-methoxyethyl-5-methyluridine" instead of "5′-*O*-(DMTr)-2-*N*-(phenoxyacetyl)-6-*O*-(cyanoethyl)guanosine" in a similar manner to compound **12a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.62-7.18 (21H, m), 6.84 (4H, d, J = 8.7Hz), 6.07 (1H, d, J = 5.7Hz), 4.86-4.76 (1H, m), 4.63-4.54 (1H, m), 4.20 (1H, t, J = 5.4Hz), 3.95-3.89 (1H, m), 3.78 (6H, s), 3.78-3.71 (2H, m), 3.60-3.48 (2H, m), 3.44-3.02 (5H, m), 3.31 (3H, s), 1.88-1.15 (6H, m), 1.35 (3H, s), 0.58 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 156.3 (1P, s).

[00808] Example Z-30.

[00809] Oxazaphospholidine monomer 21b.



21b

[00810] Compound **21b** was obtained by using **3b** instead of **3a** in a similar manner to compound **21a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.71 (1H, d, J = 1.2Hz), 7.55-7.22 (20H, m), 6.86-6.78 (4H, m), 5.99 (1H, d, J = 3.9Hz), 4.78-4.62 (2H, m), 4.13-4.08 (1H, m), 4.07-4.02 (1H, m), 3.77 (6H, s), 3.77-3.70 (1H, m), 3.65-3.56 (1H, m), 3.52-3.36 (4H, m), 3.33-3.14 (2H, m),

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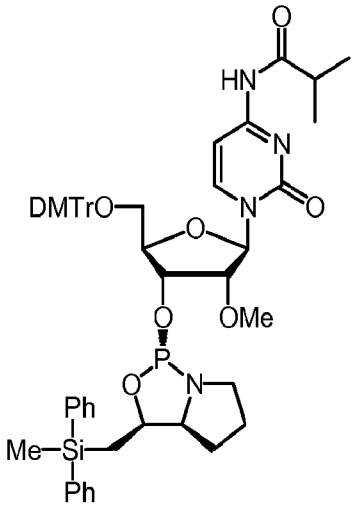
3.29 (3H, s), 3.08-2.94 (1H, m), 1.86-1.72 (1H, m), 1.71-1.55 (2H, m), 1.30 (3H, d, J = 1.2Hz), 1.47-1.16 (3H, m) 0.64 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 155.6 (1P, s).

[00811] Example Z-31.

[00812] Oxazaphospholidine monomer 22a.

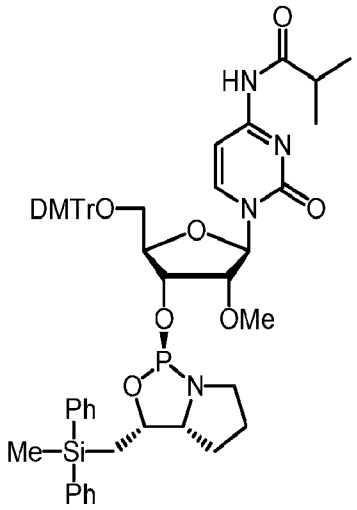


22a

[00813] Compound **22a** was obtained by using "5′-*O*-(DMTr)-2′-*O*-methyl-4-*N*-(isobutyryl)cytidine" instead of "5′-*O*-(DMTr)-2-*N*-(phenoxyacetyl)-6-*O*-(cyanoethyl)guanosine" in a similar manner to compound **12a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.49 (1H, d, J = 7.2Hz), 7.58-7.20 (19H, m), 6.96 (1H, d, J =7.2Hz), 6.90-6.82 (4H, m), 5.98 (1H, s), 4.84 (1H, dd, J = 13.1, 7.5Hz), 4.59 (1H, dt, J = 8.3, 4.5Hz), 4.19-4.13 (1H, m), 3.79 (6H, s), 3.78-3.72 (1H, m), 3.63-3.40 (3H, m), 3.55 (3H, s), 3.36-3.24 (1H, m), 3.09-2.95 (1H, m), 2.59 (1H, septet, J = 6.9Hz), 1.85-1.53 (5H, m), 1.48-1.37 (1H, m), 1.24-1.17 (6H, m), 0.59 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 155.2 (1P, s).

[00814] Example Z-32.

[00815] Oxazaphospholidine monomer 22b.

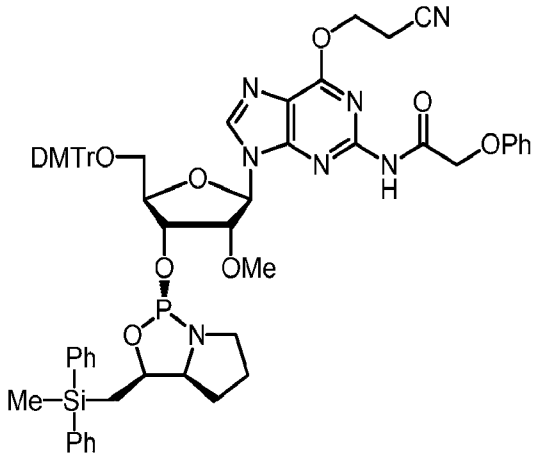


22b

[00816] Compound **22b** was obtained by using **3b** instead of **3a** in a similar manner to compound **22a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.62 (1H, d, *J* = 7.5Hz), 7.57-7.23 (19H, m), 7.02 (1H, d, *J* =7.5Hz), 6.89-6.81 (4H, m), 5.92 (1H, s), 4.90 (1H, dt, *J* = 9.0, 5.7Hz), 4.61 (1H, dt, *J* = 8.7, 4.8Hz), 4.25-4.17 (1H, m), 3.81 (6H, s), 3.67 (1H, d, *J* = 4.5Hz), 3.62-3.25 (4H, m), 3.38 (3H, s), 3.16-3.02 (1H, m), 2.58 (1H, septet, *J* = 6.9Hz), 1.87-1.40 (6H, m), 1.26-1.14 (6H, m), 0.64 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 158.2 (1P, s).

[00817] Example Z-33.

[00818] Oxazaphospholidine monomer 23a.



23a

[00819] Compound **23a** was obtained by using "5′-*O*-(DMTr)-2′-*O*-methyl-2-*N*-(phenoxyacetyl)-6-*O*-(cyanoethyl)guanosine" instead of "5′-*O*-(DMTr)-2-*N*-(phenoxyacetyl)-6-*O*-(cyanoethyl)guanosine" in a similar manner to compound **12a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.67 (1H, brs), 8.01 (1H, s), 7.56-7.16 (24H, m), 6.83-6.74 (4H, m), 6.08 (1H, d, *J* = 6.9Hz),

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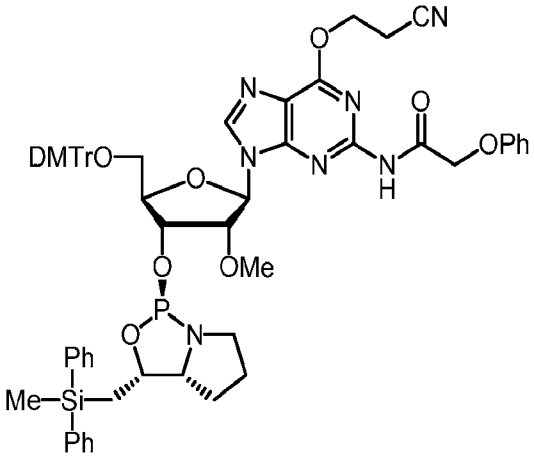
4.85-4.76 (1H, m), 4.84 (2H, t, J = 6.6Hz), 4.65-4.56 (1H, m), 4.59 (2H, brs), 4.48 (1H, dd, J = 6.6, 5.1Hz), 4.09-4.05 (1H, m), 3.75 (6H, s), 3.60-3.42 (2H, m), 3.40-3.26 (2H, m), 3.35 (3H, s), 3.18-3.05 (1H, m), 3.08 (2H, t, J = 6.6Hz), 1.89-1.49 (3H, m), 1.48-1.16 (3H, m), 0.59 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 156.9 (1P, s).

[00820] Example Z-34.

[00821] Oxazaphospholidine monomer 23b.

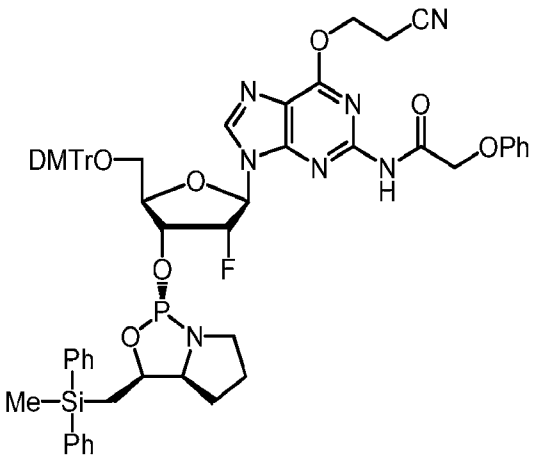


23b

[00822] Compound **23b** was obtained by using **3b** instead of **3a** in a similar manner to compound **23a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.74 (1H, brs), 8.09 (1H, s), 7.56-6.94 (24H, m), 6.84-6.71 (4H, m), 6.09 (1H, d, J = 4.8Hz), 4.83-4.70 (2H, m), 4.83 (2H, t, J = 6.6Hz), 4.63 (2H, brs), 4.35 (1H, t, J = 5.0Hz), 4.23-4.16 (1H, m), 3.75 (6H, s), 3.58-3.19 (4H, m), 3.32 (3H, s), 3.16-3.04 (1H, m), 3.07 (2H, t, J = 6.6Hz), 1.90-1.55 (3H, m), 1.48-1.15 (3H, m), 0.64 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 154.6 (1P, s).

[00823] Example Z-35.

[00824] Oxazaphospholidine monomer 24a.

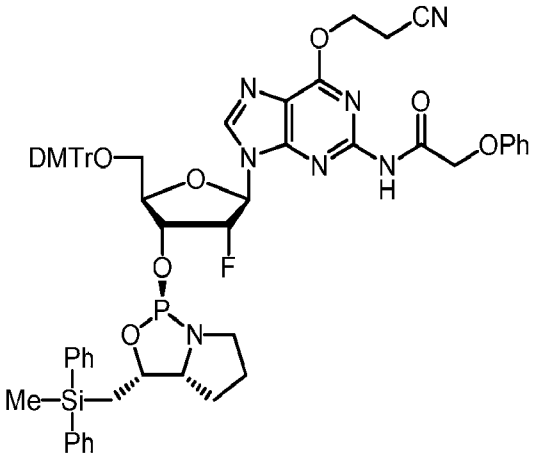


24a

[00825] Compound **24a** was obtained by using "5′-*O*-(DMTr)-2′-deoxy-2′-fluoro-2-*N*-(phenoxyacetyl)-6-*O*-(cyanoethyl)guanosine" instead of "5′-*O*-(DMTr)-2-*N*-(phenoxyacetyl)-6-*O*-(cyanoethyl)guanosine" in a similar manner to compound **12a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.74 (1H, brs), 8.03 (1H, s), 7.55-6.94 (24H, m), 6.80-6.69 (4H, m), 6.21 (1H, dd, J = 14.9, 3.6Hz), 5.34 (1H, dt, J = 52.3, 3.6Hz), 5.01-4.75 (2H, m), 4.84 (1H, t, J = 6.6Hz), 4.62 (2H, brs), 4.15-4.07 (1H, m), 3.73 (6H, s), 3.59-3.29 (4H, m), 3.15-3.00 (1H, m), 3.07 (2H, t, J = 6.6Hz), 1.90-1.49 (3H, m), 1.47-1.12 (3H, m), 0.58 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 155.6 (1P, d, J = 10.9Hz).

[00826] Example Z-36.

[00827] Oxazaphospholidine monomer 24b.



24b

[00828] Compound **24b** was obtained by using **3b** instead of **3a** in a similar manner to compound **24a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.81 (1H, brs), 8.06 (1H, s), 7.55-6.95 (24H, m), 6.77-6.69 (4H, m), 6.06 (1H, d, J = 17.1Hz), 5.24-5.08 (1H, m), 5.04-4.80 (2H, m), 4.87 (1H, t, J

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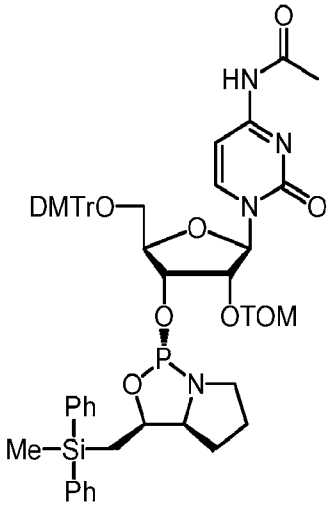
= 6.6Hz), 4.62 (2H, brs), 4.25-4.19 (1H, m), 3.73 (6H, s), 3.58-3.02 (5H, m), 3.10 (2H, t, *J* = 6.6Hz), 1.90-1.56 (3H, m), 1.50-1.15 (3H, m), 0.63 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 158.0 (1P, d, *J* = 4.4Hz).

[00829] Example Z-37.

[00830] Oxazaphospholidine monomer 25a.



25a

[00831] Compound **25a** was obtained by using "5′-*O*-(DMTr)-2′-*O*-TOM-4-*N*-(acetyl)cytidine" instead of "5′-*O*-(DMTr)-2-*N*-(phenoxyacetyl)-6-*O*-(cyanoethyl)guanosine" in a similar manner to compound **12a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.04 (1H, brs), 8.30 (1H, d, *J* = 7.5Hz), 7.51-7.21 (19H, m), 6.99 (1H, d, *J* = 7.5Hz), 6.89-6.81 (4H, m), 6.12 (1H, d, *J* = 3.3Hz), 5.07 (1H, d, *J* = 4.8Hz), 5.05 (1H, d, *J* = 4.8Hz), 4.84-4.75 (1H, m), 4.62-4.52 (1H, m), 4.31-4.25 (1H, m), 4.08-4.01 (1H, m), 3.78 (6H, d, *J* = 3.0Hz), 3.55-3.23 (4H, m), 3.10-2.96 (1H, m), 2.24 (3H, s), 1.84-1.49 (3H, m), 1.46-0.96 (24H, m), 0.58 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 156.5 (1P, s).

[00832] Example Z-38.

[00833] Oxazaphospholidine monomer 25b.

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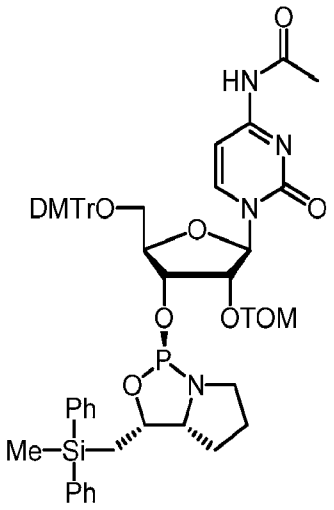
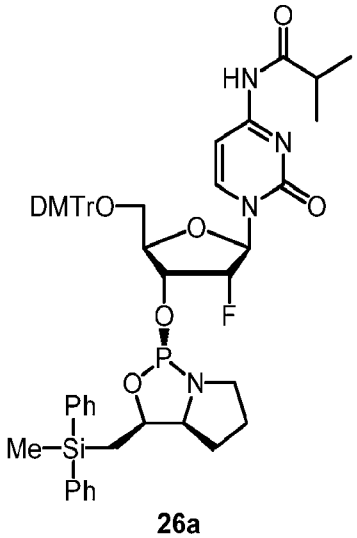


25b

[00834] Compound **25b** was obtained by using **3b** instead of **3a** in a similar manner to compound **25a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.19 (1H, brs), 8.46 (1H, d, *J* = 7.5Hz), 7.54-7.23 (19H, m), 7.01 (1H, d, *J* = 7.5Hz), 6.88-6.79 (4H, m), 6.19 (1H, d, *J* = 1.8Hz), 5.11 (1H, d, *J* = 4.8Hz), 5.07 (1H, d, *J* = 4.8Hz), 4.81-4.71 (1H, m), 4.60-4.51 (1H, m), 4.26-4.18 (2H, m), 3.79 (6H, s), 3.63-3.55 (1H, m), 3.48-3.28 (2H, m), 3.21-2.94 (2H, m), 2.26 (3H, s), 1.81-1.49 (3H, m), 1.43-0.96 (24H, m), 0.62 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 156.4 (1P, s).

[00835] Example Z-39.

[00836] Oxazaphospholidine monomer 26a.



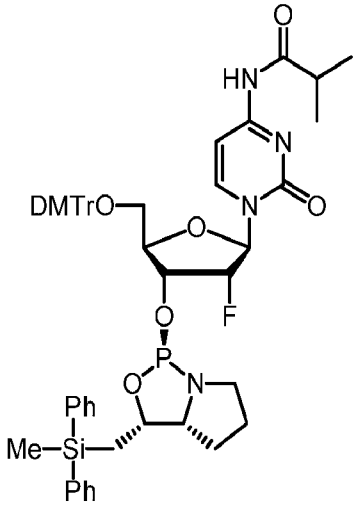
26a

[00837] Compound **26a** was obtained by using "5′-*O*-(DMTr)-2′-deoxy-2′-fluoro-4-*N*-(isobutyryl)cytidine" instead of "5′-*O*-(DMTr)-2-*N*-(phenoxyacetyl)-6-*O*-(cyanoethyl)guanosine" in a similar manner to compound **12a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.66 (1H, brs), 8.41 (1H, d, *J* = 7.5Hz), 7.55-7.20 (19H, m), 7.01 (1H, d, *J* = 7.5Hz), 6.89-6.81

(4H, m), 6.06 (1H, d, J = 15.9Hz), 4.85 (1H, dd, J = 51.4, 3.9Hz), 4.84 (1H, dd, J = 12.9, 7.5Hz), 4.77-4.59 (1H, m), 4.15-4.08 (1H, m), 3.79 (6H, s), 3.63-3.29 (4H, m), 3.10-2.96 (1H, m), 2.65 (1H, septet, J = 6.9Hz), 1.85-1.53 (3H, m), 1.48-1.17 (3H, m), 1.21 (3H, d, J = 4.8Hz), 1.19 (3H, d, J = 4.8Hz), 0.59 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 155.5 (1P, d, J = 6.6Hz).

[00838] Example Z-40.

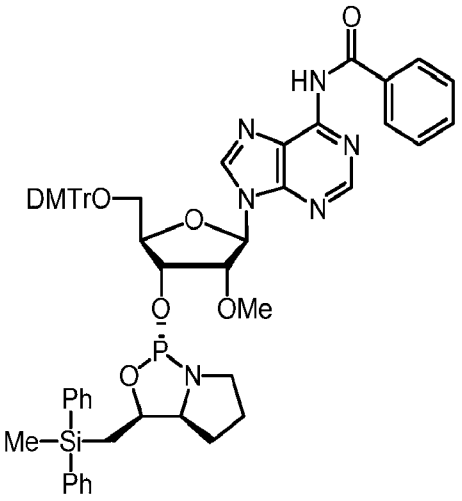
[00839] Oxazaphospholidine monomer 26b.



26b

[00840] Compound **26b** was obtained by using **3b** instead of **3a** in a similar manner to compound **26a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.53 (1H, d, J = 7.5Hz), 7.57-7.23 (20H, m), 7.10 (1H, d, J = 7.5Hz), 6.89-6.81 (4H, m), 6.10 (1H, d, J = 15.9Hz), 5.00-4.92 (1H, m), 4.84 (1H, dd, J = 51.5, 3.3Hz), 4.75-4.58 (1H, m), 4.24 (1H, d, J = 9.3Hz), 3.81 (6H, s), 3.65-3.39 (3H, m), 3.32-3.06 (2H, m), 2.59 (1H, septet, J = 6.9Hz), 1.88-1.53 (4H, m), 1.49-1.34 (2H, m), 1.27-1.18 (6H, m), 0.65 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 159.0 (1P, d, J = 4.4).

[00841] Oxazaphospholidine monomer 27a

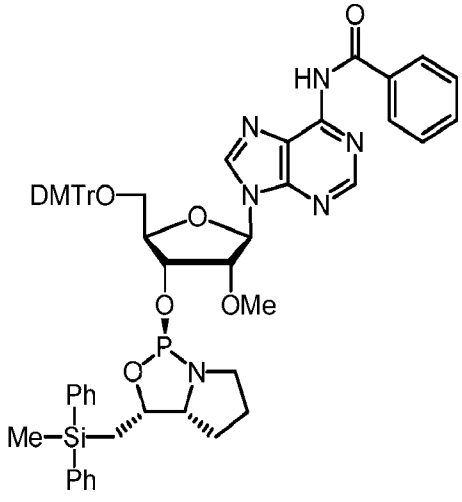


27a

[00842] Compound **27a** was obtained by using "5′-*O*-(DMTr)-2′-*O*-methyl-6-*N*-(benzoyl)adenosine" instead of "5′-*O*-(DMTr)-2-*N*-(phenoxyacetyl)-6-*O*-(cyanoethyl)guanosine" in a similar manner to compound **12a**.

[00843] ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.66 (1H, s), 8.13 (1H, s), 8.03 (2H, d, J = 7.2Hz), 7.64-7.16 (23H, m), 6.79 (4H, d, J = 8.7Hz), 6.08 (1H, d, J = 6.3Hz), 4.91-4.81 (1H, m), 4.77-4.69 (1H, m), 4.64-4.57 (1H, m), 4.15-4.10 (1H, m), 3.76 (6H, s), 3.60-3.23 (4H, m), 3.35 (3H, s), 3.14-3.00 (1H, m), 1.90-1.19 (6H, m), 0.62 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 155.8 (1P, s).

[00844] Oxazaphospholidine monomer 27b.



27b

[00845] Compound **27b** was obtained by using **3b** instead of **3a** in a similar manner to compound **27a**.

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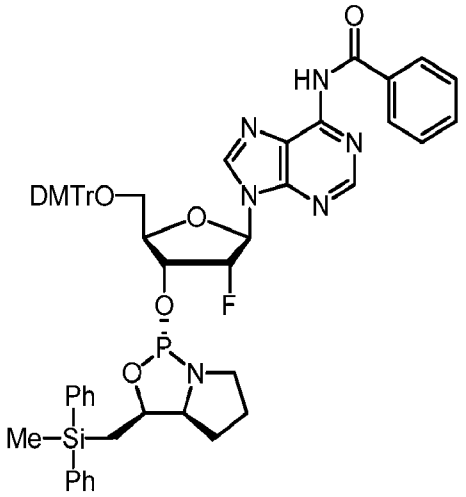
[00846] ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.12 (1H, brs), 8.73 (1H, s), 8.24 (1H, s), 8.07-8.01 (2H, m), 7.62-7.17 (22H, m), 6.83-6.77 (4H, m), 6.12 (1H, d, *J* = 4.8Hz), 4.84-4.73 (2H, m), 4.43 (1H, t, *J* = 4.8Hz), 4.25-4.19 (1H, m), 3.77 (6H, s), 3.55-3.20 (4H, m), 3.28 (3H, s), 3.16-3.03 (1H, m), 1.90-1.17 (6H, m), 0.65 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 155.0 (1P, s).

[00847] Oxazaphospholidine monomer 28a.



28a

[00848] Compound **28a** was obtained by using "5′-*O*-(DMTr)-2′-deoxy-2′-fluoro-6-*N*-(benzoyl)adenosine" instead of "5′-*O*-(DMTr)-2-*N*-(phenoxyacetyl)-6-*O*-(cyanoethyl)guanosine" in a similar manner to compound **12a**.

[00849] ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.64 (1H, s), 8.14 (1H, s), 8.06-8.01 (2H, m), 7.63-7.07 (23H, m), 6.78-6.70 (4H, m), 6.12 (1H, dd, *J* = 18.0, 2.4Hz), 5.24-5.01 (2H, m), 4.94-4.84 (1H, m), 4.17-4.06 (1H, m), 3.73 (6H, s), 3.55-3.40 (3H, m), 3.30-3.22 (1H, m), 3.03-2.88 (1H, m), 1.92-1.19 (6H, m), 0.62 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 150.5 (1P, d, *J* = 7.7Hz).

[00850] Oxazaphospholidine monomer 28b.

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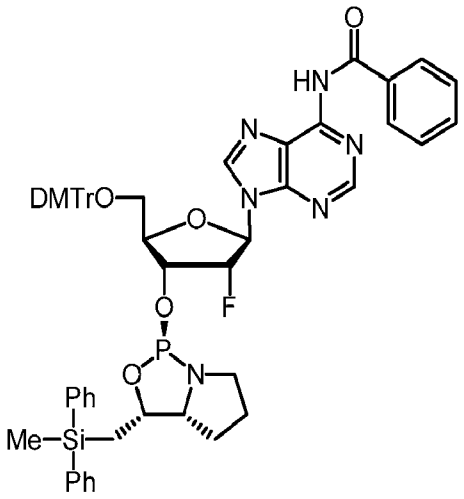
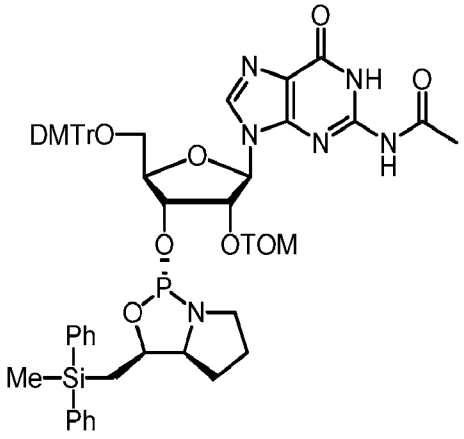


28b

[00851] Compound **28b** was obtained by using **3b** instead of **3a** in a similar manner to compound **28a**.

[00852] ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.07 (1H, brs), 8.80 (1H, s), 8.24 (1H, s), 8.08-8.01 (2H, m), 7.66-7.15 (22H, m), 6.81-6.75 (4H, m), 6.14 (1H, dd, *J* = 18.0, 1.8Hz), 5.16-4.91 (3H, m), 4.28-4.21 (1H, m), 3.76 (6H, s), 3.57-3.11 (5H, m), 1.82-1.16 (6H, m), 0.65 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 157.8 (1P, d, *J* = 5.6Hz).

[00853] Oxazaphospholidine monomer 29a.



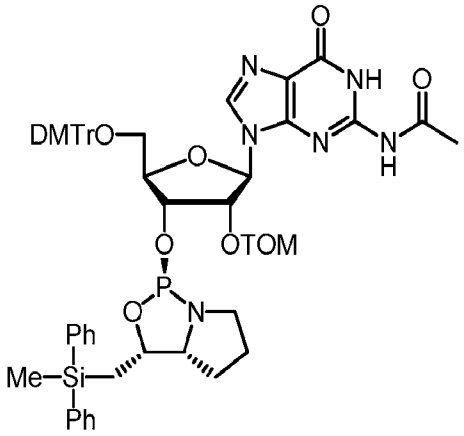
29a

[00854] Compound **29a** was obtained by using "5′-*O*-(DMTr)-2′-*O*-TOM-2-*N*-(acetyl)guanosine" instead of "5′-*O*-(DMTr)-2-*N*-(phenoxyacetyl)-6-*O*-(cyanoethyl)guanosine" in a similar manner to compound **12a**.

[00855] ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.70 (1H, s), 7.63-7.13 (21H, m), 6.84-6.76 (4H, m), 5.77 (1H, d, *J* = 8.4Hz), 5.41-5.33 (1H, m), 4.90 (2H, s), 4.78-4.68 (2H, m), 3.86 (1H, brs),

3.75 (3H, s), 3.74 (3H, s), 3.56-3.41 (2H, m), 3.32-2.90 (3H, m), 1.92-1.10 (9H, m), 0.97-0.87 (21H, m), 0.52 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 158.1 (1P, s).

[00856] Oxazaphospholidine monomer 29b.

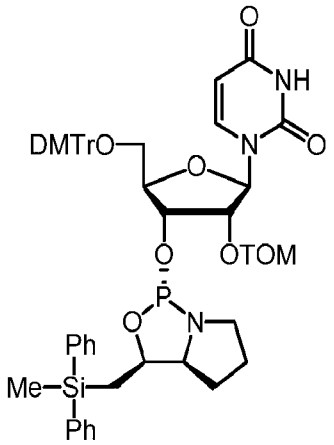


29b

[00857] Compound **29b** was obtained by using **3b** instead of **3a** in a similar manner to compound **29a**.

[00858] ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.77 (1H, s), 7.56-7.15 (21H, m), 6.82-6.75 (4H, m), 5.86 (1H, d, *J* = 7.5Hz), 5.26-5.17 (1H, m), 4.95 (1H, d, *J* =5.4Hz), 4.85 (1H, d, *J* =5.4Hz), 4.78-4.71 (1H, m), 4.59-4.49 (1H, m), 4.10-4.05 (1H, m), 3.74 (6H, s), 3.52-3.37 (2H, m), 3.30-3.18 (1H, m), 3.11-2.85 (2H, m), 1.85-1.15 (9H, m), 0.93-0.84 (21H, m), 0.62 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 152.3 (1P, s).

[00859] Oxazaphospholidine monomer 30a.

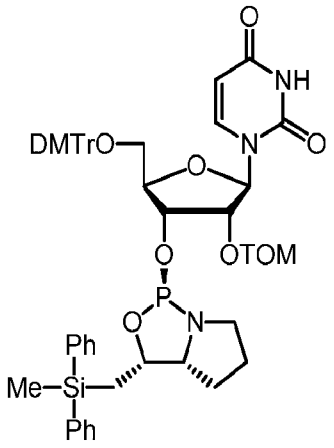


30a

[00860] Compound **30a** was obtained by using "5′-*O*-(DMTr)-2′-*O*-TOM-uridine" instead of "5′-*O*-(DMTr)-2-*N*-(phenoxyacetyl)-6-*O*-(cyanoethyl)guanosine" in a similar manner to compound **12a**.

[00861] ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.76 (1H, d, *J* = 8.1Hz), 7.55-7.18 (20H, m), 6.88-6.80 (4H, m), 6.11 (1H, d, *J* = 6.0Hz), 5.32 (1H, d, *J* = 8.1Hz), 4.99 (1H, d, *J* = 5.1Hz), 4.93 (1H, d, *J* = 5.1Hz), 4.84-4.75 (1H, m), 4.54-4.46 (1H, m), 4.38 (1H, t, *J* = 5.7Hz), 3.87-3.83 (1H, m), 3.78 (3H, s), 3.77 (3H, s), 3.56-3.42 (1H, m), 3.39-3.28 (1H, m), 3.36 (1H, dd, *J* = 11.0, 2.7Hz), 3.25 (1H, dd, *J* = 11.0, 2.7Hz), 3.16-3.03 (1H, m), 1.88-1.12 (6H, m), 1.08-0.97 (21H, m), 0.59 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 156.6 (1P, s).

[00862] Oxazaphospholidine monomer 30b.

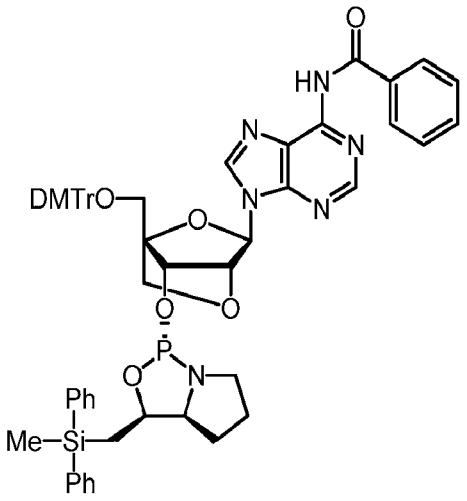


30b

[00863] Compound **30b** was obtained by using **3b** instead of **3a** in a similar manner to compound **30a**.

[00864] ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.87 (1H, d, *J* = 7.8Hz), 7.52-7.48 (4H, m), 7.38-7.21 (16H, m), 6.83-6.79 (4H, m), 6.14 (1H, d, *J* = 4.8Hz), 5.33 (1H, d, *J* = 7.8Hz), 4.99 (1H, d, *J* = 5.4Hz), 4.89 (1H, d, *J* = 5.4Hz), 4.67 (1H, dd, *J* = 13.8, 7.2Hz), 4.52 (1H, dt, *J* = 10.4, 4.8Hz), 4.31 (1H, t, *J* = 4.8Hz), 4.06-4.03 (1H, m), 3.78 (3H, s), 3.77 (3H, s), 3.47 (1H, dd, *J* = 10.4, 2.4Hz), 3.47-3.39 (1H, m), 3.22-3.17 (2H, m), 3.00 (1H, ddd, *J* = 19.5, 10.4, 4.8Hz), 1.82-1.74 (1H, m), 1.68-1.58 (1H, m), 1.56 (1H, dd, *J* = 14.4, 8.4Hz), 1.38 (1H, dd, *J* = 14.4, 7.2Hz), 1.31-1.25 (1H, m), 1.26-1.17 (1H, m), 1.08-0.98 (21H, m), 0.63 (3H, s); ^{31}P NMR (243.0 MHz, $CDCl_3$) δ 154.3 (1P, s).

[00865] Oxazaphospholidine monomer 31a.

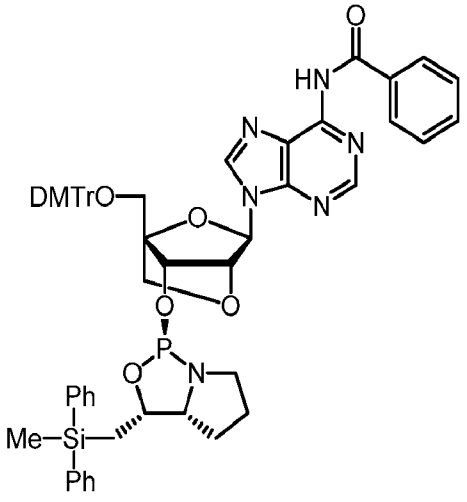


31a

[00866] Compound **31a** was obtained by using "5′-*O*-(DMTr)-2′-*O*,4′-*C*-methylene-6-*N*-(benzoyl)adenosine" instead of "5′-*O*-(DMTr)-2-*N*-(phenoxyacetyl)-6-*O*-(cyanoethyl)guanosine" in a similar manner to compound **12a**.

[00867] ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.10 (1H, brs), 8.76 (1H, s), 8.32 (1H, s), 8.04 (2H, d, *J* = 7.2Hz), 7.64-7.18 (22H, m), 6.84 (4H, d, *J* = 8.7Hz), 6.10 (1H, s), 4.76 (1H, d *J* = 6.9Hz), 4.58 (1H, s), 4.61-4.51 (1H, m), 3.91 (1H, d, *J* = 7.8Hz), 3.77 (1H, d, *J* = 7.8Hz), 3.75 (6H, s), 3.50 (1H, s), 3.47-3.33 (1H, m), 3.31-3.19 (1H, m), 3.03-2.88 (1H, m), 1.84-1.09 (6H, m), 0.51 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 152.9 (1P, s).

[00868] Oxazaphospholidine monomer 31b.



31b

[00869] Compound **31b** was obtained by using **3b** instead of **3a** in a similar manner to compound **31a**.

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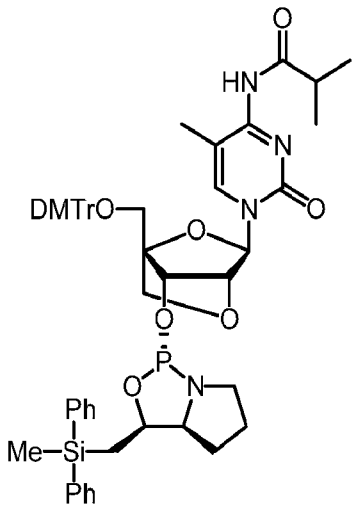
[00870] ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.81 (1H, s), 8.30 (1H, s), 8.07-8.00 (2H, m), 7.64-7.17 (22H, m), 6.86-6.79 (4H, m), 6.12 (1H, s), 4.81-4.72 (1H, m), 4.62 (1H, d *J* = 7.2Hz), 4.57 (1H, s), 3.94 (1H, d, *J* = 7.8Hz), 3.89 (1H, d, *J* = 7.8Hz), 3.77 (6H, s), 3.48 (2H, s), 3.46-3.32 (1H, m), 3.24-3.13 (1H, m), 3.10-2.97 (1H, m), 1.84-1.49 (3H, m), 1.42-1.09 (3H, m), 0.58 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 157.3 (1P, s).

[00871] Oxazaphospholidine monomer 32a.



32a

[00872] Compound **32a** was obtained by using "5′-*O*-(DMTr)-2′-*O*,4′-*C*-methylene-4-*N*-(isobutyryl)-5-methylcytidine" instead of "5′-*O*-(DMTr)-2-*N*-(phenoxyacetyl)-6-*O*-(cyanoethyl)guanosine" in a similar manner to compound **12a**.

[00873] ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.88 (1H, brs), 7.58-7.18 (20H, m), 6.88-6.80 (4H, m), 5.65 (1H, s), 4.69-4.60 (1H, m), 4.52 (1H, d, *J* = 6.6Hz), 4.49 (1H, s), 3.81-3.74 (1H, m), 3.75 (3H, s), 3.73 (3H, s), 3.64 (1H, d, *J* = 8.1Hz), 3.56 (1H, d, *J* = 11.1Hz), 3.53 (1H, d, *J* = 8.1Hz), 3.46 (1H, d, *J* = 11.1Hz), 3.56-3.40 (1H, m), 3.32-3.20 (1H, m), 3.14-3.00 (1H, m), 1.85-1.12 (6H, m), 1.60 (3H, s), 1.19 (6H, d, *J* = 6.9Hz), 0.55 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 155.9 (1P, s).

[00874] Oxazaphospholidine monomer 32b.

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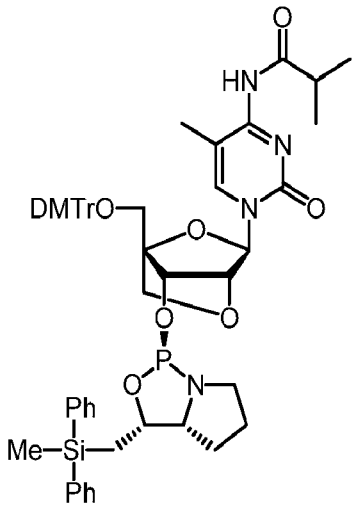
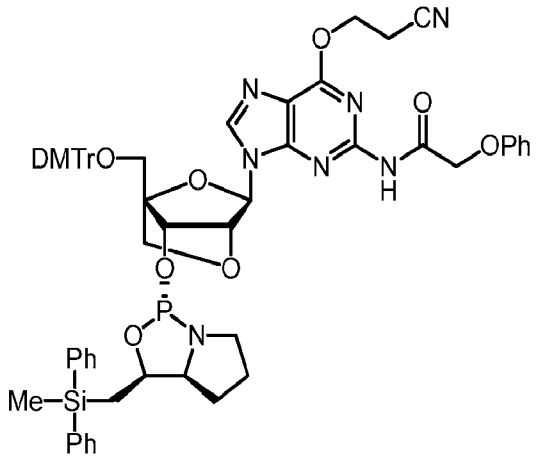


32b

[00875] Compound **32b**was obtained by using **3b** instead of **3a** in a similar manner to compound **32a**.

[00876] ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.86 (1H, brs), 7.56-7.19 (20H, m), 6.88-6.79 (4H, m), 5.69 (1H, s), 4.86-4.76 (1H, m), 4.46 (1H, s), 4.45 (1H, d, *J* = 7.5Hz), 3.80-3.75 (1H, m), 3.79 (6H, s), 3.74 (1H, d, *J* = 8.1Hz), 3.69 (1H, d, *J* = 8.1Hz), 3.51 (1H, d, *J* = 11.1Hz), 3.44-3.30 (1H, m), 3.39 (1H, d, *J* = 11.1Hz), 3.29-3.17 (1H, m), 3.11-2.97 (1H, m), 1.86-1.52 (3H, m), 1.64 (3H, s), 1.45-1.10 (3H, m), 1.21 (6H, d, *J* = 6.6Hz), 0.62 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 158.2 (1P, s).

[00877] Oxazaphospholidine monomer 33a.

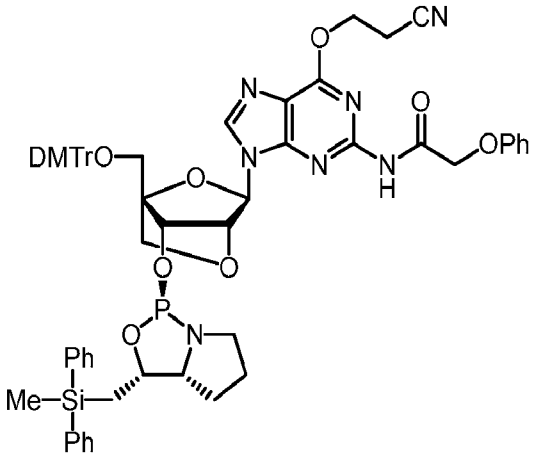


33a

[00878] Compound **33a** was obtained by using "5′-*O*-(DMTr)-2′-*O*,4′-*C*-methylene-2-*N*-(phenoxyacetyl)-6-*O*-(cyanoethyl)guanosine" instead of "5′-*O*-(DMTr)-2-*N*-(phenoxyacetyl)-6-*O*-(cyanoethyl)guanosine" in a similar manner to compound **12a**.

[00879] ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.71 (1H, brs), 8.16 (1H, s), 7.50-7.17 (21H, m), 7.09-7.01 (3H, m), 6.86-6.79 (4H, m), 6.03 (1H, s), 4.84 (2H, t, *J* = 6.6Hz), 4.72 (2H, s), 4.68 (1H, d, *J* = 7.2Hz), 4.55-4.46 (1H, m), 4.50 (1H, s), 3.90 (1H, d, *J* = 7.8Hz), 3.77 (1H, d, *J* = 7.8Hz), 3.75 (6H, s), 3.51 (1H, d, *J* = 10.8Hz), 3.47 (1H, d, *J* = 10.8Hz), 3.45-3.21 (2H, m), 3.08 (2H, t, *J* =6.6Hz), 3.03-2.89 (1H, m), 1.80-1.08 (6H, m), 0.47 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 153.2 (1P, s).

[00880] Oxazaphospholidine monomer 33b.

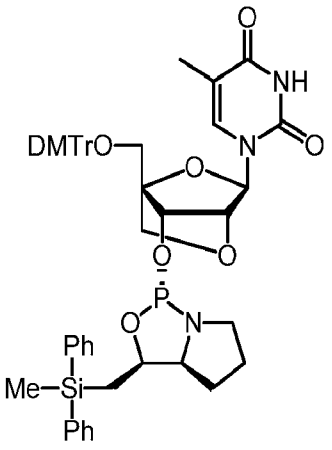


33b

[00881] Compound **33b** was obtained by using **3b** instead of **3a** in a similar manner to compound **33a**.

[00882] ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.86 (1H, brs), 8.13 (1H, s), 7.55-7.17 (21H, m), 7.08-6.98 (3H, m), 6.95-6.78 (4H, m), 6.01 (1H, s), 4.86 (2H, t, *J* = 6.6Hz), 4.82-4.73 (1H, m), 4.70 (2H, s), 4.64 (1H, d, *J* = 7.5Hz), 4.49 (1H, s), 3.94 (1H, d, *J* = 7.8Hz), 3.89 (1H, d, *J* = 7.8Hz), 3.77 (6H, s), 3.46 (2H, s), 3.45-3.30 (1H, m), 3.24-3.12 (1H, m), 3.09 (2H, t, *J* =6.6Hz), 3.09-2.96 (1H, m), 1.81-1.50 (3H, m), 1.41-1.06 (3H, m), 0.58 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 157.4 (1P, s).

[00883] Oxazaphospholidine monomer 34a.

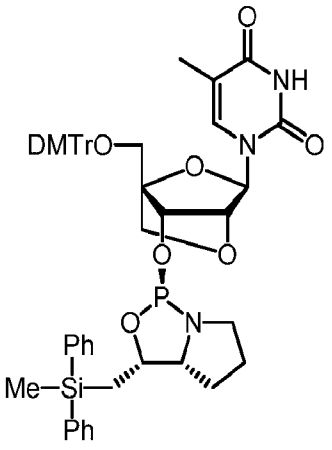


34a

[00884] Compound **34a** was obtained by using "5′-*O*-(DMTr)-2′-*O*,4′-*C*-methylene-5-methyluridine" instead of "5′-*O*-(DMTr)-2-*N*-(phenoxyacetyl)-6-*O*-(cyanoethyl)guanosine" in a similar manner to compound **12a**.

[00885] ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.71 (1H, d, J = 0.9Hz), 7.50-7.17 (20H, m), 6.87-6.80 (4H, m), 5.61 (1H, s), 4.69-4.60 (1H, m), 4.55 (1H, d, J = 6.9Hz), 4.41 (1H, s), 3.74 (3H, s), 3.73 (3H, s), 3.64 (1H, d, J = 7.8Hz), 3.55 (1H, d, J = 7.8Hz), 3.53 (1H, d, J = 10.8Hz), 3.46 (1H, d, J = 10.8Hz), 3.56-3.42 (1H, m), 3.35-3.24 (1H, m), 3.13-3.00 (1H, m), 1.85-1.45 (3H, m), 1.55 (3H, d, J = 0.9Hz), 1.41-1.12 (3H, m), 0.56 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 155.1 (1P, s).

[00886] Oxazaphospholidine monomer 34b.



34b

[00887] Compound **34b** was obtained by using **3b** instead of **3a** in a similar manner to compound **34a**.

[00888] ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.69 (1H, s), 7.56-7.19 (20H, m), 6.88-6.79 (4H, m), 5.66 (1H, s), 4.87-4.77 (1H, m), 4.47 (1H, d, J = 7.8Hz), 4.40 (1H, s), 3.78 (6H, s), 3.74 (1H,

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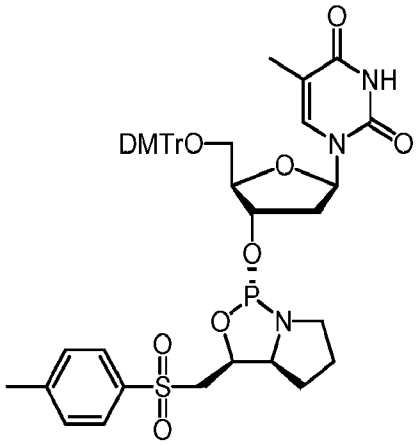
d, J = 7.8Hz), 3.68 (1H, d, J = 7.8Hz), 3.50 (1H, d, J = 10.8Hz), 3.46-3.32 (1H, m), 3.39 (1H, d, J = 10.8Hz), 3.30-3.19 (1H, m), 3.12-2.98 (1H, m), 1.85-1.56 (3H, m), 1.59 (3H, s), 1.46-1.12 (3H, m), 0.63 (3H, s); ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 158.1 (1P, s).

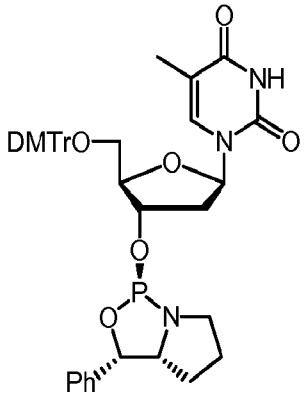
[00889] Oxazaphospholidine monomer 35a.



35a

[00890] Compound **35a** was obtained by using **13a'** instead of **3a** in a similar manner to compound **13a**. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.76 (2H, d, J = 9.0Hz), 7.62 (1H, d, J = 1.2Hz), 7.40 (2H, d, J = 7.2Hz), 7.32-7.23 (10H, m), 6.85 (4H, d, J = 8.4Hz), 6.41 (1H, dd, J = 8.4, 5.4Hz), 4.94 (1H, dd, J = 12.3, 5.4Hz), 4.84-4.79 (1H, m), 4.03-4.01 (1H, m), 3.79 (6H, s), 3.59-3.53 (1H, m), 3.52-3.44 (2H, m), 3.41 (1H, dd, J = 14.7, 7.2Hz), 3.37-3.30 (2H, m), 3.13 (1H, ddd, J = 19.3, 10.3, 4.1Hz), 2.50-2.44 (1H, m), 2.39 (3H, s), 2.35-2.29 (1H, m), 1.91-1.72 (2H, m), 1.64-1.59 (1H, m), 1.40 (3H, s), 1.12-1.05 (1H, m); ^{31}P NMR (243.0 MHz, $CDCl_3$) δ 154.2 (1P, s).

[00891] Example Z-41.



Z-27

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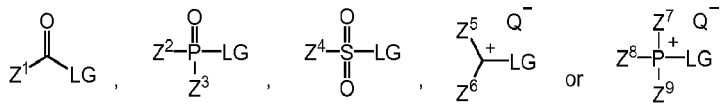
[00892] The above Compound **Z-27**, which represents a conventional monomer, was used to produce oligos. Figure 70 shows a chart of products obtained through Comparison Example Z-1. As shown in Figures 69 and 70, the present monomers provided more complete deprotection and less side product, which makes product isolation and/or purification easier.

[00893] In some embodiments, the present invention provides chemically stable monomers. Exemplary such monomers are depicted in the Examples above. In some embodiments, the present invention provides monomers with high isolated yield. In some embodiments, the present invention provides monomers with isolated yield higher than conventional method. In some embodiments, the isolated yield is more than 80%. Exemplary such monomers are depicted in the Examples above.

Condensing reagent

[00894] Condensing reagents (C_R) useful in accordance with methods of the present invention are of any one of the following general formulae:



C_R1 C_R2 C_R3 C_R4 C_R5

wherein Z^1, Z^2, Z^3, Z^4, Z^5, Z^6, Z^7, Z^8, and Z^9 are independently optionally substituted group selected from alkyl, aminoalkyl, cycloalkyl, heterocyclic, cycloalkylalkyl, heterocycloalkyl, aryl, heteroaryl, alkyloxy, aryloxy, or heteroaryloxy, or wherein any of Z^2 and Z^3, Z^5 and Z^6, Z^7 and Z^8, Z^8 and Z^9, Z^9 and Z^7, or Z^7 and Z^8 and Z^9 are taken together to form a 3 to 20 membered alicyclic or heterocyclic ring; Q^- is a counter anion; and LG is a leaving group.

[00895] In some embodiments, a counter ion of a condensing reagent C_R is Cl^-, Br^-, BF_4^-, PF_6^-, TfO^-, Tf_2N^-, AsF_6^-, ClO_4^-, or SbF_6^-, wherein Tf is CF_3SO_2. In some embodiments, a leaving group of a condensing reagent C_R is F, Cl, Br, I, 3-nitro-1,2,4-triazole, imidazole, alkyltriazole, tetrazole, pentafluorobenzene, or 1-hydroxybenzotriazole.

[00896] Examples of condensing reagents used in accordance with methods of the present invention include, but are not limited to, pentafluorobenzoyl chloride, carbonyldiimidazole (CDI), 1-mesitylenesulfonyl-3-nitrotriazole (MSNT), 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide hydrochloride (EDCI-HCl), benzotriazole-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (PyBOP), *N,N'*-bis(2-oxo-3-

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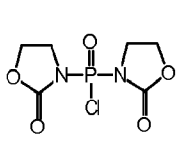
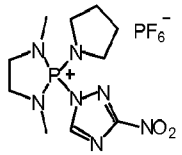
oxazolidinyl)phosphinic chloride (BopCl), 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), and *O*-benzotriazole-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate (HBTU), DIPCDI; *N*,*N'*-bis(2-oxo-3-oxazolidinyl)phosphinic bromide (BopBr), 1,3-dimethyl-2-(3-nitro-1,2,4-triazol-1-yl)-2-pyrrolidin-1-yl-1,3,2-diazaphospholidinium hexafluorophosphate (MNTP), 3-nitro-1,2,4-triazol-1-yl-tris(pyrrolidin-1-yl)phosphonium hexafluorophosphate (PyNTP), bromotripyrrolidinophosphonium hexafluorophosphate (PyBrOP); *O*-(benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium tetrafluoroborate (TBTU); and tetramethylfluoroformamidinium hexafluorophosphate (TFFH). In certain embodiments, a counter ion of the condensing reagent C_R is Cl^-, Br^-, BF_4^-, PF_6^-, TfO^-, Tf_2N^-, AsF_6^-, ClO_4^-, or SbF_6^-, wherein Tf is CF_3SO_2.

[00897] In some embodiments, a condensing reagent is 1-(2,4,6-triisopropylbenzenesulfonyl)-5-(pyridin-2-yl) tetrazolide, pivaloyl chloride, bromotrispyrrolidinophosphonium hexafluorophosphate, *N*,*N'*-bis(2-oxo-3-oxazolidinyl) phosphinic chloride (BopCl), or 2-chloro-5,5-dimethyl-2-oxo-1,3,2-dioxaphosphinane. In some embodiment, a condensing reagent is *N*,*N'*-bis(2-oxo-3-oxazolidinyl)phosphinic chloride (BopCl). In some embodiments, a condensing reagent is selected from those described in WO/2006/066260).

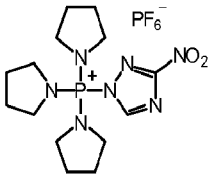
[00898] In some embodiments, a condensing reagent is 1,3-dimethyl-2-(3-nitro-1,2,4-triazol-1-yl)-2-pyrrolidin-1-yl-1,3,2-diazaphospholidinium hexafluorophosphate (MNTP), or 3-nitro-1,2,4-triazol-1-yl-tris(pyrrolidin-1-yl)phosphonium hexafluorophosphate (PyNTP):



BopCl



MNTP



PyNTP

Selection of base and sugar of nucleoside coupling partner

[00899] As described herein, nucleoside coupling partners for use in accordance with methods of the present invention can be the same as one another or can be different from one another. In some embodiments, nucleoside coupling partners for use in the synthesis of a provided oligonucleotide are of the same structure and/or stereochemical configuration as one another. In some embodiments, each nucleoside coupling partner for use in the synthesis of a

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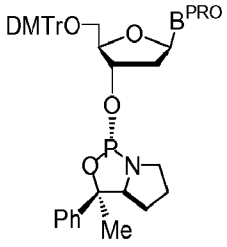
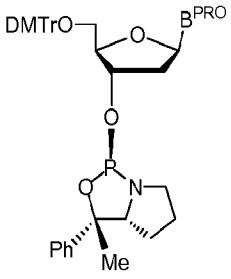
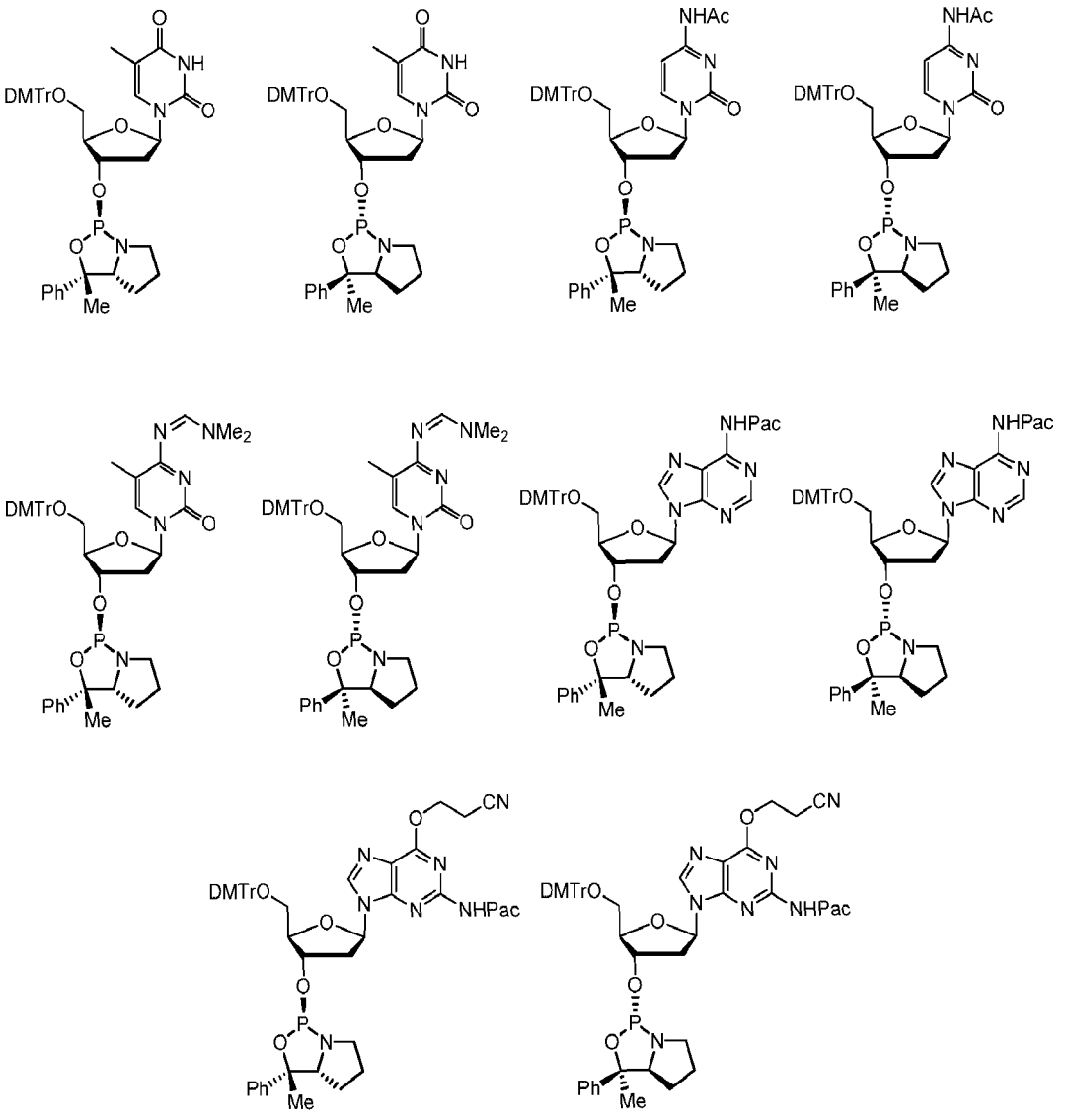
provided oligonucleotide is not of the same structure and/or stereochemical configuration as certain other nucleoside coupling partners of the oligonucleotide. Exemplary nucleobases and sugars for use in accordance with methods of the present invention are described herein. One of skill in the relevant chemical and synthetic arts will recognize that any combination of nucleobases and sugars described herein are contemplated for use in accordance with methods of the present invention.

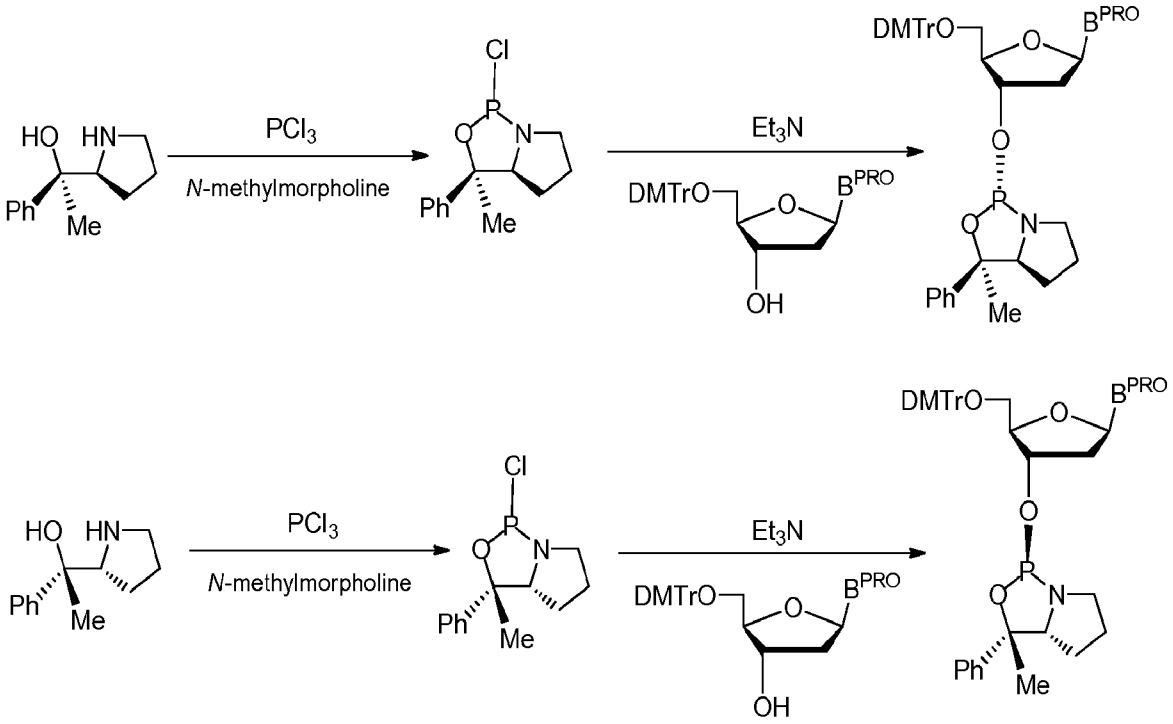
Coupling step:

[00900] Exemplary coupling procedures and chiral reagents and condensing reagents for use in accordance with the present invention are outlined in, *inter alia*, Wada I (JP4348077; WO2005/014609; WO2005/092909), Wada II (WO2010/064146), and Wada III (WO2012/039448). Chiral nucleoside coupling partners for use in accordance with the present invention are also referred to herein as "Wada amidites." In some embodiments, a coupling partner has the structure of , wherein B^{PRO} is a protected nucleobase. In some embodiments, a coupling partner has the structure of , wherein B^{PRO} is a protected nucleobase. Exemplary chiral phosphoramidites as coupling partner are depicted below:



[00901] One of the methods used for synthesizing the coupling partner is depicted in Scheme II, below.

Scheme II. Exemplary synthesis of coupling partner.



[00902] In some embodiments, the step of coupling comprises reacting a free hydroxyl group of a nucleotide unit of an oligonucleotide with a nucleoside coupling partner under suitable conditions to effect the coupling. In some embodiments, the step of coupling is preceded by a step of deblocking. For instance, in some embodiments, the 5' hydroxyl group of the growing oligonucleotide is blocked (i.e., protected) and must be deblocked in order to subsequently react with a nucleoside coupling partner.

[00903] Once the appropriate hydroxyl group of the growing oligonucleotide has been deblocked, the support is washed and dried in preparation for delivery of a solution comprising a chiral reagent and a solution comprising an activator. In some embodiments, a chiral reagent and an activator are delivered simultaneously. In some embodiments, co-delivery comprises delivering an amount of a chiral reagent in solution (e.g., a phosphoramidite solution) and an amount of activator in a solution (e.g., a CMPT solution) in a polar aprotic solvent such as a nitrile solvent (e.g., acetonitrile).

[00904] In some embodiments, the step of coupling provides a crude product composition in which the chiral phosphite product is present in a diastereomeric excess of > 95%. In some embodiments, the chiral phosphite product is present in a diastereomeric excess of > 96%. In some embodiments, the chiral phosphite product is present in a diastereomeric excess of > 97%. In some embodiments, the chiral phosphite product is present in a diastereomeric excess of >

98%. In some embodiments, the chiral phosphite product is present in a diastereomeric excess of > 99%.

Capping step:

[00905] Provided methods for making chirally controlled oligonucleotides comprise a step of capping. In some embodiments, a step of capping is a single step. In some embodiments, a step of capping is two steps. In some embodiments, a step of capping is more than two steps.

[00906] In some embodiments, a step of capping comprises steps of capping the free amine of the chiral auxiliary and capping any residual unreacted 5' hydroxyl groups. In some embodiments, the free amine of the chiral auxiliary and the unreacted 5' hydroxyl groups are capped with the same capping group. In some embodiments, the free amine of the chiral auxiliary and the unreacted 5' hydroxyl groups are capped with different capping groups. In certain embodiments, capping with different capping groups allows for selective removal of one capping group over the other during synthesis of the oligonucleotide. In some embodiments, the capping of both groups occurs simultaneously. In some embodiments, the capping of both groups occurs iteratively.

[00907] In certain embodiments, capping occurs iteratively and comprises a first step of capping the free amine followed by a second step of capping the free 5' hydroxyl group, wherein both the free amine and the 5' hydroxyl group are capped with the same capping group. For instance, in some embodiments, the free amine of the chiral auxiliary is capped using an anhydride (e.g., phenoxyacetic anhydride, i.e., Pac_2O) prior to capping of the 5' hydroxyl group with the same anhydride. In certain embodiments, the capping of the 5' hydroxyl group with the same anhydride occurs under different conditions (e.g., in the presence of one or more additional reagents). In some embodiments, capping of the 5' hydroxyl group occurs in the presence of an amine base in an etherial solvent (e.g., NMI (N-methylimidazole) in THF). The phrase "capping group" is used interchangeably herein with the phrases "protecting group" and "blocking group".

[00908] In some embodiments, an amine capping group is characterized in that it effectively caps the amine such that it prevents rearrangement and/or decomposition of the intermediate phosphite species. In some embodiments, a capping group is selected for its ability to protect the amine of the chiral auxiliary in order to prevent intramolecular cleavage of the internucleotide linkage phosphorus.

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[00909] In some embodiments, a 5' hydroxyl group capping group is characterized in that it effectively caps the hydroxyl group such that it prevents the occurrence of "shortmers," e.g., "n-m" (m and n are integers and m<n; n is the number of bases in the targeted oligonucleotide) impurities that occur from the reaction of an oligonucleotide chain that fails to react in a first cycle but then reacts in one or more subsequent cycles. The presence of such shortmers, especially "n-1", has a deleterious effect upon the purity of the crude oligonucleotide and makes final purification of the oligonucleotide tedious and generally low-yielding.

[00910] In some embodiments, a particular cap is selected based on its tendency to facilitate a particular type of reaction under particular conditions. For instance, in some embodiments, a capping group is selected for its ability to facilitate an E1 elimination reaction, which reaction cleaves the cap and/or auxiliary from the growing oligonucleotide. In some embodiments, a capping group is selected for its ability to facilitate an E2 elimination reaction, which reaction cleaves the cap and/or auxiliary from the growing oligonucleotide. In some embodiments, a capping group is selected for its ability to facilitate a β-elimination reaction, which reaction cleaves the cap and/or auxiliary from the growing oligonucleotide.

Modifying step:

[00911] As used herein, the phrase "modifying step", "modification step" and "P-modification step" are used interchangeably and refer generally to any one or more steps used to install a modified internucleotidic linkage. In some embodiments, the modified internucleotidic linkage having the structure of formula I. A P-modification step of the present invention occurs during assembly of a provided oligonucleotide rather than after assembly of a provided oligonucleotide is complete. Thus, each nucleotide unit of a provided oligonucleotide can be individually modified at the linkage phosphorus during the cycle within which the nucleotide unit is installed.

[00912] In some embodiments, a suitable P-modification reagent is a sulfur electrophile, selenium electrophile, oxygen electrophile, boronating reagent, or an azide reagent.

[00913] For instance, in some embodiments, a selemium reagent is elemental selenium, a selenium salt, or a substituted diselenide. In some embodiments, an oxygen electrophile is elemental oxygen, peroxide, or a substituted peroxide. In some embodiments, a boronating reagent is a borane-amine (e.g., *N*,*N*-diisopropylethylamine (BH_3·DIPEA), borane-pyridine

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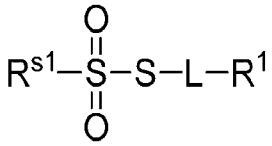
($BH_3 \cdot Py$), borane-2-chloropyridine ($BH_3 \cdot CPy$), borane-aniline ($BH_3 \cdot An$)), a borane-ether reagent (e.g., borane-tetrahydrofuran ($BH_3 \cdot THF$)), a borane-dialkylsulfide reagent (e.g., $BH_3 \cdot Me_2S$), aniline-cyanoborane, or a triphenylphosphine-carboalkoxyborane. In some embodiments, an azide reagent is comprises an azide group capable of undergoing subsequent reduction to provide an amine group.

[00914] In some embodiments, a P-modification reagent is a sulfurization reagent as described herein. In some embodiments, a step of modifying comprises sulfurization of phosphorus to provide a phosphorothioate linkage or phosphorothioate triester linkage. In some embodiments, a step of modifying provides an oligonucleotide having an internucleotidic linkage of formula I.

[00915] In some embodiments, the present invention provides sulfurizing reagents, and methods of making, and use of the same.

[00916] In some embodiments, such sulfurizing reagents are thiosulfonate reagents. In some embodiments, a thiosulfonate reagent has a structure of formula **S-I**:



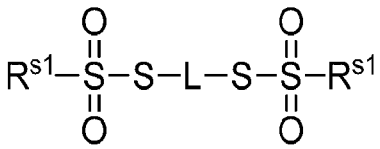
S-I

wherein:

R^{s1} is R; and

each of R, L and R^1 is independently as defined and described above and herein.

[00917] In some embodiments, the sulfurizing reagent is a bis(thiosulfonate) reagent. In some embodiments, the bis(thiosulfonate) reagent has the structure of formula **S-II**:



S-II

wherein each of R^{s1} and L is independently as defined and described above and herein.

[00918] As defined generally above, R^{s1} is R, wherein R is as defined and described above and herein. In some embodiments, R^{s1} is optionally substituted aliphatic, aryl, heterocyclyl or heteroaryl. In some embodiments, R^{s1} is optionally substituted alkyl. In some embodiments, R^{s1}

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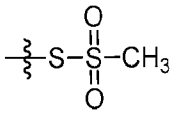
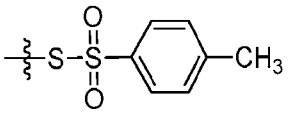
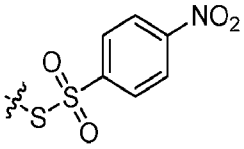
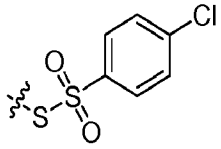
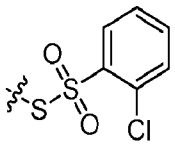
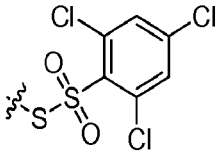
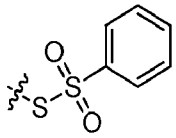
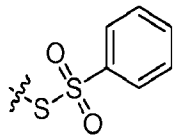
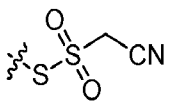
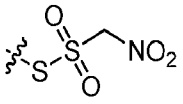
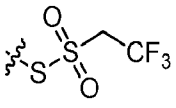
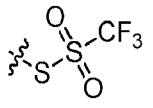
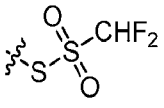
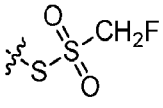
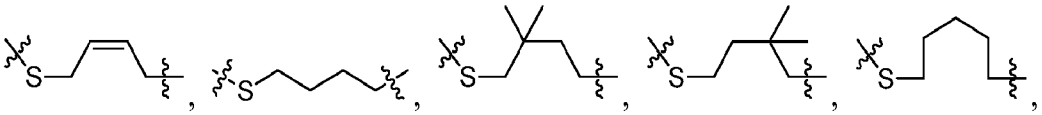
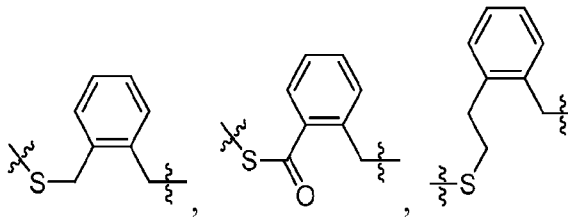
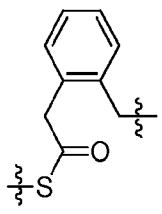
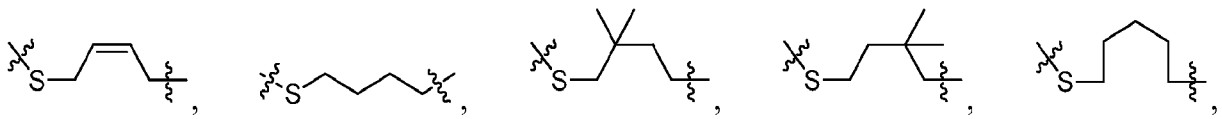
is optionally substituted alkyl. In some embodiments, R^{s1} is methyl. In some embodiments, R^{s1} is cyanomethyl. In some embodiments, R^{s1} is nitromethyl. In some embodiments, R^{s1} is optionally substituted aryl. In some embodiments, R^{s1} is optionally substituted phenyl. In some embodiments, R^{s1} is phenyl. In some embodiments, R^{s1} is *p*-nitrophenyl. In some embodiments, R^{s1} is *p*-methylphenyl. In some embodiments, R^{s1} is *p*-chlorophenyl. In some embodiments, R^{s1} is *o*-chlorophenyl. In some embodiments, R^{s1} is 2,4,6-trichlorophenyl. In some embodiments, R^{s1} is pentafluorophenyl. In some embodiments, R^{s1} is optionally substituted heterocyclyl. In some embodiments, R^{s1} is optionally substituted heteroaryl.

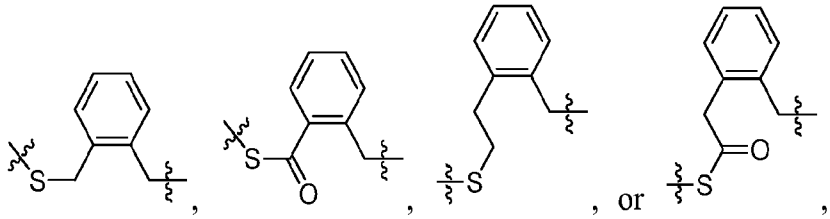
[00919] In some embodiments, R^{s1}–$S(O)_2S$– is  (MTS). In some embodiments, R^{s1}–$S(O)_2S$– is  (TTS). In some embodiments, R^{s1}–$S(O)_2S$– is  (NO_2PheTS). In some embodiments, R^{s1}–$S(O)_2S$– is  (p-ClPheTS). In some embodiments, R^{s1}–$S(O)_2S$– is  (o-ClPheTS). In some embodiments, R^{s1}–$S(O)_2S$– is  (2,4,6-TriClPheTS). In some embodiments, R^{s1}–$S(O)_2S$– is  (PheTS). In some embodiments, R^{s1}–$S(O)_2S$– is  (PFPheTS). In some embodiments, R^{s1}–$S(O)_2S$– is  (a-CNMTS). In some embodiments, R^{s1}–$S(O)_2S$– is  (a-NO_2MTS). In some embodiments, R^{s1}–

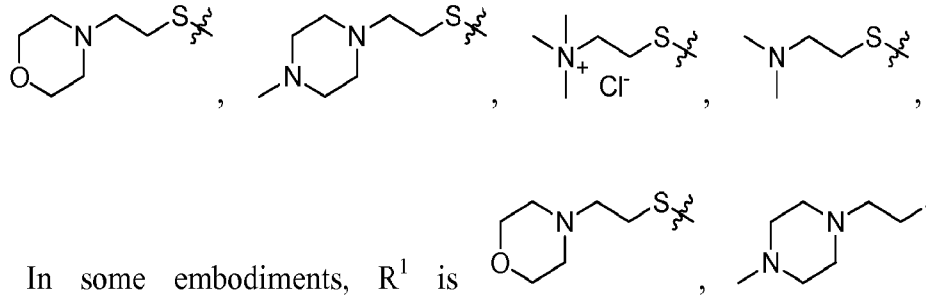
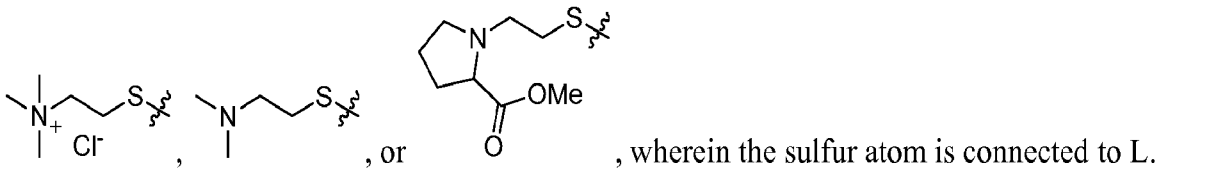
$S(O)_2S$– is  (a-CF_3MTS). In some embodiments, R^{s1}–$S(O)_2S$– is  (a-CF_3TS). In some embodiments, R^{s1}–$S(O)_2S$– is  (a-CHF_2TS). In some embodiments, R^{s1}–$S(O)_2S$– is  (a-CH_2FTS).

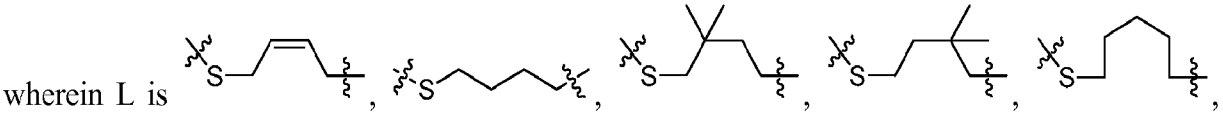
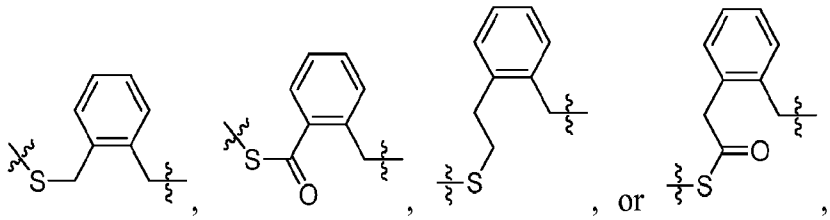
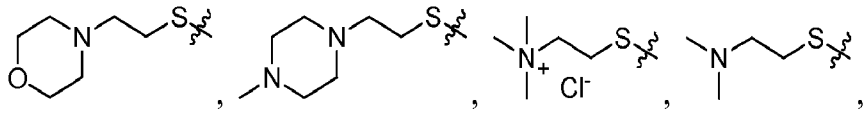
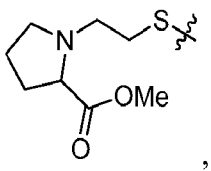
[00920] In some embodiments, the sulfurizing reagent has the structure of **S-I** or **S-II**, wherein L is –S–R^{L3}– or –S–C(O)–R^{L3}–. In some embodiments, L is –S–R^{L3}– or –S–C(O)–R^{L3}–, wherein R^{L3} is an optionally substituted C_1–C_6 alkylene. In some embodiments, L is –S–R^{L3}– or –S–C(O)–R^{L3}–, wherein R^{L3} is an optionally substituted C_1–C_6 alkenylene. In some embodiments, L is –S–R^{L3}– or –S–C(O)–R^{L3}–, wherein R^{L3} is an optionally substituted C_1-C_6 alkylene wherein one or more methylene units are optionally and independently replaced by an optionally substituted C_1–C_6 alkenylene, arylene, or heteroarylene. In some embodiments, In some embodiments, R^{L3} is an optionally substituted –S–(C_1–C_6 alkenylene)–, –S–(C_1–C_6 alkylene)–, –S–(C_1–C_6 alkylene)–arylene–(C_1–C_6 alkylene)–, –S–CO–arylene–(C_1–C_6 alkylene)–, or –S–CO–(C_1–C_6 alkylene)–arylene–(C_1–C_6 alkylene)–. In some embodiments, the sulfurizing reagent has the structure of **S-I** or **S-II**, wherein L is –S–R^{L3}– or –S–C(O)–R^{L3}–, and the sulfur atom is connected to R^1.

[00921] In some embodiments, the sulfurizing reagent has the structure of **S-I** or **S-II**, wherein L is alkylene, alkenylene, arylene or heteroarylene.

[00922] In some embodiments, the sulfurizing reagent has the structure of **S-I** or **S-II**, wherein L is , , or . In some embodiments, L is ,


, wherein the sulfur atom is connected to R^1.

[00923] In some embodiments, the sulfurizing reagent has the structure of **S-I** or **S-II**, wherein R^1 is 
. In some embodiments, R^1 is 
, wherein the sulfur atom is connected to L.

[00924] In some embodiments, the sulfurizing reagent has the structure of **S-I** or **S-II**, wherein L is 

, wherein the sulfur atom is connected to R^1; and R^1 is 
or 
, wherein the sulfur atom is connected to L.

[00925] In some embodiments, the sulfurizing reagent has the structure of **S-I** or **S-II**, wherein R^1 is $-S-R^{L2}$, wherein R^{L2} is as defined and described above and herein. In some embodiments, R^{L2} is an optionally substituted group selected from $-S-(C_1-C_6$ alkylene)–

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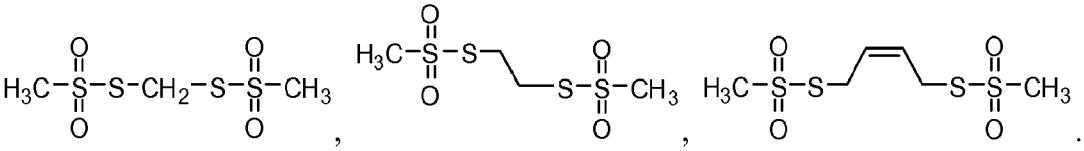
heterocyclyl, –S–(C_1–C_6 alkenylene)–heterocyclyl, –S–(C_1–C_6 alkylene)–N(R')$_2$, –S–(C_1–C_6 alkylene)–N(R')$_3$, wherein each R' is as defined above and described herein.

[00926] In some embodiments, –L–R^1 is –R^{L3}–S–S–R^{L2}, wherein each variable is independently as defined above and described herein. In some embodiments, –L–R^1 is –R^{L3}–C(O)–S–S–R^{L2}, wherein each variable is independently as defined above and described herein.

[00927] Exemplary bis(thiosulfonate) reagents of formula **S-II** are depicted below:



[00928] In some embodiments, the sulfurization reagent is a compound having one of the following formulae:

S_8, R^{s2}–S–S–R^{s3}, or R^{s2}–S–X^s–R^{s3},

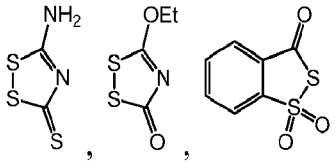
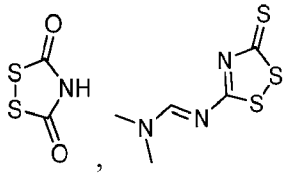
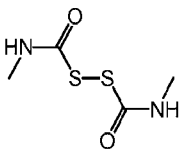
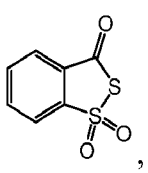
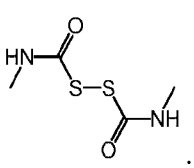
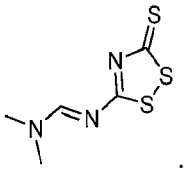
wherein:

each of R^{s2} and R^{s3} is independently an optionally substituted group selected from aliphatic, aminoalkyl, carbocyclyl, heterocyclyl, heterocycloalkyl, aryl, heteroaryl, alkyloxy, aryloxy, heteroaryloxy, acyl, amide, imide, or thiocarbonyl; or

R^{s2} and R^{s3} are taken together with the atoms to which they are bound to form an optionally substituted heterocyclic or heteroaryl ring;

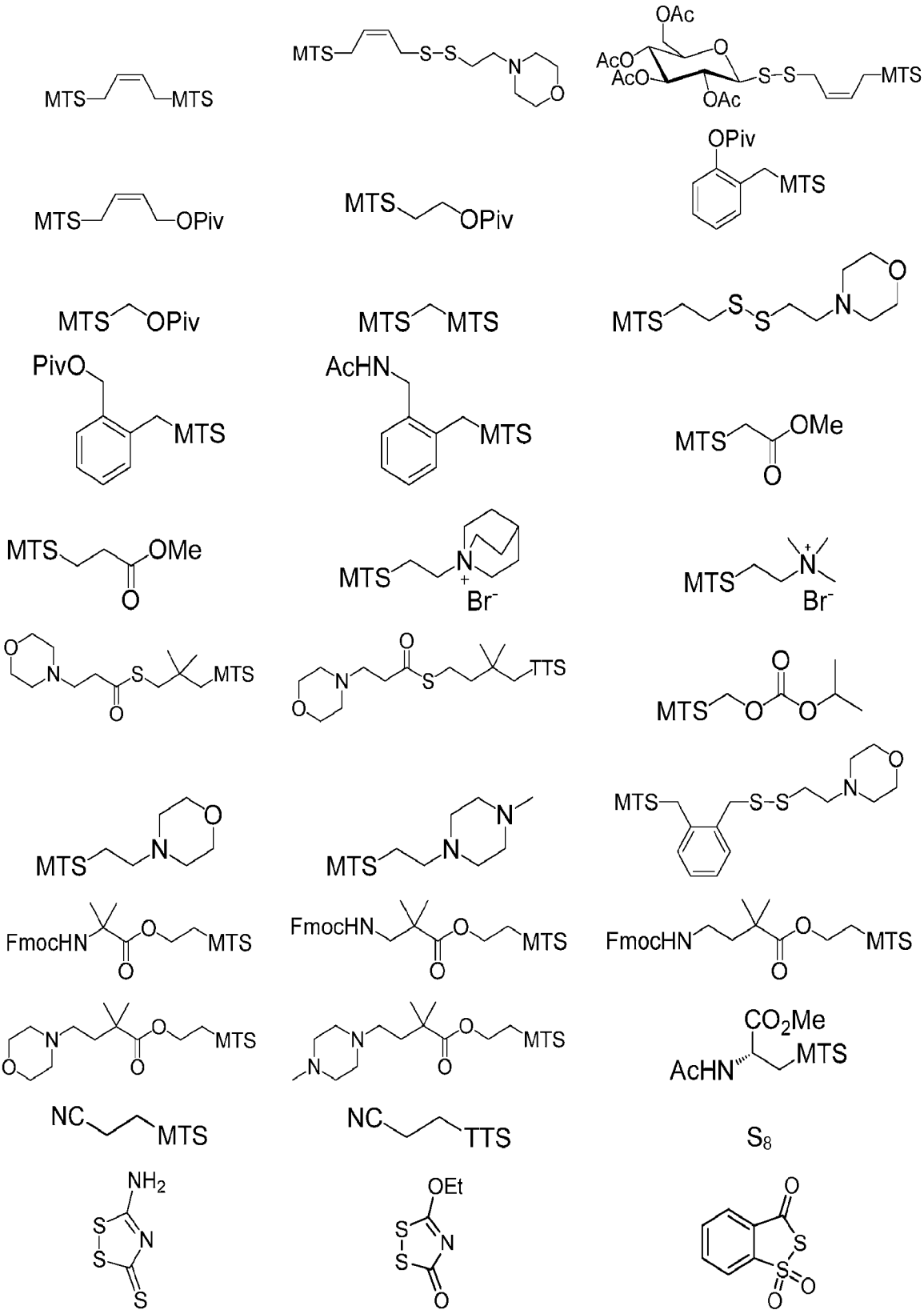
X^s is –S(O)$_2$–, –O–, or –N(R')–; and

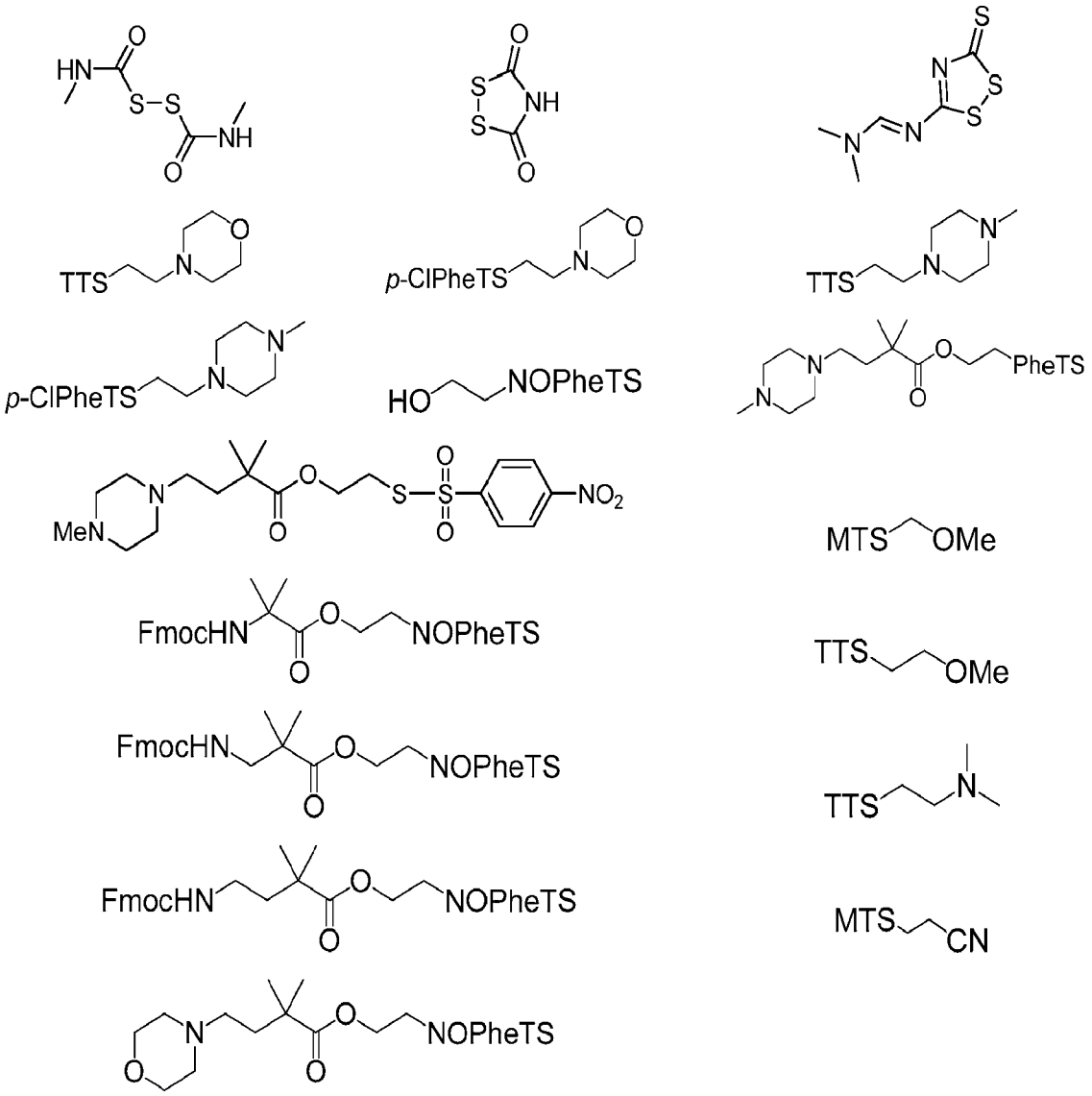
R' is as defined and described above and herein.

[00929] In some embodiments, the sulfurization reagent is S_8, , , , or . In some embodiments, the sulfurization reagent is S_8, , or . In some embodiments, the sulfurization reagent is .

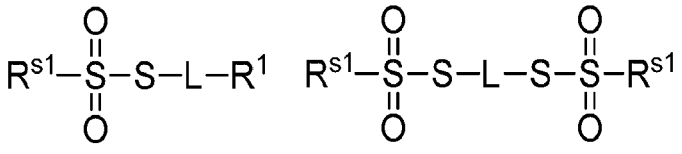
[00930] Exemplary sulfuring reagents are depicted in Table 5 below.

Table 5. Exemplary sulfurization reagents.





[00931] In some embodiments, a provided sulfurization reagent is used to modify an H-phosphonate. For instance, in some embodiments, an H-phosphonate oligonucleotide is synthesized using, e.g., a method of Wada I or Wada II, and is modified using a sulfurization reagent of formula **S-I** or **S-II**:



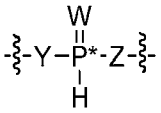
S-I S-II

,

wherein each of R^{S1}, L, and R^1 are as described and defined above and herein.

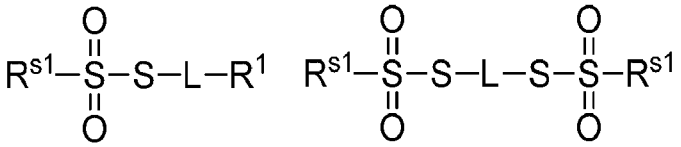
[00932] In some embodiments, the present invention provides a process for synthesizing a phosphorothioate triester, comprising steps of:

i) reacting an H-phosphonate of structure:



wherein each of W, Y, and Z are as described and defined above and herein, with a silylating reagent to provide a silyloxyphosphonate; and

ii) reacting the silyloxyphosphonate with a sulfurization reagent of structure **S-I** or **S-II**:



S-I S-II ,

to provide a phosphorothiotriester.

[00933] In some embodiments, a selenium electrophile is used instead of a sulfurizing reagent to introduce modification to the internucleotidic linkage. In some embodiments, a selenium electrophile is a compound having one of the following formulae:

Se, R^{s2}–Se–Se– R^{s3}, or R^{s2}–Se–X^s–R^{s3},

wherein:

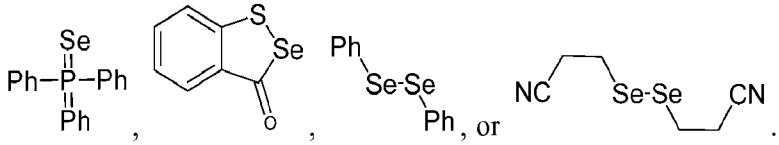
each of R^{s2} and R^{s3} is independently an optionally substituted group selected from aliphatic, aminoalkyl, carbocyclyl, heterocyclyl, heterocycloalkyl, aryl, heteroaryl, alkyloxy, aryloxy, heteroaryloxy, acyl, amide, imide, or thiocarbonyl; or

R^{s2} and R^{s3} are taken together with the atoms to which they are bound to form an optionally substituted heterocyclic or heteroaryl ring;

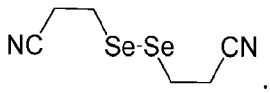
X^s is –$S(O)_2$–, –O–, or –N(R')–; and

R' is as defined and described above and herein.

[00934] In other embodiments, the selenium electrophile is a compound of Se, KSeCN,



. In some embodiments, the selenium electrophile is Se or



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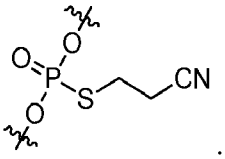
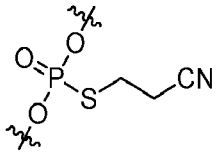
[00935] In some embodiments, a sulfurization reagent for use in accordance with the present invention is characterized in that the moiety transferred to phosphorus during sulfurization is a substituted sulfur (e.g., -SR) as opposed to a single sulfur atom (e.g., $-S^-$ or =S).

[00936] In some embodiments, a sulfurization reagent for use in accordance with the present invention is characterized in that the activity of the reagent is tunable by modifying the reagent with a certain electron withdrawing or donating group.

[00937] In some embodiments, a sulfurization reagent for use in accordance with the present invention is characterized in that it is crystalline. In some embodiments, a sulfurization reagent for use in accordance with the present invention is characterized in that it has a high degree of crystallinity. In certain embodiments, a sulfurization reagent for use in accordance with the present invention is characterized by ease of purification of the reagent via, e.g., recrystallization. In certain embodiments, a sulfurization reagent for use in accordance with the present invention is characterized in that it is substantially free from sulfur-containing impurities. In some embodiments, sulfurization reagents which are substantially free from sulfur-containing impurities show increased efficiency.

[00938] In some embodiments, the provided chirally controlled oligonucleotide comprises one or more phosphate diester linkages. To synthesize such chirally controlled oligonucleotides, one or more modifying steps are optionally replaced with an oxidation step to install the corresponding phosphate diester linkages. In some embodiments, the oxidation step is performed in a fashion similar to ordinary oligonucleotide synthesis. In some embodiments, an oxidation step comprises the use of I_2. In some embodiments, an oxidation step comprises the use of I_2 and pyridine. In some embodiments, an oxidation step comprises the use of 0.02 M I_2 in a THF/pyridine/water (70:20:10 – v/v/v) co-solvent system. An exemplary cycle is depicted in Scheme I-c.

[00939] In some embodiments, a phosphorothioate precursor is used to synthesize chirally controlled oligonucleotides comprising phosphorothioate linkages. In some embodiments, such a phosphorothioate precursor is . In some embodiments,  is converted into phosphorothioate diester linkages during standard deprotection/release procedure after cycle exit. Examples are further depicted below.

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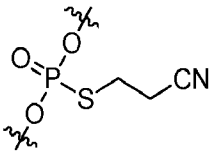
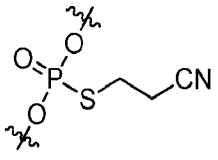
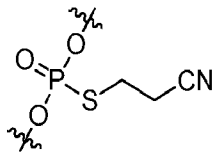
[00940] In some embodiments, the provided chirally controlled oligonucleotide comprises one or more phosphate diester linkages and one or more phosphorothioate diester linkages. In some embodiments, the provided chirally controlled oligonucleotide comprises one or more phosphate diester linkages and one or more phosphorothioate diester linkages, wherein at least one phosphate diester linkage is installed after all the phosphorothioate diester linkages when synthesized from 3' to 5'. To synthesize such chirally controlled oligonucleotides, in some embodiments, one or more modifying steps are optionally replaced with an oxidation step to install the corresponding phosphate diester linkages, and a phosphorothioate precursor is installed for each of the phosphorothioate diester linkages. In some embodiments, a phosphorothioate precursor is converted to a phosphorothioate diester linkage after the desired oligonucleotide length is achieved. In some embodiments, the deprotection/release step during or after cycle exit converts the phosphorothioate precursors into phosphorothioate diester linkages. In some embodiments, a phosphorothioate precursor is characterized in that it has the ability to be removed by a beta-elimination pathway. In some embodiments, a phosphorothioate precursor is . As understood by one of ordinary skill in the art, one of the benefits of using a phosphorothioate precursor, for instance, , during synthesis is that  is more stable than phosphorothioate in certain conditions.

[00941] In some embodiments, a phosphorothioate precursor is a phosphorus protecting group as described herein, e.g., 2-cyanoethyl (CE or Cne), 2-trimethylsilylethyl, 2-nitroethyl, 2-sulfonylethyl, methyl, benzyl, *o*-nitrobenzyl, 2-(*p*-nitrophenyl)ethyl (NPE or Npe), 2-phenylethyl, 3-(*N*-*tert*-butylcarboxamido)-1-propyl, 4-oxopentyl, 4-methylthio-1-butyl, 2-cyano-1,1-dimethylethyl, 4-*N*-methylaminobutyl, 3-(2-pyridyl)-1-propyl, 2-[*N*-methyl-*N*-(2-pyridyl)]aminoethyl, 2-(*N*-formyl,*N*-methyl)aminoethyl, 4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl. Examples are further depicted below.

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[00942] Methods for synthesizing a desired sulfurization reagent are described herein and in the examples section.

[00943] As noted above, in some embodiments, sulfurization occurs under conditions which cleave the chiral reagent from the growing oligonucleotide. In some embodiments, sulfurization occurs under conditions which do not cleave the chiral reagent from the growing oligonucleotide.

[00944] In some embodiments, a sulfurization reagent is dissolved in a suitable solvent and delivered to the column. In certain embodiments, the solvent is a polar aprotic solvent such as a nitrile solvent. In some embodiments, the solvent is acetonitrile. In some embodiments, a solution of sulfurization reagent is prepared by mixing a sulfurization reagent (e.g., a thiosulfonate derivative as described herein) with BSTFA (N,O-bis-trimethylsilyl-trifluoroacetamide) in a nitrile solvent (e.g., acetonitrile). In some embodiments, BSTFA is not included. For example, the present inventors have found that relatively more reactive sulfurization reagents of general formula R^{s2}–S–$S(O)_2$–R^{s3} can often successfully participate in sulfurization reactions in the absence of BSTFA. To give but one example, the inventors have demonstrated that where R^{s2} is *p*-nitrophenyl and R^{s3} is methyl then no BSTFA is required. In light of this disclosure, those skilled in the art will readily be able to determine other situations and/or sulfurization reagents that do not require BSTFA.

[00945] In some embodiments, the sulfurization step is performed at room temperature. In some embodiments, the sulfurization step is performed at lower temperatures such as about 0 °C, about 5 °C, about 10 °C, or about 15 °C. In some embodiments, the sulfurization step is performed at elevated temperatures of greater than about 20 °C.

[00946] In some embodiments, a sulfurization reaction is run for about 1 minute to about 120 minutes. In some embodiments, a sulfurization reaction is run for about 1 minute to about 90 minutes. In some embodiments, a sulfurization reaction is run for about 1 minute to about 60 minutes. In some embodiments, a sulfurization reaction is run for about 1 minute to about 30 minutes. In some embodiments, a sulfurization reaction is run for about 1 minute to about 25 minutes. In some embodiments, a sulfurization reaction is run for about 1 minute to about 20 minutes. In some embodiments, a sulfurization reaction is run for about 1 minute to about 15 minutes. In some embodiments, a sulfurization reaction is run for about 1 minute to about 10

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minutes. In some embodiments, a sulfurization reaction is run for about 5 minute to about 60 minutes.

[00947] In some embodiments, a sulfurization reaction is run for about 5 minutes. In some embodiments, a sulfurization reaction is run for about 10 minutes. In some embodiments, a sulfurization reaction is run for about 15 minutes. In some embodiments, a sulfurization reaction is run for about 20 minutes. In some embodiments, a sulfurization reaction is run for about 25 minutes. In some embodiments, a sulfurization reaction is run for about 30 minutes. In some embodiments, a sulfurization reaction is run for about 35 minutes. In some embodiments, a sulfurization reaction is run for about 40 minutes. In some embodiments, a sulfurization reaction is run for about 45 minutes. In some embodiments, a sulfurization reaction is run for about 50 minutes. In some embodiments, a sulfurization reaction is run for about 55 minutes. In some embodiments, a sulfurization reaction is run for about 60 minutes.

[00948] It was unexpectedly found that certain of the sulfurization modification products made in accordance with methods of the present invention are unexpectedly stable. In some embodiments, it the unexpectedly stable products are phosphorothioate triesters. In some embodiments, the unexpectedly stable products are chirally controlled oligonucleotides comprising one or more internucleotidic linkages having the structure of formula I-c.

[00949] One of skill in the relevant arts will recognize that sulfurization methods described herein and sulfurization reagents described herein are also useful in the context of modifying H-phosphonate oligonucleotides such as those described in Wada II (WO2010/064146).

[00950] In some embodiments, the sulfurization reaction has a stepwise sulfurization efficiency that is at least about 80%, 85%, 90%, 95%, 96%, 97%, or 98%. In some embodiments, the sulfurization reaction provides a crude dinucleotide product compositon that is at least 98% pure. In some embodiments, the sulfurization reaction provides a crude tetranucleotide product compositon that is at least 90% pure. In some embodiments, the sulfurization reaction provides a crude dodecanucleotide product compositon that is at least 70% pure. In some embodiments, the sulfurization reaction provides a crude icosanucleotide product compositon that is at least 50% pure.

[00951] Once the step of modifying the linkage phosphorus is complete, the oligonucleotide undergoes another deblock step in preparation for re-entering the cycle. In some

embodiments, a chiral auxiliary remains intact after sulfurization and is deblocked during the subsequent deblock step, which necessarily occurs prior to re-entering the cycle. The process of deblocking, coupling, capping, and modifying, are repeated until the growing oligonucleotide reaches a desired length, at which point the oligonucleotide can either be immediately cleaved from the solid support or left attached to the support for purification purposes and later cleaved. In some embodiments, one or more protecting groups are present on one or more of the nucleotide bases, and cleaveage of the oligonucleotide from the support and deprotection of the bases occurs in a single step. In some embodiments, one or more protecting groups are present on one or more of the nucleotide bases, and cleaveage of the oligonucleotide from the support and deprotection of the bases occurs in more than one steps. In some embodiments, deprotection and cleavage from the support occurs under basic conditions using, e.g., one or more amine bases. In certain embodiments, the one or more amine bases comprise propyl amine. In certain embodiments, the one or more amine bases comprise pyridine.

[00952] In some embodiments, cleavage from the support and/or deprotection occurs at elevated temperatures of about 30 °C to about 90 °C. In some embodiments, cleavage from the support and/or deprotection occurs at elevated temperatures of about 40 °C to about 80 °C. In some embodiments, cleavage from the support and/or deprotection occurs at elevated temperatures of about 50 °C to about 70 °C. In some embodiments, cleavage from the support and/or deprotection occurs at elevated temperatures of about 60 °C. In some embodiments, cleavage from the support and/or deprotection occurs at ambient temperatures.

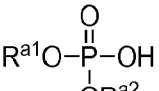
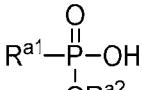
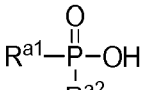
[00953] Exemplary purification procedures are described herein and/or are known generally in the relevant arts.

[00954] Noteworthy is that the removal of the chiral auxiliary from the growing oligonucleotide during each cycle is beneficial for at least the reasons that (1) the auxiliary will not have to be removed in a separate step at the end of the oligonucleotide synthesis when potentially sensitive functional groups are installed on phosphorus; and (2) unstable phosphorus-auxiliary intermediates prone to undergoing side reactions and/or interfering with subsequent chemistry are avoided. Thus, removal of the chiral auxiliary during each cycle makes the overall synthesis more efficient.

[00955] While the step of deblocking in the context of the cycle is described above, additional general methods are included below.

Deblocking Step

[00956] In some embodiments, the step of coupling is preceded by a step of deblocking. For instance, in some embodiments, the 5' hydroxyl group of the growing oligonucleotide is blocked (i.e., protected) and must be deblocked in order to subsequently react with a nucleoside coupling partner.

[00957] In some embodiments, acidification is used to remove a blocking group. In some embodiments, the acid is a Brønsted acid or Lewis acid. Useful Brønsted acids are carboxylic acids, alkylsulfonic acids, arylsulfonic acids, phosphoric acid and its derivatives, phosphonic acid and its derivatives, alkylphosphonic acids and their derivatives, arylphosphonic acids and their derivatives, phosphinic acid, dialkylphosphinic acids, and diarylphosphinic acids which have a pKa (25 °C in water) value of -0.6 (trifluoroacetic acid) to 4.76 (acetic acid) in an organic solvent or water (in the case of 80% acetic acid). The concentration of the acid (1 to 80%) used in the acidification step depends on the acidity of the acid. Consideration to the acid strength must be taken into account as strong acid conditions will result in depurination/depyrimidination, wherein purinyl or pyrimidinyl bases are cleaved from ribose ring and or other sugar ring. In some embodiments, an acid is selected from $R^{a1}COOH$, $R^{a1}SO_3H$, $R^{a3}SO_3H$, , , , or , wherein each of R^{a1} and R^{a2} is independently hydrogen or an optionally substituted alkyl or aryl, and R^{a3} is an optionally substituted alkyl or aryl.

[00958] In some embodiments, acidification is accomplished by a Lewis acid in an organic solvent. Exemplary such useful Lewis acids are $Zn(X^a)_2$ wherein X^a is Cl, Br, I, or CF_3SO_3.

[00959] In some embodiments, the step of acidifying comprises adding an amount of a Brønsted or Lewis acid effective to remove a blocking group without removing purine moieties from the condensed intermediate.

[00960] Acids that are useful in the acidifying step also include, but are not limited to 10% phosphoric acid in an organic solvent, 10% hydrochloric acid in an organic solvent, 1% trifluoroacetic acid in an organic solvent, 3% dichloroacetic acid or trichloroacetic acid in an

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organic solvent or 80% acetic acid in water. The concentration of any Brønsted or Lewis acid used in this step is selected such that the concentration of the acid does not exceed a concentration that causes cleavage of a nucleobase from a sugar moiety.

[00961] In some embodiments, acidification comprises adding 1% trifluoroacetic acid in an organic solvent. In some embodiments, acidification comprises adding about 0.1% to about 8% trifluoroacetic acid in an organic solvent. In some embodiments, acidification comprises adding 3% dichloroacetic acid or trichloroacetic acid in an organic solvent. In some embodiments, acidification comprises adding about 0.1% to about 10% dichloroacetic acid or trichloroacetic acid in an organic solvent. In some embodiments, acidification comprises adding 3% trichloroacetic acid in an organic solvent. In some embodiments, acidification comprises adding about 0.1% to about 10% trichloroacetic acid in an organic solvent. In some embodiments, acidification comprises adding 80% acetic acid in water. In some embodiments, acidification comprises adding about 50% to about 90%, or about 50% to about 80%, about 50% to about 70%, about 50% to about 60%, about 70% to about 90% acetic acid in water. In some embodiments, the acidification comprises the further addition of cation scavengers to an acidic solvent. In certain embodiments, the cation scavengers can be triethylsilane or triisopropylsilane. In some embodiments, a blocking group is deblocked by acidification, which comprises adding 1% trifluoroacetic acid in an organic solvent. In some embodiments, a blocking group is deblocked by acidification, which comprises adding 3% dichloroacetic acid in an organic solvent. In some embodiments, a blocking group is deblocked by acidification, which comprises adding 3% trichloroacetic acid in an organic solvent. In some embodiments, a blocking group is deblocked by acidification, which comprises adding 3% trichloroacetic acid in dichloromethane.

[00962] In certain embodiments, methods of the present invention are completed on a synthesizer and the step of deblocking the hydroxyl group of the growing oligonucleotide comprises delivering an amount solvent to the synthesizer column, which column contains a solid support to which the oligonucleotide is attached. In some embodiments, the solvent is a halogenated solvent (e.g., dichloromethane). In certain embodiments, the solvent comprises an amount of an acid. In some embodiments, the solvent comprises an amount of an organic acid such as, for instance, trichloroacetic acid. In certain embodiments, the acid is present in an amount of about 1% to about 20% w/v. In certain embodiments, the acid is present in an amount

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of about 1% to about 10% w/v. In certain embodiments, the acid is present in an amount of about 1% to about 5% w/v. In certain embodiments, the acid is present in an amount of about 1 to about 3% w/v. In certain embodiments, the acid is present in an amount of about 3% w/v. Methods for deblocking a hydroxyl group are described further herein. In some embodiments, the acid is present in 3% w/v is dichloromethane.

[00963] In some embodiments, the chiral auxiliary is removed before the deblocking step. In some embodiments, the chiral auxiliary is removed during the deblocking step.

[00964] In some embodiments, cycle exit is performed before the deblocking step. In some embodiments, cycle exit is preformed after the deblocking step.

General conditions for blocking group/protecting group removal

[00965] Functional groups such as hydroxyl or amino moieties which are located on nucleobases or sugar moieties are routinely blocked with blocking (protecting) groups (moieties) during synthesis and subsequently deblocked. In general, a blocking group renders a chemical functionality of a molecule inert to specific reaction conditions and can later be removed from such functionality in a molecule without substantially damaging the remainder of the molecule (see *e.g.*, Green and Wuts, Protective Groups in Organic Synthesis, 2nd Ed., John Wiley & Sons, New York, 1991). For example, amino groups can be blocked with nitrogen blocking groups such as phthalimido, 9-fludrenylmethoxycarbonyl (FMOC), triphenylmethylsulfenyl, *t*-BOC, 4,4'-dimethoxytrityl (DMTr), 4-methoxytrityl (MMTr), 9-phenylxanthin-9-yl (Pixyl), trityl (Tr), or 9-(*p*-methoxyphenyl)xanthin-9-yl (MOX). Carboxyl groups can be protected as acetyl groups. Hydroxy groups can be protected such as tetrahydropyranyl (THP), *t*-butyldimethylsilyl (TBDMS), 1-[(2-chloro-4-methyl)phenyl]-4-methoxypiperidin-4-yl (Ctmp), 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl (Fpmp), 1-(2-chloroethoxy)ethyl, 3-methoxy-1,5-dicarbomethoxypentan-3-yl (MDP), bis(2-acetoxyethoxy)methyl (ACE), triisopropylsilyloxymethyl (TOM), 1-(2-cyanoethoxy)ethyl (CEE), 2-cyanoethoxymethyl (CEM), [4-(*N*-dichloroacetyl-*N*-methylamino)benzyloxy]methyl, 2-cyanoethyl (CN), pivaloyloxymethyl (PivOM), levunyloxymethyl (ALE). Other representative hydroxyl blocking groups have been described (see *e.g.*, Beaucage *et al.*, *Tetrahedron*, **1992**, *46*, 2223). In some embodiments, hydroxyl blocking groups are acid-labile groups, such as the trityl, monomethoxytrityl, dimethoxytrityl, trimethoxytrityl, 9-phenylxanthin-9-yl (Pixyl) and 9-(p-

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methoxyphenyl)xanthin-9-yl (MOX). Chemical functional groups can also be blocked by including them in a precursor form. Thus an azido group can be considered a blocked form of an amine as the azido group is easily converted to the amine. Further representative protecting groups utilized in nucleic acid synthesis are known (see e.g. Agrawal et al., Protocols for Oligonucleotide Conjugates, Eds., Humana Press, New Jersey, 1994, Vol. 26, pp. 1-72).

[00966] Various methods are known and used for removal of blocking groups from nucleic acids. In some embodiments, all blocking groups are removed. In some embodiments, a portion of blocking groups are removed. In some embodiments, reaction conditions can be adjusted to selectively remove certain blocking groups.

[00967] In some embodiments, nucleobase blocking groups, if present, are cleavable with an acidic reagent after the assembly of a provided oligonucleotide. In some embodiment, nucleobase blocking groups, if present, are cleavable under neither acidic nor basic conditions, *e.g.* cleavable with fluoride salts or hydrofluoric acid complexes. In some embodiments, nucleobase blocking groups, if present, are cleavable in the presence of base or a basic solvent after the assembly of a provided oligonucleotide. In certain embodiments, one or more of the nucleobase blocking groups are characterized in that they are cleavable in the presence of base or a basic solvent after the assembly of a provided oligonucleotide but are stable to the particular conditions of one or more earlier deprotection steps occurring during the assembly of the provided oligonucleotide.

[00968] In some embodiments, blocking groups for nucleobases are not required. In some embodiments, blocking groups for nucleobases are required. In some embodiments, certain nucleobases require one or more blocking groups while other nucleobases do not require one or more blocking groups.

[00969] In some embodiments, the oligonucleotide is cleaved from the solid support after synthesis. In some embodiments, cleavage from the solid support comprises the use of propylamine. In some embodiments, cleavage from the solid support comprises the use of propylamine in pyridine. In some embodiments, cleavage from the solid support comprises the use of 20% propylamine in pyridine. In some embodiments, cleavage from the solid support comprises the use of propylamine in anhydrous pyridine. In some embodiments, cleavage from the solid support comprises the use of 20% propylamine in anhydrous pyridine. In some embodiments, cleavage from the solid support comprises use of a polar aprotic solvent such as

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acetonitrile, NMP, DMSO, sulfone, and/or lutidine. In some embodiments, cleavage from the solid support comprises use of solvent, e.g., a polar aprotic solvent, and one or more primary amines (e.g., a C_{1-10} amine), and/or one or more of methoxylamine, hydrazine, and pure anhydrous ammonia.

[00970] In some embodiments, deprotection of oligonucleotide comprises the use of propylamine. In some embodiments, deprotection of oligonucleotide comprises the use of propylamine in pyridine. In some embodiments, deprotection of oligonucleotide comprises the use of 20% propylamine in pyridine. In some embodiments deprotection of oligonucleotide comprises the use of propylamine in anhydrous pyridine. In some embodiments, deprotection of oligonucleotide comprises the use of 20% propylamine in anhydrous pyridine.

[00971] In some embodiments, the oligonucleotide is deprotected during cleavage.

[00972] In some embodiments, cleavage of oligonucleotide from solid support, or deprotection of oligonucleotide, is performed at about room temperature. In some embodiments, cleavage of oligonucleotide from solid support, or deprotection of oligonucleotide, is performed at elevated temperature. In some embodiments, cleavage of oligonucleotide from solid support, or deprotection of oligonucleotide, is performed at above about 30 °C, 40 °C, 50 °C, 60 °C, 70°C, 80 °C 90 °C or 100 °C. In some embodiments, cleavage of oligonucleotide from solid support, or deprotection of oligonucleotide, is performed at about 30 °C, 40 °C, 50 °C, 60 °C, 70°C, 80 °C 90 °C or 100 °C. In some embodiments, cleavage of oligonucleotide from solid support, or deprotection of oligonucleotide, is performed at about 40-80 °C. In some embodiments, cleavage of oligonucleotide from solid support, or deprotection of oligonucleotide, is performed at about 50-70 °C. In some embodiments, cleavage of oligonucleotide from solid support, or deprotection of oligonucleotide, is performed at about 60 °C.

[00973] In some embodiments, cleavage of oligonucleotide from solid support, or deprotection of oligonucleotide, is performed for more than 0.1 hr, 1 hr, 2 hrs, 5 hrs, 10 hrs, 15 hrs, 20 hrs, 24 hrs, 30 hrs, or 40 hrs. In some embodiments, cleavage of oligonucleotide from solid support, or deprotection of oligonucleotide, is performed for about 0.1-5 hrs. In some embodiments, cleavage of oligonucleotide from solid support, or deprotection of oligonucleotide, is performed for about 3-10 hrs. In some embodiments, cleavage of oligonucleotide from solid support, or deprotection of oligonucleotide, is performed for about 5-15 hrs. In some embodiments, cleavage of oligonucleotide from solid support, or deprotection of oligonucleotide,

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is performed for about 10-20 hrs. In some embodiments, cleavage of oligonucleotide from solid support, or deprotection of oligonucleotide, is performed for about 15-25 hrs. In some embodiments, cleavage of oligonucleotide from solid support, or deprotection of oligonucleotide, is performed for about 20-40 hrs. In some embodiments, cleavage of oligonucleotide from solid support, or deprotection of oligonucleotide, is performed for about 2 hrs. In some embodiments, cleavage of oligonucleotide from solid support, or deprotection of oligonucleotide, is performed for about 5 hrs. In some embodiments, cleavage of oligonucleotide from solid support, or deprotection of oligonucleotide, is performed for about 10 hrs. In some embodiments, cleavage of oligonucleotide from solid support, or deprotection of oligonucleotide, is performed for about 15 hrs. In some embodiments, cleavage of oligonucleotide from solid support, or deprotection of oligonucleotide, is performed for about 18 hrs. In some embodiments, cleavage of oligonucleotide from solid support, or deprotection of oligonucleotide, is performed for about 24 hrs.

[00974] In some embodiments, cleavage of oligonucleotide from solid support, or deprotection of oligonucleotide, is performed at room temperature for more than 0.1 hr, 1 hr, 2 hrs, 5 hrs, 10 hrs, 15 hrs, 20 hrs, 24 hrs, 30 hrs, or 40 hrs. In some embodiments, cleavage of oligonucleotide from solid support, or deprotection of oligonucleotide, is performed at room temperature for about 5-48 hrs. In some embodiments, cleavage of oligonucleotide from solid support, or deprotection of oligonucleotide, is performed at room temperature for about 10-24 hrs. In some embodiments, cleavage of oligonucleotide from solid support, or deprotection of oligonucleotide, is performed at room temperature for about 18 hrs. In some embodiments, cleavage of oligonucleotide from solid support, or deprotection of oligonucleotide, is performed at elevated temperature for more than 0.1 hr, 1 hr, 2 hrs, 5 hrs, 10 hrs, 15 hrs, 20 hrs, 24 hrs, 30 hrs, or 40 hrs. In some embodiments, cleavage of oligonucleotide from solid support, or deprotection of oligonucleotide, is performed at elevated temperature for about 0.5-5 hrs. In some embodiments, cleavage of oligonucleotide from solid support, or deprotection of oligonucleotide, is performed at about 60 °C for about 0.5-5 hrs. In some embodiments, cleavage of oligonucleotide from solid support, or deprotection of oligonucleotide, is performed at about 60 °C for about 2 hrs.

[00975] In some embodiments, cleavage of oligonucleotide from solid support, or deprotection of oligonucleotide comprises the use of propylamine and is performed at room

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temperature or elevated temperature for more than 0.1 hr, 1 hr, 2 hrs, 5 hrs, 10 hrs, 15 hrs, 20 hrs, 24 hrs, 30 hrs, or 40 hrs. Exemplary conditions are 20% propylamine in pyridine at room temperature for about 18 hrs, and 20% propylamine in pyridine at 60 °C for about 18 hrs,

[00976] In some embodiments, the present invention provides methods for making a chirally controlled oligonucleotide comprising steps of:

(1) coupling;

(2) capping;

(3) modifying;

(4) deblocking; and

(5) repeating steps (1) – (4) until a desired length is achieved;

wherein the chirally controlled oligonucleotide comprises at least one phosphorothioate diester linkage or at least one internucleotidic linkage having the structure of formula I-c.

[00977] In some embodiments, the present invention provides methods for making a chirally controlled oligonucleotide comprising steps of:

(1) coupling;

(2) capping;

(3) modifying;

(4) deblocking; and

(5) repeating steps (1) – (4) until a desired length is achieved;

wherein:

at least one cycle of (1) to (4) forms an phosphorothioate diester linkage.

[00978] In some embodiments, the present invention provides methods for making a chirally controlled oligonucleotide comprising steps of:

(1) coupling;

(2) capping;

(3) modifying;

(4) deblocking; and

(5) repeating steps (1) – (4) until a desired length is achieved;

wherein:

at least one cycle of (1) to (4) forms an internucleotidic linkage having the structure of formula I-c.

[00979] In some embodiments, the present invention provides methods for making a chirally controlled oligonucleotide comprising steps of:

(1) coupling;

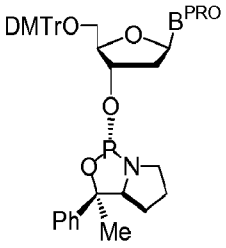
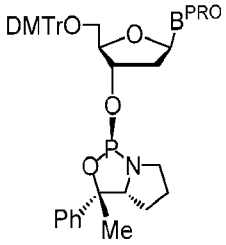
(2) capping;

(3) modifying;

(4) deblocking; and

(5) repeating steps (1) – (4) until a desired length is achieved;

wherein:

the coupling step comprises the use of an activating group and  or , wherein B^{PRO} is a protected nucleobase;

the capping step comprising capping of the amino group in the chiral auxiliary and the capping of unreacted 5'–OH;

the modifying step comprising installation of –S–L–R^1 group to the linkage phosphorus, wherein each of L and R^1 is independently as defined above and described herein;

the delocking step comprising the use of an acid.

[00980] In some embodiments, the present invention provides methods for making a chirally controlled oligonucleotide comprising steps of:

(1) coupling;

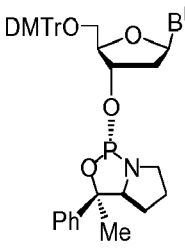
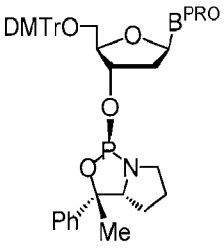
(2) capping;

(3) modifying;

(4) deblocking; and

(5) repeating steps (1) – (4) until a desired length is achieved;

wherein:

the coupling step comprises the use of CMPT and  or ,
wherein B^{PRO} is a protected nucleobase;

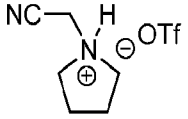
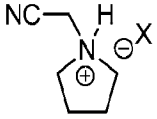
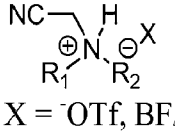
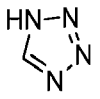
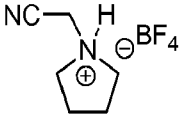
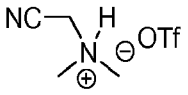
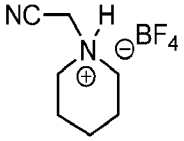
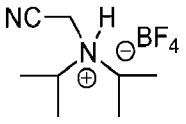
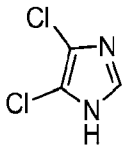
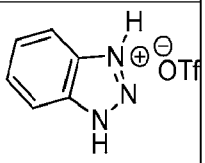
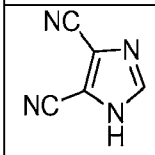
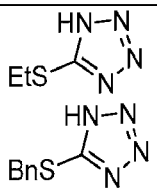
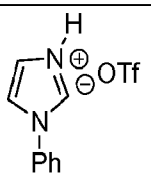
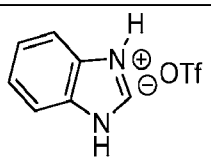
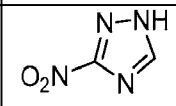
the capping step comprising capping of the amino group in the chiral auxiliary and the capping of unreacted 5'–OH;

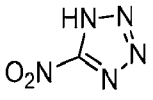
the modifying step comprising installation of –S–L–R^1 group to the linkage phosphorus, wherein each of L and R^1 is independently as defined above and described herein;

the delocking step comprising the use of an acid.

[00981] In some embodiments, an activator is a "Wada" activator, i.e., the activator is from any one of Wada I, II, or III documents cited above.

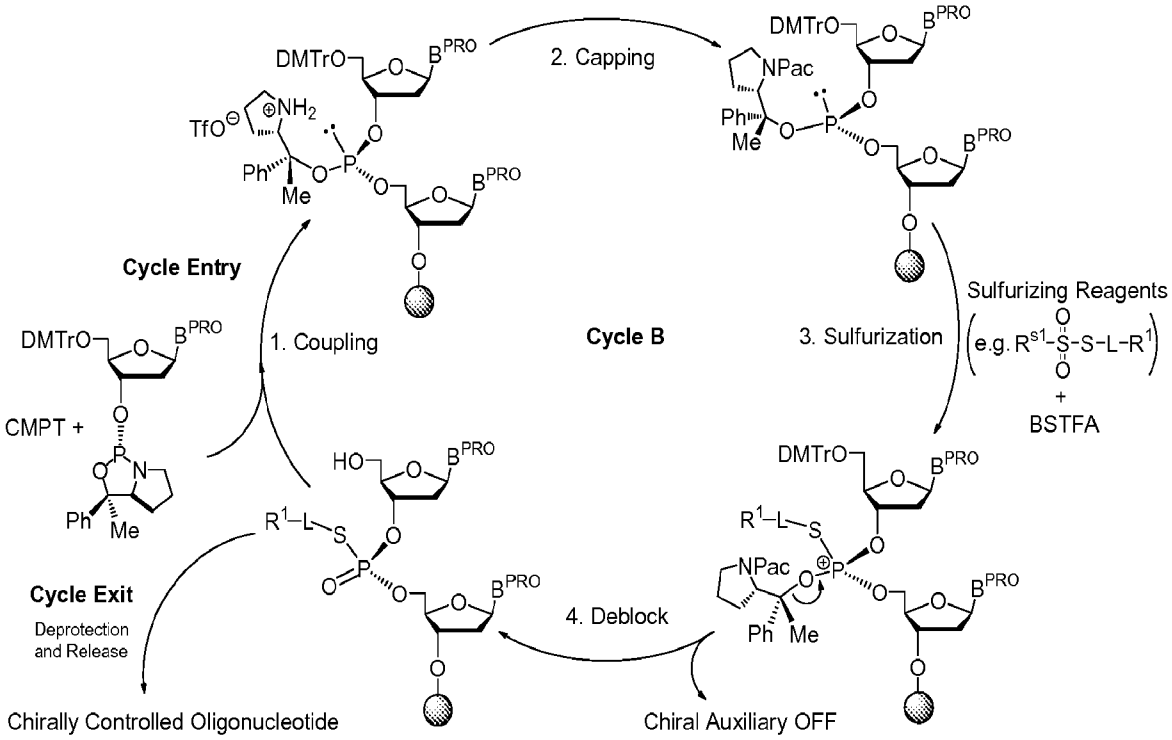
[00982] Exemplary activating groups are depicted below:

	 X = non nucleophilic anion	 X = $^-$OTf, BF_4^-, PF6$^-$, TfN$^-$		
				
				

[00983] An exemplary cycle is depicted in Scheme I-b.

Scheme I-b. Installation of phosphorothioate linkages.



[00984] In some embodiments, the present invention provides methods for making a chirally controlled oligonucleotide comprising steps of:

(1) coupling;

(2) capping;

(3) modifying;

(4) deblocking; and

(5) repeating steps (1) – (4) until a desired length is achieved;

wherein:

the chirally controlled oligonucleotide comprises at least one phosphorothioate diester linkage or at least one internucleotidic linkage of formula I-c, and at least one phosphate diester internucleotidic linkage; and

at least one modifying step is replaced by an oxidaztion step.

[00985] In some embodiments, the present invention provides methods for making a chirally controlled oligonucleotide comprising steps of:

(1) coupling;

(2) capping;

(3) modifying;

(4) deblocking; and

(5) repeating steps (1) – (4) until a desired length is achieved;

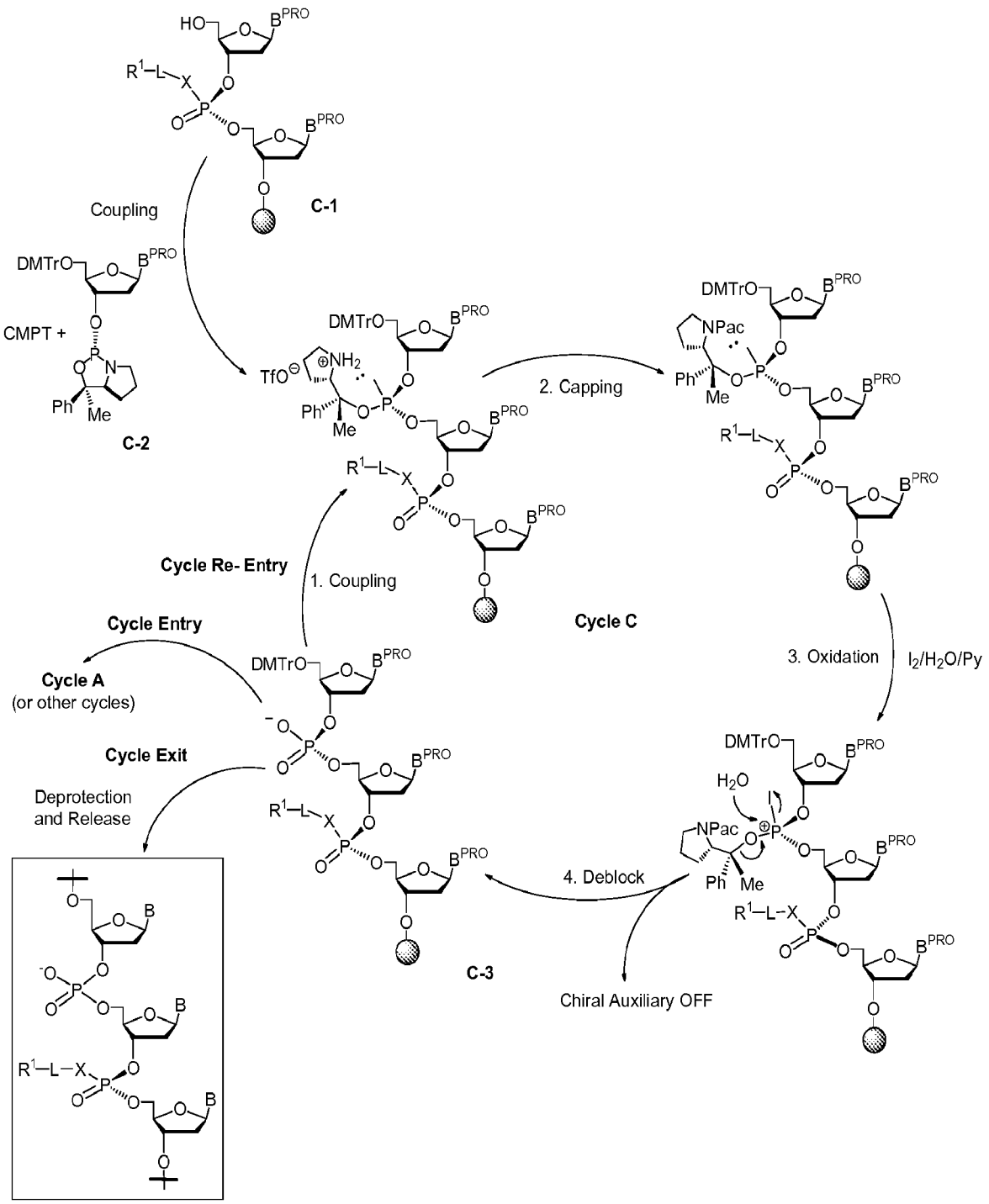
wherein:

the chirally controlled oligonucleotide comprises at least one phosphorothioate diester linkage or at least one internucleotidic linkage of formula I-c, and at least one phosphate diester internucleotidic linkage; and

at least one modifying step is replaced by an oxidaztion step comprising the use of I_2.

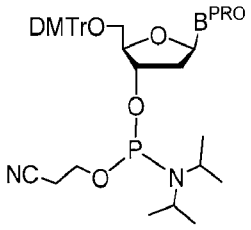
[00986] An exemplary cycle is illustrated in Scheme I-c.

Scheme I-c. Installation of both phosphate diester and modified internucleotidic linkages in a chirally controlled oligonucleotide.



[00987] In Scheme I-c, oligonucleotide (or nucleotide, or oligonucleotide with modified internucleotidic linkage) on solid support (C-1) is coupled with phosphoramidite C-2. After coupling and capping, an oxidation step is performed. After deblocking, a phosphate diester linkage is formed. The cycle product C-3 can either re-enter cycle C to install more phosphate diester linkage, or enter other cycles to install other types of internucleotidic linkages, or go to cycle exit.

[00988] In some embodiments, non-chirally pure phosphoramidite can be used instead of C-2 in Scheme I-c. In some embodiments, β-cyanoethylphosphoramidites protected with DMTr is used. In some embodiments, the phosphoramidite being used has the structure of



[00989] In some embodiments, the present invention provides methods for making a chirally controlled oligonucleotide comprising steps of:

(1) coupling;

(2) capping;

(3) modifying;

(4) deblocking; and

(5) repeating steps (1) – (4) until a desired length is achieved;

wherein:

the chirally controlled oligonucleotide comprises one or more phosphorothioate diester linkages; and

one or more phosphorothioate diester precursors are formed for each of the corresponding phosphorothioate diester linkage.

[00990] In some embodiments, the present invention provides methods for making a chirally controlled oligonucleotide comprising steps of:

(1) coupling;

(2) capping;

(3) modifying;

(4) deblocking; and

(5) repeating steps (1) – (4) until a desired length is achieved;

wherein:

the chirally controlled oligonucleotide comprises at least one phosphorothioate diester linkages;

one or more phosphorothioate diester precursors are formed for each of the corresponding phosphorothioate diester linkage; and

each phosphorothioate diester precursor is converted to a phosphorothioate diester linkage after the desired length is achieved.

[00991] In some embodiments, the present invention provides methods for making a chirally controlled oligonucleotide comprising steps of:

(1) coupling;

(2) capping;

(3) modifying;

(4) deblocking; and

(5) repeating steps (1) – (4) until a desired length is achieved;

wherein:

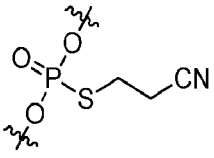
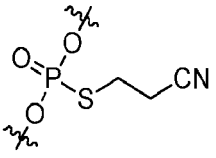
the chirally controlled oligonucleotide comprises at least one phosphorothioate diester linkages and at least one phosphate diester internucleotidic linkage;

at least one modifying step is replaced by an oxidization step; and

at least one modifying step is performed to install a phosphorothioate diester precursor for each of the phosphorothioate diester linkages; and

each phosphorothioate diester precursor is converted to a phosphorothioate diester linkage after the desired length is achieved.

[00992] In some embodiments, the use of a phosphorothioate diester precursor increases the stability of oligonucleotide during synthesis. In some embodiments, the use of a phosphorothioate diester precursor improves the efficiency of chirally controlled oligonucleotide synthesis. In some embodiments, the use of a phosphorothioate diester precursor improves the yield of chirally controlled oligonucleotide synthesis. In some embodiments, the use of a phosphorothioate diester precursor improves the product purity of chirally controlled oligonucleotide synthesis.

[00993] In some embodiments, the phosphorothioate diester precursor in the above-mentioned methods is . In some embodiments,  is converted to a phosphorothioate diester linkage during deprotection/release. An exemplary cycle is depicted in Scheme I-d. More examples are depicted below.

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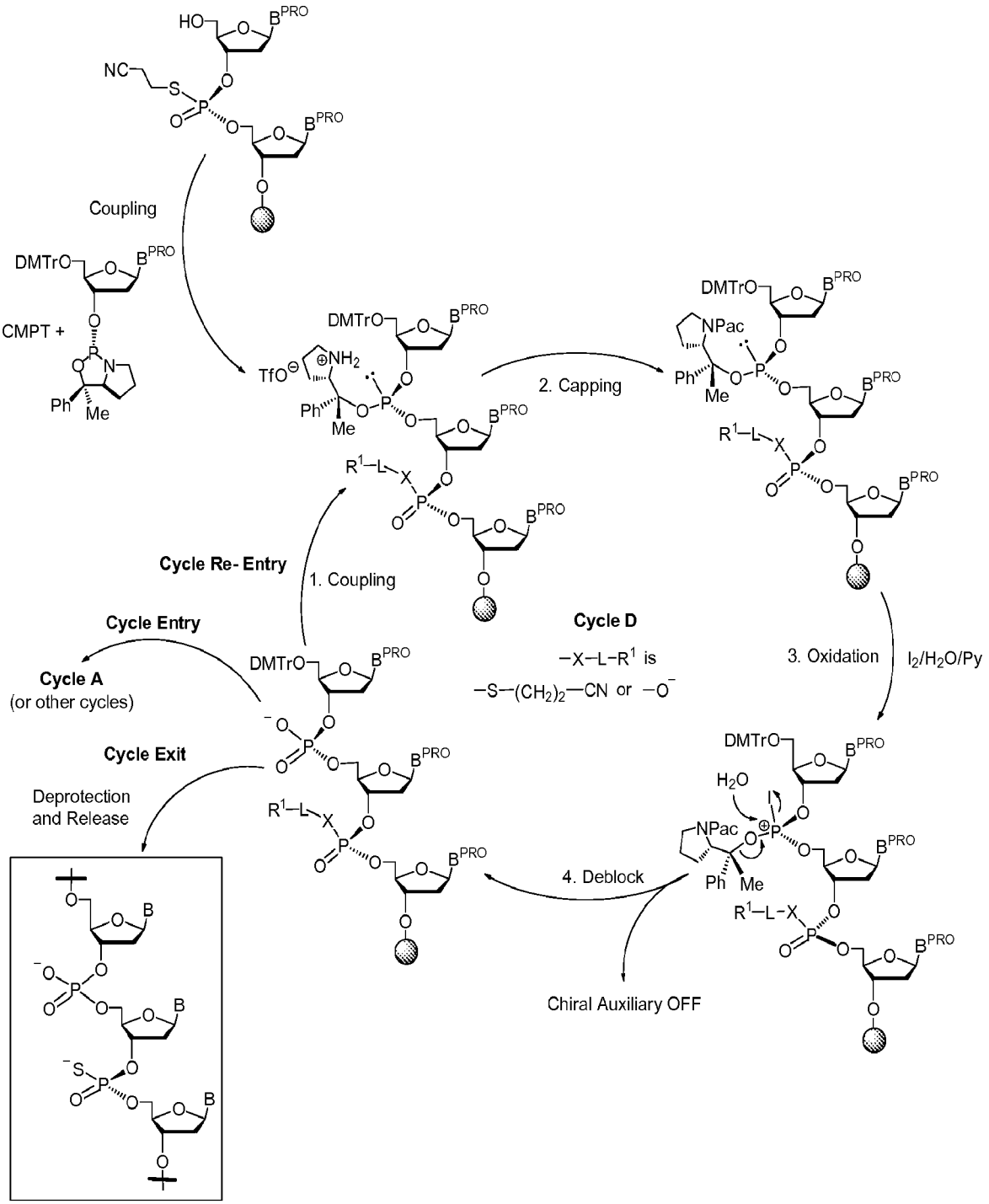
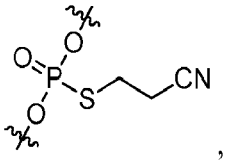
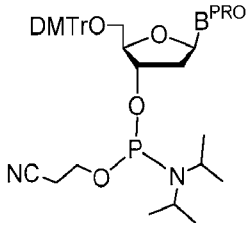
Scheme I-d. Phosphorothioate diester precursor in chirally controlled oligonucleotide synthesis.



[00994] As illustrated in Scheme I-d, both phosphorothioate and phosphate diester linkages can be incorporated into the same chirally controlled oligonucleotide. As understood by a person of ordinary skill in the art, the provided methods do not require that the phosphorothioate diester and the phosphate diester to be consecutive – other internucleotidic linkages can form between them using a cycle as described above. In Scheme I-d,

phosphorothioate diester precursors, , are installed in place of the phosphorothioate diester linkages. In some embodiments, such replacement provided increased synthesis efficiency during certain steps, for instance, the oxidation step. In some embodiments, the use of phosphorothioate diester precursors generally improve the stability of chirally controlled oligonucleotides during synthesis and/or storage. After cycle exit, during deprotection/release, the phosphorothioate diester precursor is converted to phosphorothioate diester linkage. In some embodiments, it is benefical to use phosphorothioate diester precursor even when no phosphate diester linkage is present in the chirally controlled oligonucleotide, or no oxidation step is required during synthesis.

[00995] As in Scheme I-c, in some embodiments, non-chirally pure phosphoramidite can be used for cycles comprising oxidation steps. In some embodiments, β-cyanoethylphosphoramidites protected with DMTr is used. In some embodiments, the phosphoramidite being used has the structure of 

[00996] In some embodiments, methods of the present invention provide chirally controlled oligonucleotide compositions that are enriched in a particular oligonucleotide type.

[00997] In some embodiments, at least about 10% of a provided crude composition is of a particular oligonucleotide type. In some embodiments, at least about 20% of a provided crude composition is of a particular oligonucleotide type. In some embodiments, at least about 30% of a provided crude composition is of a particular oligonucleotide type. In some embodiments, at least about 40% of a provided crude composition is of a particular oligonucleotide type. In some embodiments, at least about 50% of a provided crude composition is of a particular oligonucleotide type. In some embodiments, at least about 60% of a provided crude composition is of a particular oligonucleotide type. In some embodiments, at least about 70% of a provided crude composition is of a particular oligonucleotide type. In some embodiments, at least about 80% of a provided crude composition is of a particular oligonucleotide type. In some embodiments, at least about 90% of a provided crude composition is of a particular

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oligonucleotide type. In some embodiments, at least about 95% of a provided crude composition is of a particular oligonucleotide type.

[00998] In some embodiments, at least about 1% of a provided composition is of a particular oligonucleotide type. In some embodiments, at least about 2% of a provided composition is of a particular oligonucleotide type. In some embodiments, at least about 3% of a provided composition is of a particular oligonucleotide type. In some embodiments, at least about 4% of a provided composition is of a particular oligonucleotide type. In some embodiments, at least about 5% of a provided composition is of a particular oligonucleotide type. In some embodiments, at least about 10% of a provided composition is of a particular oligonucleotide type. In some embodiments, at least about 20% of a provided composition is of a particular oligonucleotide type. In some embodiments, at least about 30% of a provided composition is of a particular oligonucleotide type. In some embodiments, at least about 40% of a provided composition is of a particular oligonucleotide type. In some embodiments, at least about 50% of a provided composition is of a particular oligonucleotide type. In some embodiments, at least about 60% of a provided composition is of a particular oligonucleotide type. In some embodiments, at least about 70% of a provided composition is of a particular oligonucleotide type. In some embodiments, at least about 80% of a provided composition is of a particular oligonucleotide type. In some embodiments, at least about 90% of a provided composition is of a particular oligonucleotide type. In some embodiments, at least about 95% of a provided composition is of a particular oligonucleotide type.

Biological applications

[00999] As discussed in detail herein, the present invention provides, among other things, a chirally controlled oligonucleotide composition, meaning that the composition contains a plurality of oligonucleotides of at least one type. Each oligonucleotide molecule of a particular "type" is comprised of preselected (e.g., predetermined) structural elements with respect to: (1) base sequence; (2) pattern of backbone linkages; (3) pattern of backbone chiral centers; and (4) pattern of backbone P-modification moieties. In some embodiments, provided oligonucloetide compositions contain oligonucleotides that are prepared in a single synthesis process. In some embodiments, provided compositions contain oligonucloetides having more than one chiral configuration within a single oligonucleotide molecule (e.g., where different residues along the

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oligonucleotide have different stereochemistry); in some such embodiments, such oligonucleotides may be obtained in a single synthesis process, without the need for secondary conjugation steps to generate individual oligonucleotide molecules with more than one chiral configuration.

[001000] Oligonucleotide compositions as provided herein can be used as agents for modulating a number of cellular processes and machineries, including but not limited to, transcription, translation, immune responses, epigenetics, etc. In addition, oligonucleotide compositions as provided herein can be used as reagents for research and/or diagnostic purposes. One of ordinary skill in the art will readily recognize that the present invention disclosure herein is not limited to particular use but is applicable to any situations where the use of synthetic oligonucleitides is desirable. Among other things, provided compositions are useful in a variety of therapeutic, diagnostic, agricultural, and/or research applications.

[001001] In some embodiments, provided oligonucloetide compositions comprise oligonucleotides and/or residues thereof that include one or more structural modifications as described in detail herein. In some embodiments, provided oligonucleotide compositions comprise oligonucleoties that contain one or more nucleic acid analogs. In some embodiments, provided oligonucleotide compositions comprise oligonucleotides that contain one or more artificial nucleic acids or residues, including but not limited to: peptide nucleic acids (PNA), Morpholino and locked nucleic acids (LNA), glycon nucleic acids (GNA), threose nucleic acids (TNA), Xeno nucleic acids (ZNA), and any combination thereof.

[001002] In any of the embodiments, the invention is useful for oligonucleotide-based modulation of gene expression, immune response, etc. Accordingly, stereo-defined,oligonucleotide compositions of the invention, which contain oligonucleotides of predetermined type (i.e., which are chirally controlled, and optionally chirally pure), can be used in lieu of conventional stereo-random or chirally impure counterparts. In some embodiments, provided compositions show enhanced intended effects and/or reduced unwanted side effects. Certain embodimetns of biological and clinical/therapeutic applications of the invention are discussed explicitly below.

[001003] Various dosing regimens can be utilized to administer .provided chirally controlled oligonucleotide compositions. In some embodiments, multiple unit doses are administered, separated by periods of time. In some embodiments, a given composition has a

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recommended dosing regimen, which may involve one or more doses. In some embodiments, a dosing regimen comprises a plurality of doses each of which are separated from one another by a time period of the same length; in some embodiments, a dosing regimen comprises a plurality of doses and at least two different time periods separating individual doses. In some embodiments, all doses within a dosing regimen are of the same unit dose amount. In some embodiments, different doses within a dosing regimen are of different amounts. In some embodiments, a dosing regimen comprises a first dose in a first dose amount, followed by one or more additional doses in a second dose amount different from the first dose amount. In some embodiments, a dosing regimen comprises a first dose in a first dose amount, followed by one or more additional doses in a second (or subsequent) dose amount that is same as or different from the first dose (or another prior dose) amount. In some embodiments, a dosing regimen comprises administering at least one unit dose for at least one day. In some embodiments, a dosing regimen comprises administering more than one dose over a time period of at least one day, and sometimes more than one day. In some embodiments, a dosing regimen comprises administering multiple doses over a time period of at least week. In some embodiments, the time period is at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40 or more (e.g., about 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more) weeks. In some embodiments, a dosing regimen comprises administering one dose per week for more than one week. In some embodiments, a dosing regimen comprises administering one dose per week for 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40 or more (e.g., about 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more) weeks. In some embodiments, a dosing regimen comprises administering one dose every two weeks for more than two week period. In some embodiments, a dosing regimen comprises administering one dose every two weeks over a time period of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40 or more (e.g., about 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more) weeks. In some embodiments, a dosing regimen comprises administering one dose per month for one month. In some embodiments, a dosing regimen comprises administering one dose per month for more than one month. In some embodiments, a dosing regimen comprises administering one dose per month for 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more months. In some embodiments, a dosing regimen comprises administering one dose per

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week for about 10 weeks. In some embodiments, a dosing regimen comprises administering one dose per week for about 20 weeks. In some embodiments, a dosing regimen comprises administering one dose per week for about 30 weeks. In some embodiments, a dosing regimen comprises administering one dose per week for 26 weeks. In some embodiments, a chirally controlled oligonucleotide composition is administered according to a dosing regimen that differs from that utilized for a chirally uncontrolled (e.g., stereorandom) oligonucleotide composition of the same sequence, and/or of a different chirally controlled oligonucleotide composition of the same sequence. In some embodiments, a chirally controlled oligonucleotide composition is administered according to a dosing regimen that is reduced as compared with that of a chirally uncontrolled (e.g., sterorandom) oligonucleotide composition of the same sequence in that it achieves a lower level of total exposure over a given unit of time, involves one or more lower unit doses, and/or includes a smaller number of doses over a given unit of time. In some embodiments, a chirally controlled oligonucleotide composition is administered according to a dosing regimen that extends for a longer period of time than does that of a chirally uncontrolled (e.g., stereorandom) oligonucleotide composition of the same sequence Without wishing to be limited by theory, Applicant notes that in some embodiments, the shorter dosing regimen, and/or longer time periods between doses, may be due to the improved stability, bioavailability, and/or efficacy of a chirally controlled oligonucleotide composition. In some embodiments, a chirally controlled oligonucleotide composition has a longer dosing regimen compared to the corresponding chirally uncontrolled oligonucleotide composition. In some embodiments, a chirally controlled oligonucleotide composition has a shorter time period between at least two doses compared to the corresponding chirally uncontrolled oligonucleotide composition. Without wishing to be limited by theory, Applicant notes that in some embodiments longer dosing regimen, and/or shorter time periods between doses, may be due to the improved safety of a chirally controlled oligonucleotide composition.

[001004] A single dose can contain various amounts of a type of chirally controlled oligonucleotide, as desired suitable by the application. In some embodiments, a single dose contains about 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300 or more (e.g., about 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000 or more) mg of a type of chirally controlled oligonucleotide. In some embodiments, a single dose contains about 1 mg of a type of

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chirally controlled oligonucleotide. In some embodiments, a single dose contains about 5 mg of a type of chirally controlled oligonucleotide. In some embodiments, a single dose contains about 10 mg of a type of chirally controlled oligonucleotide. In some embodiments, a single dose contains about 15 mg of a type of chirally controlled oligonucleotide. In some embodiments, a single dose contains about 20 mg of a type of chirally controlled oligonucleotide. In some embodiments, a single dose contains about 50 mg of a type of chirally controlled oligonucleotide. In some embodiments, a single dose contains about 100 mg of a type of chirally controlled oligonucleotide. In some embodiments, a single dose contains about 150 mg of a type of chirally controlled oligonucleotide. In some embodiments, a single dose contains about 200 mg of a type of chirally controlled oligonucleotide. In some embodiments, a single dose contains about 250 mg of a type of chirally controlled oligonucleotide. In some embodiments, a single dose contains about 300 mg of a type of chirally controlled oligonucleotide. In some embodiments, a chirally controlled oligonucleotide is administered at a lower amount in a single dose, and/or in total dose, than a chirally uncontrolled oligonucleotide. In some embodiments, a chirally controlled oligonucleotide is administered at a lower amount in a single dose, and/or in total dose, than a chirally uncontrolled oligonucleotide due to improved efficacy. In some embodiments, a chirally controlled oligonucleotide is administered at a higher amount in a single dose, and/or in total dose, than a chirally uncontrolled oligonucleotide. In some embodiments, a chirally controlled oligonucleotide is administered at a higher amount in a single dose, and/or in total dose, than a chirally uncontrolled oligonucleotide due to improved safety.

Biologically Active Oligonucleotides

[001005] A provided oligonucleotide composition as used herein may comprise single stranded and/or multiply stranded oligonucleotides. In some embodiments, single-stranded oligonucleotides contain self-complementary portions that may hybridize under relevant conditions so that, as used, even single-stranded oligonucleotides may have at least partially double-stranded character. In some embodiments, an oligonucleotide included in a provided composition is single-stranded, double-stranded, or triple-stranded. In some embodiments, an oligonucleotide included in a provided composition comprises a single-stranded portion and a multiple-stranded portion within the oligonucleotide. In some embodiments, as noted above,

individual single-stranded oligonucleotides can have double-stranded regions and single-stranded regions.

[001006] In some embodiments, provided compositions include one or more oligonucleotides fully or partially complementary to strand of: structural genes, genes control and/or termination regions, and/or self-replicating systems such as viral or plasmid DNA. In some embodiments, provided compositions include one or more oligonucleotides that are or act as siRNAs or other RNA interference reagents (RNAi agents or iRNA agents), shRNA, antisense oligonucleotides, self-cleaving RNAs, ribozymes, fragment thereof and/or variants thereof (such as Peptidyl transferase 23S rRNA, RNase P, Group I and Group II introns, GIR1 branching ribozymes, Leadzyme, Hairpin ribozymes, Hammerhead ribozymes, HDV ribozymes, Mammalian CPEB3 ribozyme, VS ribozymes, glmS ribozymes, CoTC ribozyme, etc.), microRNAs, microRNA mimics, supermirs, aptamers, antimirs, antagomirs, Ul adaptors, triplex-forming oligonucleotides, RNA activators, long non-coding RNAs, short non-coding RNAs (e.g., piRNAs), immunomodulatory oligonucleotides (such as immunostimulatory oligonucleotides, immunoinhibitory oligonucleotides), GNA, LNA, ENA, PNA, TNA, morpholinos, G-quadruplex (RNA and DNA), antiviral oligonucleotides, and decoy oligonucleotides.

[001007] In some embodiments, provided compositions include one or more hybrid (e.g., chimeric) oligonucleotides. In the context of the present disclosure, the term "hybrid" broadly refers to mixed structural components of oligonucloetides. Hybrid oliogonucleotides may refer to, for example, (1) an oligonucleotide molecule having mixed classes of nucleotides, e.g., part DNA and part RNA within the single molecule (e.g., DNA-RNA); (2) complementary pairs of nucleic acids of different classes, such that DNA:RNA base pairing occurs either intramolecularly or intermolecularly; or both; (3) an oligonucleotide with two or more kinds of the backbone or internucleotide linkages.

[001008] In some embodiments, provided compositions include one or more oligonucleotide that comprises more than one classes of nucleic acid residues within a single molecule. For example, in any of the embodiments described herein, an oligonucleotide may comprise a DNA portion and an RNA portion. In some embodiments, an oligonucleotide may comprise a unmodified portion and modified portion.

[001009] Provided oligonucleotide compositions can include oligonucleotides containing any of a variety of modifications, for example as described herein. In some embodiments,

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particular modifications are selected, for example, in light of intended use. In some embodiments, it is desirable to modify one or both strands of a double-stranded oligonucleotide (or a double-stranded portion of a single-stranded oligonucleotie). In some embodiments, the two strands (or portions) include different modifications. In some embodiments, the two strands include the same modificatinons. One of skill in the art will appreciate that the degree and type of modifications enabled by methods of the present invention allow for numerous permutations of modifications to be made. Exemplary such modifications are described herein and are not meant to be limiting.

RNA interference

[001010] Provided oligonucleotide compositions are useful, among other things, for applications in RNA interference.

[001011] RNA interference (RNAi) refers to the inhibition of gene expression by RNA molecules. Typically, these are small, double-stranded RNA molecules. Since gene expression controls most cellular processes, the ability to inhibit gene expression provides a potentially powerful tool for modulating biological conditions, including treating human and/or animal (e.g., livestock or pet) diseases. A number of studies have been conducted to demonstrate the use of RNAi in regulating or controlling disease-associated gene expression. See, for example: Cullen, K.A., Hall, M.J. & Golosinskiy, A. Ambulatory surgery in the United States, 2006. *Natl Health Stat Report* 2009; 1-25; Elbashir S, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 2001; 411: 494-498; Fire A, Xu S, Montgomery MK, Kostas SA, Driveri SE & Mello C. Potent and specific RNA interference by double-stranded RNA in Caenorhadbditis elegans. *Nature* 1998; 391 (6669):806-811; Gauglitz, G.G., Kortin, H.C., Pavicic, T., Ruzicka, T., & Jeschke, M.G. Hypertrophic scarring and keloids: pathomechanisms and current emerging treatment strategies. *Mol Med* 2011; 17(1-2): 113-125; Li-Tsang, C.W., Lau, J.C. & Chan, C.C. Prevalence of hypertrophic scar formation and its characteristics among the Chinese population. *Burns* 2005; 31, 610-616; Wang H, Ghosh A, Baigude H, Yang C, Qui L, Xia L, et al. Therapeutic gene silencing delivered by a chemically modified siRNA against mutant SOD1 slows ALS progression. *JBC* 2008; 283 (23):15845-15852; Weiser, T.G., Regenbogen, S.E., Thompson,

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K.D., Haynes, A.B., Lipsitz, S.R., Berry, W.R. & Gawande, A.A. An estimation of the global volume of surgery: a modeling strategy based on available data. *Lancet* 2008; 372(9633):139-44.

[001012] The phenomenon of RNA interference was initially demonstrated in *C. elegans*, in which the injection of dsRNA molecules inhibited complementary gene expression. Though the use of siRNA has become a widely used tool for down-regulating gene expression, the existence of a naturally occurring pathway in eukaryotes has been well described. The origin of endogenous siRNA (or miRNA) may be transposons, viruses, repetitive sequences and genes. The process of producing effective endogenous siRNA is regulated by three enzymes. RNA-dependent RNA polymerases convert single-stranded RNA into double-stranded RNA. Alternatively, DNA-dependent RNA polymerases produce dsRNA by transcribing inverted DNA repeats. The resulting large RNA molecules are subject to digestion by ribonuclease III (Dicer) to produce short double-stranded siRNA molecules. Argonaute proteins are then required to bind siRNA molecules to form a complex known as RISC (RNA-induced silencing complex). RISC recognizes double-stranded RNA fragment and splits the double-strands apart, retaining one strand in the RISC complex. RISCs may then promote epigentic silencing through RNA-directed DNA methylation or by target RNA cleavage. Though protein translation may be knocked down considerably, siRNA does not normally eliminate the expression of a gene target completely. RISC can therefore help the guide strand of RNA bind to and destroy its corresponding cellular messenger RNA target. Thus, RNAi provides a method to potentially block the creation of the proteins that cause disease.

[001013] siRNA technology represents a useful molecular tool. The use of RNA interference for artificially manipulating gene expression was initially limited by the activation of cellular antiviral mechanisms. Exposure of cells to sequences longer than 30 nucleotides has been shown to induce interferon gene expression resulting in non-specific RNA degradation and reduced protein synthesis. However, this problem can be circumvented by designing short (e.g., 19 to 22 nucleotide) siRNA sequences. Methods for siRNA delivery into cells include, without limitation, liposome-based addition of purified ribonucleotides to the media or transfection of plasmid vectors designed to express siRNA molecules. Plasmid vectors rely on the use of two RNA Polymerase III promoters (U6 and H1) to drive transcription of the siRNA molecule. The target sequence (19 to 29 nucleotides) is placed in a sense and antisense orientation with a small spacer group in between (short hairpin RNA or shRNA). Once transcribed, a hairpin structure is

formed that can be recognized and cleaved by Dicer. Alternatively, RNA duplexes may be transcribed without hairpin structures and directly process by the RISC. Currently, there are a variety of plasmid and viral vectors that utilize similar concepts to produce siRNA, shRNA, or single stranded siRNA (ss-siRNA) molecules (See, e.g., 2012 Cell-150-883 Walt Lima et al. ssRNAi activate RNAi in animals).

[001014] In some embodiments, a provided oligonucleotide or oligonucleotide composition is useful as ss-siRNA or GalNAc conjugated siRNA.

[001015] The art is familiar with certain structural features that affect siRNA as a tool. Following the discovery of siRNA, several studies attempted to identify the optimal characteristics required for siRNA design. Some of the requirements include using sequences shorter than 30 nucleotides to avoid PKR activation, sequence stability at the 5' end of the antisense strand relative to the 3' terminus and inserting a TT overhang. Based on studies like these, a number of algorithms have been developed by academic and industrial labs to predict the most effective target sequences for a given gene. Though most of these programs are not perfect, the likelihood of obtaining a predicted sequence is superior to designing sequences without consideration of the recommended features. Synthesis and testing of multiple sequences may be required. The design of siRNA experiments may contain some potential pitfalls, thus the design should be done to include appropriate controls and measurable endpoints. A negative control may include a non-complementary sequence with thermodynamically similar properties as the effective siRNA sequence. When transfecting a plasmid vector to introduce siRNA or shRNA, the ratio of lipid to nucleic acid may be equal and the control vector may contain a sequence that is transcribed and processed intracellularly. Validation of the siRNA effect may also be carried out by measuring both RNA and protein expression.

[001016] In some embodiments, a provided oligonucleotide as used herein is double-stranded. Typically, double-stranded oligonucleotides comprising a duplex structure of between 20 and 23, but specifically 21, base pairs have been hailed as particularly effective in inducing RNA interference (Elbashir ct al., EMBO 2001, 20:6877-6888). However, others have found that shorter or longer double-stranded oligonucleotides can be effective as well.

[001017] In some embodiments, a double-stranded oligonucleotide utilized in accordance with the present invention comprises two oligonucleotide strands that are sufficiently complementary to hybridize to form a duplex structure. In some embodiments, a duplex

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structure is between about 12 to about 45 base pairs in length. In some embodiments, a duplex structure is between about 18 to about 25 base pairs in length. In some embodiments, a duplex structure is between about 19 to about 24 base pairs in length. In some embodiments, a duplex structure is between about 19 to about 21 base pairs in length. In some embodiments, a duplex structure is a double-stranded oligonucleotides of between about 25 to about 30 base pairs in length. In some embodiments, a duplex structure is a double-stranded oligonucleotide of between about 10 to about 15 base pairs in length. In some embodiments, a double-stranded oligonucleotide is at least about 21 nucleotides long.

[001018] In some embodiments, a double-stranded oligonucleotide utilized in accordance with the present invention comprises a sense strand and an antisense strand, wherein the antisense RNA strand has a region of complementarity which is complementary to at least a part of a target sequence, and the duplex region is about 14 to about 30 nucleotides in length. In some embodiments, a region of complementarity to the target sequence is between about 14 to about 30 nucleotides in length. In some embodiments, a region of complementarity to the target sequence is between about 18 to about 25 nucleotides in length. In some embodiments, a region of complementarity to the target sequence is between about 19 to about 24 nucleotides in length. In some embodiments, a region of complementarity to the target sequence is about 19 to about 21 nucleotides in length.

[001019] The phrase "antisense strand" as used herein, refers to an oligonucleotide that is substantially or 100% complementary to a target sequence of interest. The phrase "antisense strand" includes the antisense region of both oligonucleotides that are formed from two separate strands, as well as unimolecular oligonucleotides that are capable of forming hairpin or dumbbell type structures. The terms "antisense strand" and "guide strand" are used interchangeably herein.

[001020] The phrase "sense strand" refers to an oligonucleotide that has the same nucleoside sequence, in whole or in part, as a target sequence such as a messenger RNA or a sequence of DNA. The terms "sense strand" and "passenger strand" are used interchangeably herein.

[001021] By "target sequence" is meant any nucleic acid sequence whose expression or activity is to be modulated. The target nucleic acid can be DNA or RNA, such as endogenous DNA or RNA, viral DNA or viral RNA, or other RNA encoded by a gene, virus, bacteria,

fungus, mammal, or plant. In some embodiments, a target sequence is associated with a disease or disorder.

[001022] By "specifically hybridizable" and "complementary" is meant that a nucleic acid can form hydrogen bond(s) with another nucleic acid sequence by either traditional Watson-Crick or other non- traditional types. In reference to the nucleic molecules of the present invention, the binding free energy for a nucleic acid molecule with its complementary sequence is sufficient to allow the relevant function of the nucleic acid to proceed, e.g., RNAi activity. Determination of binding free energies for nucleic acid molecules is well known in the art (see, e.g., Turner et al, 1987, *CSH Symp. Quant. Biol.* LIT pp.123-133; Frier et al., 1986, *Proc. Nat. Acad. Sci.* USA83:9373-9377; Turner et al., 1987, /. *Ain. Chem. Soc.* 109:3783-3785)

[001023] A percent complcmentarity indicates the percentage of contiguous residues in a nucleic acid molecule that can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9,10 out of 10 being 50%, 60%, 70%, 80%, 90%, and 100% complementary). "Perfectly complementary" or 100% complementarity means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence. Less than perfect complementarity refers to the situation in which some, but not all, nucleoside units of two strands can hydrogen bond with each other. "Substantial complementarity" refers to polynucleotide strands exhibiting 90% or greater complementarity, excluding regions of the polynucleotide strands, such as overhangs, that are selected so as to be noncomplementary. Specific binding requires a sufficient degree of complementarity to avoid non-specific binding of the oligomeric compound to non-target sequences under conditions in which specific binding is desired, e.g., under physiological conditions in the case of *in vivo* assays or therapeutic treatment, or in the case of *in vitro* assays, under conditions in which the assays are performed. In some embodiments, non-target sequences differ from corresponding target sequences by at least 5 nucleotides.

Double stranded oligonucleotides

[001024] In some embodiments, a double-stranded oligonucleotide utilized in accordance with the present invention is sufficiently large that it can be cleaved by an endogenous molecule, e.g., by Dicer, to produce smaller double-stranded oligonucleotides, e.g., RNAi agents. In some

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embodiments, a provided double-stranded oligonucleotide modulates the expression of a target gene via RISC mediated cleavage of the target sequence.

[001025] In some embodiments, a double-stranded region of a double-stranded oligonucleotide is equal to or at least, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotide pairs in length.

[001026] In some embodiments, an antisense strand of a double-stranded oligonucleotide is equal to or at least 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length.

[001027] In some embodiments, a sense strand of a double-stranded oligonucleotidc is equal to or at least 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length.

[001028] In some embodiments, one strand has at least one stretch of 1-5 single-stranded nucleotides in the double-stranded region. By "stretch of single-stranded nucleotides in the double-stranded region" is meant that there is present at least one nucleotide base pair at both ends of the single-stranded stretch. In some embodiments, both strands have at least one stretch of 1-5 (e.g., 1, 2, 3, 4, or 5) single-stranded nucleotides in the double stranded region. When both strands have a stretch of 1-5 (e.g., 1, 2, 3, 4, or 5) single-stranded nucleotides in the double stranded region, such single-stranded nucleotides can be opposite to each other (e.g., a stretch of mismatches) or they can be located such that the second strand has no single-stranded nucleotides opposite to the single-stranded oligonucleotides of the first strand and vice versa (e.g., a single-stranded loop). In some embodiments, the single-stranded nucleotides are present within 8 nucleotides from either end, for example 8, 7, 6, 5, 4, 3, or 2 nucleotide from either the 5' or 3' end of the region of complementarity between the two strands.

[001029] In some embodiments, each strand of a double-stranded oligonucleotide utilized in accordance with the present invention has a ZXY structure, such as is described in International Application No. PCT/US2004/07070 filed on March 8, 2004, contents of which are hereby incorporated in their entireties.

Hairpins and dumbbells

[001030] In some embodiments, a double-stranded oligonucleotide utilized in accordance with the present invention is a single molecule that comprises self-complementary regions; thus

the two "strands" of a double-stranded regions are in fact covalentley linked to one another. Such two strands can be linked to each other at both ends, or at one end only. By linking at one end is meant that 5'-end of first strand is linked to the 3'-end of the second strand or 3'-end of first strand is linked to 5'-end of the second strand. When the two strands are linked to each other at both ends, 5'-end of first strand is linked to 3'-end of second strand and 3'-end of first strand is linked to 5'-end of second strand. In some embodiments, two strands are linked together by an oligonucleotide linker including, but not limited to, $(N)_n$; wherein N is independently a modified or unmodified nucleotide and n is 3-23. In some embodiemtns, n is 3-10, e.g., 3, 4, 5, 6, 7, 8, 9, or 10. In some embodiments, an oligonucleotide linker is selected from the group consisting of GNRA, $(G)_4$, $(U)_4$, and $(dT)_4$, wherein N is a modified or unmodified nucleotide and R is a modified or unmodified purine nucleotide. In some embodiments, some of the nucleotides in the linker are involved in base-pair interactions with other nucleotides in the linker. In some embodiments, the two strands are linked together by a non-nucleosidic linker, e.g.

[001031] In some embodiments, hairpin and dumbbell type RNAi agents have a duplex region equal to or at least 14, 15, 15, 16, 17, 18, 19, 29, 21, 22, 23, 24, or 25 nucleotide pairs. In some embodiments, the duplex region is equal to or fewer than 200, 100, or 50, nucleotide pairs in length. In some embodiments, ranges for the duplex region are about 15 to about 30, about 17 to about 23, about 19 to about 23, and about 19 to about 21 nucleotides pairs in length. In some embodiments, hairpin oligonucleotides mimic the natural precursors of microRNAs.

[001032] In some embodiments, hairpin RNAi agents can have a single strand overhang or terminal unpaired region, e.g., at the 3' end on the antisense side of the hairpin, etc.. In some embodiments, the overhangs are about 1 to about 4 nucleotides in length. In some embodiments, the overhangs are about 2 to about 3 nucleotides in length.

[001033] In some embodiments, a hairpin RNAi agent is characterized in that the 3'-end of an antisense strand is linked to 5'-end of a sense strand. In some embodiments, a hairpin RNAi agent is characterized in that the 5'-end of an antisense strand is linked to the 3'- end of a sense strand. Provided hairpin oligonucleotides are also referred to herein as "shRNA".

Single-stranded oligonucleotides

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[001034] In some embodiments, a single-stranded oligonucleotide utilized in accordance with the present invention comprises a nucleotide sequence that is substantially complementary to a "sense" nucleic acid encoding a gene expression product, e.g., complementary to the coding strand of a double-stranded cDNA molecule or complementary to an RNA sequence, e.g., a pre-mRNA, mRNA, miRNA, or premiRNA. Provided single-stranded oligonucleotides include, but are not limited to, antisense oligonucleotides and single-stranded RNAi agents. In some embodiments, the region of complementarity is less than about 30 nucleotides in length. In some embodiments, the region of complementarity is at least about 15 nucleotides in length. In some embodiments, a provided single stranded oligonucleotide is about 10 to about 25 nucleotides in length (e.g., about 11, 12, 13, 14, 15, 16, 18, 19, 20, 21, 22, 23, or 24 nucleotides in length). In some embodiments, a provided single stranded oligonucleotide is about 25 to about 30 nucleotides in length. In some embodiments, a provided single stranded oligonucleotide is about 15 to about 29 nucleotides in length. In some embodiments, a provided single stranded oligonucleotide is characterized as having less than 100% complementarity to a mRNA, RNA or DNA arc. In some embodiments, a provided single-stranded oligonucleotide has a ZXY structure, such as is described in International Application No. PCT/US2004/07070 filed on March 8, 2004.

[001035] In some embodiments, a utilized single-stranded oligonucleotide can hybridize to a complementary RNA, e.g., mRNA, pre-mRNA, and prevent access of the translation machinery to the target RNA transcript, thereby preventing protein synthesis. In some embodiments, a provided single-stranded oligonucleotide can hybridize to a complementary RNA and the RNA target can be subsequently cleaved by an enzyme such as RNasc H, thus preventing translation of target RNA. In some embodiments, a provided single-stranded oligonucleotide modulates the expression of a target gene via RISC mediated cleavage of the target sequence.

[001036] A "single-stranded RNAi agent" as used herein, is an RNAi agent which is made up of a single molecule. In some embodiments, a single-stranded RNAi agent includes a duplexed region, formed by intra-strand pairing, e.g., it is or it includes a hairpin or pan-handle structure. In some embodiments, single-stranded RNAi agents are antisense with regard to the target molecule. In some embodiments, single-stranded RNAi agents are sufficiently long such that they are able to enter the RISC and participate in RISC mediated cleavage of a target

mRNA. Exemplary single-stranded siRNAs (ss siRNAs) are known and are described, for example, in U.S. Pat. Pub. No. 2006/0166901, the contents of which are herein incoporated by reference in its entirety.

[001037] In some embodiments, a single-stranded RNAi agent is at least about 12 nucleotides in length. In some embodiments, a single-stranded RNAi agent is at least about 15 nucleotides in length. In some embodiments, a single-stranded RNAi agent is at least about 20 nucleotides in length. In some embodiments, a single-stranded RNAi agent is at least about 25 nucleotides in length. In some embodiments, a single-stranded RNAi agent is at least about 29 nucleotides in length. In some embodiments, a single-stranded RNAi agent is at least about 30 nucleotides in length. In some embodiments, a single-stranded RNAi agent is at least about 35 nucleotides in length. In some embodiments, a single-stranded RNAi agent is at least about 40 nucleotides in length. In some embodiments, a single-stranded RNAi agent is at least about 50 nucleotides in length.

[001038] In some embodiments, a single-stranded RNAi agent is less than 200 nucleotides in length. In some embodiments, a single-stranded RNAi agent is less than 100 nucleotides in length. In some embodiments, a single-stranded RNAi agent is less than 60 nucleotides in length.

[001039] In some embodiments. a single-stranded RNAi agent is 5' phosphorylated. In some embodiments, a singlestranded RNAi agent includes a phosphoryl analog at the 5' prime terminus. In certain embodiments, a single-stranded RNAi agent has length from about 15 to about 29 nucleotides in length.

[001040] Single-stranded oligonucleotides, including those described and/or identified as single stranded siRNAs, microRNAs or mirs which may be used as targets or may serve as a template for the design of oligonucleotides of the invention are taught in, for example, Esau, *et al.* US Publication #20050261218 (USSN: 10/909125) entitled "Oligonucleotides and compositions for use in modulation small non-coding RNAs" the entire contents of which are incorporated herein by reference.

[001041] The present invention encompasses research and/or diagnostic reagents that comprise single-stranded oligonucleotides. In some embodiments, a single-stranded oligonucleotide utilized in accordance with the present invention is and/or acts as a primer. In some embodiments, primers are used in polymerase-based chain reactions (i.e., PCR) to amplify

nucleic acids. These applications include any known variations of PCR, such as reverse transcription PCR (RT-PCR) and real-time PCR.

MicroRNAs

[001042] In some embodiments provided compositions comprise one or more oligonucleotides that are or act as MicroRNAs.

[001043] MicroRNAs (miRNAs or mirs) are a highly conserved class of small RNA molecules that are transcribed from DNA in the genomes of plants and animals, but are not translated into protein. Pre-microRNAs are processed into miRNAs. Processed microRNAs are single stranded 17-25 nucleotide (nt) RNA molecules that become incorporated into the RNA-induced silencing complex (RISC) and have been identified as key regulators of development, cell proliferation, apoptosis and differentiation. They are believed to play a role in regulation of gene expression by binding to the 3'-untranslated region of specific mRNAs. RISC mediates downregulation of gene expression through translational inhibition, transcript cleavage, or both. RISC is also implicated in transcriptional silencing in the nucleus of a wide range of eukaryotes.

[001044] MicroRNAs have also been implicated in modulation of pathogens in hosts. For example, see Jopling, C.L., et al., *Science* (2005) vol. 309, pp 1577-1581. Without wishing to be bound by theory, administration of a microRNA, microRNA mimic, and/or anti microRNA oligonucleotide, leads to modulation of pathogen viability, growth, development, and/or replication. In some embodiments, a provided oligonucleotide is a microRNA, microRNA mimic, and/or anti microRNA, wherein microRNA is a host microRNA. The number of miRNA sequences identified to date is large and growing, illustrative examples of which can be found, for example, in: *"miRBase: microRIVA sequences, targets and gene nomenclature"* Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ. NAR, 2006, 34, Database Issue, D140-D144; *"The microRNA Registry"* Griffiths-Jones S. NAR, 2004, 32, Database Issue, D109-D111. Non-limiting examples of useful miRNA sequences are also provided in the accompanying **Appendix (C)**.

Ribozymes

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[001045] In some embodiments, provided compositions include one or more oligonucleotides that are or act as ribozymes.

[001046] Ribozymes are oligonucleotides having specific catalytic domains that possess endonuclease activity (Kim and Cech, Proc Natl Acad Sci U S A. 1987 Dec;84(24):8788-92; Forster and Symons, Cell. 1987 Apr 24;49(2):211-20). At least six basic varieties of naturally occurring enzymatic RNAs are known presently. In general, enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through the target binding portion of an enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

[001047] Methods of producing a ribozyme targeted to any target sequence are known in the art. Ribozymes can be designed as described in, inter alia, Int. Pat. Appl. Publ. No. WO 93/23569 and Int. Pat. Appl. Publ. No. WO 94/02595, each specifically incorporated herein by reference, and synthesized to be tested *in vitro* and *in vivo,* as described therein.

Aptamers

[001048] In some embodiments, provided compositions include one or more oligonucleotides that are or act as aptamers.

[001049] Aptamers are nucleic acid or peptide molecules that bind to a particular molecule of interest with high affinity and specificity (Tuerk and Gold, Science 249:505 (1990); Ellington and Szostak, Nature 346:818 (1990)). DNA or RNA aptamers have been successfully produced which bind many different entities from large proteins to small organic molecules. See Eaton, Curr. Opin. Chem. Biol. 1:10-16 (1997), Famulok, Curr. Opin. Struct. Biol. 9:324-9(1999), and Hermann and Patel, Science 287:820-5 (2000). Aptamers can be RNA or DNA based.
Generally, aptamers are engineered through repeated rounds of *in vitro* selection or equivalently, SELEX (systematic evolution of ligands by exponential enrichment) to bind to various molecular targets such as small molecules, proteins, nucleic acids, and even cells, tissues and organisms.

The SELEX procedure is a protocol in which single stranded oligonucleotides are selected from vast libraries of sequences, based on binding affinity at a target protein or other molecule (C. Tuerk, L. Gold, Science, 249 (1990), pp. 505–510; R. Green, A.D. Ellington, D.P. Bartel, J.W. Szostak, Methods Enzymol., 2 (1991), pp. 75–86; L. Gold, B. Polisky, O. Uhlenbeck, M. Yarus, Annu. Rev. Biochem., 64 (1995), pp. 763–797). The SELEX procedure is usually initiated with an RNA or DNA library consisting of some 10^{14}–10^{15} random oligonucleotide sequences. In a fully randomised oligonucleotide library, each molecule will exhibit a unique tertiary structure which will be dependent on the nucleotide sequence of that molecule. The binding affinity of the oligonucleotide for the target protein will be determined by the fit between moieties on the surface of the oligonucleotide and epitopes on the target protein. As a consequence of starting from a library of vast diversity it is often possible to identify aptamers of nM or sub-nM affinity for the target protein and with selectivity for that target protein over other proteins with a high degree of structural homology (K.W. Uphoff, S.D. Bell, A.D. Ellington, Curr. Opin. Struct. Biol., 6 (1996), pp. 281–288). Using SELEX methodology RNA or DNA aptamers have been generated to many proteins, peptides and small molecules including dopamine (C. Mannironi, A. Di Nardo, P. Fruscoloni, G.P. Tocchini-Valentini, Biochemistry, 36 (1997), pp. 9726–9734), substance P (D. Nieuwlandt, M. Wecker, Biochemistry, 34 (1995), pp. 5651–5659), subtilisin (H. Takeno, S. Yamamoto, T. Tanaka, Y. Sakano, Y. Kikuchi, J. Biochem., 125 (1999), pp. 1115–1119), platelet derived growth factor (L.S. Green, D. Jellinek, R. Jenison, A. Ostman, C-H. Heldin, N. Janjic Biochemistry, 35 (1996), pp. 14413–14424), vascular endothelial growth factor (L.S. Green, D. Jellinek, C. Bell, L.A. Bebe, B.D. Feistner, S.C. Gill, F.M. Jucker, N. Janjic Chem. Biol., 2 (1995), pp. 683–695), thrombin (L.C. Bock, L.C. Griffen, J.A. Latham, E.H. Vermaas, J.J. Toole, Nature, 355 (1992), pp. 564–566), and L-selectin (D. O'Connell, A. Koenig, S. Jennings, B. Hicke, H.L. Han, T. Fitzwater, Y.F. Chang, N. Varki, D. Parma Proc. Natl. Acad. Sci. USA, 93 (1996), pp. 5883–5887).

[001050] As reviewed in Dua et al. (2008) Recent Patents on DNA & Gene Sequences, 2: 172-186 (incorporated herein by reference) in more detail, over the years, a number of modified SELEX protocols have been developed. Non-limiting examples of modified SELEX methods are described in the following publications: Counter SELEX (US5580737), Flow cell SELEX (WO9833941), Truncation SELEX (WO0056930), Blended SELEX (US5683867), Transctiption free SELEX (US20026387620), Solution SELEX (US5567588), Chimeric SELEX

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(WO9604403), Tissue SELEX (US20026376474), Photo SELEX (US6001577), Toggle SELEX (US20077312325), Covalent SELEX/Chemi SELEX (US5763595), Genomic SELEX (US20016261774), SELEX without purified protein (WO0224954), CE-SELEX (WO03102212), Mirror-image SELEX – Spiegelmers (EP1386972) and Laser SELEX, DeSELEX (WO07035532), each of which is incorporated herein by reference in its entirety. Stereo-defined oligonucleotides embraced by the present invention may be used in any one or more of the variations of the SELEX methods.

[001051] An aptamer can be prepared by any known method, including synthetic, recombinant, and purification methods, and can be used alone or in combination with other aptamers specific for the same target. Further, as described more fully herein, the term "aptamer" specifically includes "secondary aptamers" containing a consensus sequence derived from comparing two or more known aptamers to a given target. Aptamers possess several characteristics that make them attractive therapeutic agents. Both DNA and RNA aptamers have been shown to bind their targets with dissociation constants (Kd) in the low picomolar to low nanomolar range. Binding of an aptamer is a highly specific interaction that can even discriminate between related proteins that share common structural domains. Though the binding affinity of both aptamers and antibodies is in the same range, aptamers have many additional features which overpower their rival in many cases. Unlike antibodies, aptamers can be targeted even against non immunogenic targets. During synthesis they can be easily subjected to a chemical modification that improves their stability and pharmacokinetics. They display nil to negligible levels of immunogenicity at therapeutic doses due to their resemblance to endogenous molecules.

[001052] In some embodiments, oligonucleotides utilized in accordance with the present invention are useful as anti-pathogenic agents, for example, antiviral agents, antibacterial agents, antifungal agents, and so on. Suitable targets for a variety of infectious agents such as viruses and bacteria have been reported. See, for example, US Patent 5726017, US Patent 5654151, US Patent 5496938, US Patent 5503978, US Patent 5587468, US Patent 5527894, US2005233317, US Patent 5496938, WO08 066231, JP2002165594, WO02081494, US Patent 5861501, WO9720072, US Patent 5475096, US Patent 6569630, CN101109013, and WO03106476, each of which is incorporated herein by reference in its entirety.

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[001053] In some embodiments, oligonucleotides utilized in accordance with the present invention are or act as anticancer agents. Any known cancer- or tumor-associated factors, such as proteins involved in the regulation of cell division or proliferation, cell cycle progression, apoptosis, cell migration, DNA repair, etc. including structural proteins and signal transduction molecules, may be targeted by an aptamer. Examples include, without limitation, See, for example, AU Patent 775412, US Patent 6232071, WO 08/028534, US Patent 6933114, WO 04/081574, AU Patent 242462, US Patent 6995249, US Patent 5668264, US Patent 6699843, WO 04/094614, and US Patent 5846713, each of which is incorporated herein by reference in its entirety.

[001054] In some embodiments, oligonucleotides utilized in accordance with the present invention are or act as antiangiogenic agents. Non-limiting examples of antiangiogenic targets include VEGF and associates receptors, as well as extracellular matrix or adhesion molecules. See, for example, US Patent 6051698, US 2003175703, WO 95/21853 and US Patent 7094535, each of which is incorporated herein by reference in its entirety.

[001055] In some embodiments, oligonucleotides utilized in accordance with the present invention are or act as anticoagulant agents. Much is known about blood coagulation factors and their function as well as proteins regulation such process. Aptamers are useful in targeting one or more of such factors in treating conditions such as cardiovascular diseases and blood coagulation disorders. See for example, WO 06/033854, US Patent 5543293, WO 07/025049, US Patent 6774118, WO 07/140000, each of which is incorporated herein by reference in its entirety.

[001056] In some embodiments, oligonucleotides utilized in accordance with the present invention are or act as immunomodulatory agents. Aptamers have been generated to target immunomodulatory molecules involved in autoimmune disorders. The present invention may be useful for targeting molecules expressed on immune cells, such as T cells and antigen presenting cells (APCs). See for example, US Patent 5869641, WO 01/09160, each of which is incorporated herein by reference in its entirety. In some embodiments, molecules involved in the complement system such as C1, C4, C2, C3 and C5 may be targeted by an aptamer. The complement system has been implicated in numerous renal, rheumatological, neurological, dermatological, hematological, allergic, infectious, and other diseases. See for example, WO 97/28178 and WO 07/103549, each of which is incorporated herein by reference in its entirety.

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Other immune-related targets include, without limitation, IL-12, IL-23 and IL-28 (see, for example, WO 07/035922, which is incorporated herein by reference), IgE (see, for example, WO 05/113813, which is incorporated herein by reference), Sp-1 and Sp1-, CD28, IL-2, GMCSF (see, for example, US Patent 6994959, which is incorporated herein by reference), SDF-1, CXCR4 (see, for example, WO 08/009437, which is incorporated herein by reference), IL-6, IL-12, IFN gamma (see, for example, US Patent 6589940 and US2003125279, each of which is incorporated herein by reference), and TLR. Stereo-defined aptamers may elicit improved efficacy against such diseases.

[001057] In some embodiments, oligonucleotides utilized in accordance with the present invention are or act as anti-inflammatory agents. Inflammatory disease such as acute respiratory distress syndrome (ARDS), septic shock, pulmonary emphysema, cystic fibrosis, rheumatoid arthritis and chronic bronchitis, have neutrophil elastase involved in their pathogenesis. Human elastate is therefore a target for treating such disorders using an aptamer that regulates inflammation. Other suitable targets include, without limitation, phospholipase A2, such as non-pancreatic secretory PLA2 (see, for example, WO 96/27604, which is incorporated by reference), selectins, E-, P- and L- (see, for example, US Patent 5780228, which is incorporated by reference), three homologous C-type lectins, other cell adhesion molecules expressed in cells such as leukocytes, endothelial cells and platelets; MCP-1 (see, for example, WO 07/093409, which is incorporated by reference), NF-kappa B and NF-IL6 (see, for example, WO 00/24404, which is incorporated by reference). Stereo-defined aptamers may elicit improved efficacy against such diseases.

[001058] In some embodiments, oligonucleotides utilized in accordance with the present invention are useful for treating certain brain diseases, including but are not limited to: Transmissible spongiform encephalopathies (TSEs) and Alzheimer's disease. Known targets are described in publications including WO 2006/138676 and DE19916417, and WO 08/008884, each of which is incorporated herein by reference. Stereo-defined aptamers may elicit improved efficacy against such diseases.

Decoy oligonucleotides

[001059] In some embodiments, provided compositions include one or more oligonucleotides that are or act as decoy oliogonucleotides.

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[001060] Because transcription factors recognize their relatively short binding sequences, even in the absence of surrounding genomic DNA, short oligonucleotides bearing the consensus binding sequence of a specific transcription factor can be used as tools for manipulating gene expression in living cells. This strategy involves the intracellular delivery of such "decoy oligonucleotides", which are then recognized and bound by the target factor. Occupation of the transcription factor's DNA-binding site by the decoy renders the transcription factor incapable of subsequently binding to the promoter regions of target genes. Decoys can be used as therapeutic agents, either to inhibit the expression of genes that are activated by a transcription factor, or to up-regulate genes that are suppressed by the binding of a transcription factor. Examples of the utilization of decoy oligonucleotides can be found in Mann et al., J. Clin. Invest., 2000, 106: 1071-1075, which is expressly incorporated by reference herein, in its entirety.

miRNA mimics

[001061] In some embodiments, provided compositions include one or more oligonucleotides that are or act as miRNA mimics.

[001062] miRNA mimics represent a class of molecules that can be used to imitate the gene modulating activity of one or more miRNAs. Thus, the term "microRNA mimic" refers to synthetic non-coding RNAs (i.e. the miRNA is not obtained by purification from a source of the endogenous miRNA) that are capable of entering the RNAi pathway and regulating gene expression. miRNA mimics can be designed as mature molecules (e.g. single stranded) or mimic precursors (e.g., pri- or pre-miRNAs).

[001063] In some embodiments, miRNA mimics are double stranded molecules (e.g., with a duplex region of between about 16 and about 31 nucleotides in length) and contain one or more sequences that have identity with the mature strand of a given miRNA.

[001064] In some embodiments, an miRNA mimic comprises a duplex region of between about 16 and about 31 nucleotides. In some embodiments, provided miRNA mimic may contain one or more of the following chemical modification patterns: the sense strand contains 2'-O-methyl modifications of nucleotides 1 and 2 (counting from the 5' end of the sense oligonucleotide), and all of the Cs and Us; the antisense strand modifications can comprise 2' F

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modification of all of the Cs and Us, phosphorylation of the 5' end of the oligonucleotide, and stabilized internucleotide linkages associated with a 2 nucleotide 3' overhang.

Supermirs

[001065] In some embodiments, provided compositions include one or more oligonucleotides that are or act as supermirs.

[001066] A supermir refers to an oligonucleotide, e.g., single stranded, double stranded or partially double stranded, which has a nucleotide sequence that is substantially identical to an miRNA and that is antisense with respect to its target. This term includes oligonucleotides which comprise at least one non-naturally-occurring portion which functions similarly. In some embodiments, the supermir does not include a sense strand. In some embodiments, the supermir does not self-hybridize to a significant extent. In some embodiments, a supermir has a secondary structure but is substantially single-stranded under physiological conditions. A supermir that is substantially single-stranded is single-stranded to the extent that less than about 50% (e.g., less than about 40%, 30%, 20%, 10%, or 5%) of the supermir is duplexed with itself. A supermir can include a hairpin segment, e.g., sequence, preferably at the 3' end can self hybridize and form a duplex region, e.g., a duplex region of at least 1, 2, 3, or 4 and preferably less than 8, 7, 6, or 5 nucleotides, e.g., 5 nucleotides. The duplexed region can be connected by a linker, e.g., a nucleotide linker, e.g., 3, 4, 5, or 6 dTs, e.g., modified dTs. In some embodiments, a supermir is duplexed with a shorter oligonucleotide, e.g., of 5, 6, 7, 8, 9, or 10 nucleotides in length, e.g., at one or both of the 3' and 5' end or at one end and in the non-terminal or middle of the supermir.

Antimers or miRNA inhibitors

[001067] In some embodiments, provided compositions include one or more oligonucleotides that are or act as antimers or miRNA inhibitors.

[001068] The terms "antimir" "microRNA inhibitor" or "miR inhibitor" are synonymous and refer to oligonucleotides or modified oligonucleotides that interfere with the activity of specific miRNAs. Inhibitors can adopt a variety of configurations including single stranded, double stranded (RNA/RNA or RNA/DNA duplexes), and hairpin designs. In some embodiments, microRNA inhibitors comprise one or more sequences or portions of sequences

that are complementary or partially complementary with the mature strand (or strands) of the miRNA to be targeted. In some embodiments, miRNA inhibitors comprise additional sequences located 5' and 3' to the sequence that is the reverse complement of the mature miRNA. Additional sequences can be the reverse complements of the sequences that are adjacent to the mature miRNA in the primiRNA from which the mature miRNA is derived, or additional sequences can be arbitrary sequences (having a mixture of A, G, C, U, or dT). In some embodiments, one or both of the additional sequences are arbitrary sequences capable of forming hairpins. Thus, in some embodiments, the sequence that is the reverse complement of the miRNA is flanked on the 5' side and on the 3' side by hairpin structures. In some embodiments, microRNA inhibitors are double stranded. In some embodiments, microRNA inhibitors are double stranded and include mismatches between nucleotides on opposite strands. In some embodiments, microRNA inhibitors are linked to conjugate moieties in order to facilitate uptake of the inhibitor into a cell.

[001069] MicroRNA inhibitors, including hairpin miRNA inhibitors, are described in detail in Vermeulen et al., "Double-Stranded Regions Are Essential Design Components Of Potent Inhibitors of RISC Function," RNA 13: 723-730 (2007) and in W02007/095387 and WO 2008/036825 each of which is incorporated herein by reference in its entirety.

[001070] An exemplary application of miRNA-based therapy is described in Pan et al. (2007) World J Gastroenterol 13(33): 4431-36, "New therapeutic opportunities for Hepatitis C based on small RNA," the contents of which are incorporated by reference. Briefly, the authors describe 22 nucleotide mature miR-122, derived from a noncoding polyadenylated RNA transcript of the hcr gene, which is a liver-specific developmental regulator. Because miR-122 is a liver specific miRNA that is involved in HCV viral replication, silencing of miR-122 can be useful for the treatment of HCV.

Antagomirs

[001071] In some embodiments, provided compositions include one or more oligonucleotides that are or act as antagomirs.

[001072] Antagomirs are RNA-like oligonucleotides that harbor various modifications for RNAse protection and pharmacologic properties, such as enhanced tissue and cellular uptake.

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They differ from normal RNA by, for example, complete 2'-0-methylation of sugar, phosphorothioatc intersugar linkage and, for example, a cholesterol-moiety at 3'-end. In some embodiments, an antagomir comprises a 2'-O-methylmodification at all nucleotides, a cholesterol moiety at 3'- end, two phosphorothioate intersugar linkages at the first two positions at the 5'-end and four phosphorothioate linkages at the 3'-end of the molecule. Antagomirs can be used to efficiently silence endogenous miRNAs by forming duplexes comprising the antagomir and endogenous miRNA, thereby preventing miRNA-induced gene silencing. An example of antagomir-mediated miRNA silencing is the silencing of miR-122, described in Krutzfeldt *et al,* Nature, 2005, 438: 685-689, which is incorporated by reference herein in its entirety.

Modified single oligonucleotide variations

[001073] Typically, it is perceived that two components are required to activate RNAi machinery—the double-stranded nucleic acid motif, which is required for recognition by RNAi-associated proteins and the guide strand which serves as the mRNA binding co-factor in the RISC's Argonaute catalytic protein. More recently, a novel type of RNAi molecules composed of a single, short (25–28 nt) oligo capable of self-dimerizing into a partially complementary duplex have been developed. These molecules were demonstrated to efficiently activate the RISC and to produce target mRNA silencing comparable to that obtained with potent conventional RNAi molecules. See: Lapierre et al., "Potent and systematic RNAi mediated silencing with single oligonucleotide compounds." RNA 2011; 17:00, the contents of which are hereby incirporated by reference. See also: WO 2010/090762 "RNA DUPLEXES WITH SINGLE STRANDED PHOSPHOROTHIOATE NUCLEOTIDE REGIONS FOR ADDITIONAL FUNCTIONALITY" (PCT/US2010/00348), the contents of which are hereby incirporated by reference.

[001074] Thus, the present invention includes RNAi constructs containing single stranded regions of phosphorothioate modified nucleotides, and the uses of such constructs in gene silencing.

[001075] In some embodiments, the invention utilizes isolated double stranded nucleic acid molecules including a guide strand and a passenger strand, wherein the passenger strand is connected through a cleavable linker to a single stranded region of at least eight phosphorothioate modified nucleotides. In some embodiments, the invention is an isolated

double stranded nucleic acid molecule having a guide strand and a passenger strand, wherein at least one of the guide strand and passenger strand is connected through a cleavable linker to a single stranded region of at least six phosphorothioate modified nucleotides.

[001076] In some embodiments, the invention utilizes an isolated double stranded nucleic acid molecule having a guide strand and a passenger strand, wherein at least one of the guide strand and passenger strand is connected through a cleavable linker to a single stranded region of at least three phosphorothioate modified nucleotides. In some embodiments, the double stranded nucleic acid molecule includes at least one of the following properties. The passenger strand may be 8-18 nucleotides in length. The nucleic acid may have at least one 2' O methyl or 2' fluoro modification. The cleavable linkage may be other than a nucleotidic linkage. The nucleic acid may include a lipophilic group. The guide strand may be 16-18 nucleotides or 26-28 nucleotides in length. In some embodiments the single stranded region is connected to the guide strand.

[001077] In some embodiments, the cleavable linker includes one or more unmodified nucleotides. In other embodiments, the cleavable linker is a phosphodiester bond. In certain embodiments, the cleavable linker is S-S. In some embodiments the cleavable linker is DNA or RNA. The single stranded region of at least eight phosphorothioate modified nucleotides can be at either the 3' or 5' end of the passenger strand. In some embodiments, the single stranded region of at least eight phosphorothioate modified nucleotides is DNA, while in other embodiments it is RNA.

[001078] In some embodiments the double stranded region of the nucleic acid molecule is a perfect duplex. In other embodiments the double stranded region contains at least one bulge region. In some embodiments the passenger strand comprises a nick within the double stranded region of the molecule. The double stranded region may contain at least one nucleotide that is phosphorothioate modified.

[001079] Nucleic acid molecules utilized in accordance with the invention may be chemically modified. In certain embodiments the chemical modification is 2'Omethyl and/or 2'Fluoro. In some embodiments more than one chemical modification is present in the same molecule. In some embodiments chemical modification increases stability, increases evasion of immune regulation, and/or prevents off-target gene silencing. Chemical modification can be present on the passenger strand and/or the guide strand. In some embodiments the single stranded region of at least eight phosphorothioate modified nucleotides is cleaved from the

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double stranded region of the nucleic acid molecule in a cell. In some embodiments the single stranded region of at least eight phosphorothioate modified nucleotides has complementarity to a mammalian gene. In certain embodiments the single stranded region of at least eight phosphorothioate modified nucleotides functions as an antisense molecule. The double stranded region may be at least 19 nucleotides long. In some embodiments the single stranded region is at least 12 nucleotides long.

[001080] In some embodiments, the invention utilizes bifunctional nucleic acid molecule including a double stranded region that functions in RNA interference and a single stranded region that functions in antisense, wherein the double stranded region comprises a guide strand and a passenger strand, and wherein the double stranded region and the single stranded region are connected through a cleavable linker. In some embodiments, the cleavable linker includes one or more unmodified nucleotides. In other embodiments, the cleavable linker is a phosphodiester bond. In certain embodiments, the cleavable linker is S-S. In some embodiments the cleavable linker is DNA or RNA.

[001081] In some embodiments, the invention relates to methods for inhibiting the expression of a target gene in a mammalian cell, including contacting the mammalian cell with an isolated double stranded nucleic acid molecule comprising a guide strand and a passenger strand, wherein the passenger strand is connected through a cleavable linker to a single stranded region of at least eight phosphorothioate modified nucleotides. In some embodiments, the cleavable linker includes one or more unmodified nucleotides. In other embodiments, the cleavable linker is a phosphodiester bond. In certain embodiments, the cleavable linker is S-S. In some embodiments the cleavable linker is DNA or RNA.

[001082] In some embodiments, the single stranded region comprises at least eight phosphorothioate modified nucleotides and can be at either the 3' or 5' end of the passenger strand. In some embodiments, the single stranded region of at least eight phosphorothioate modified nucleotides is DNA, while in other embodiments it is RNA. In some embodiments the double stranded region of the nucleic acid molecule is a perfect duplex. In other embodiments the double stranded region contains at least one bulge region. In some embodiments the passenger strand comprises a nick within the double stranded region of the molecule. The double stranded region may contain at least one nucleotide that is phosphorothioate modified.

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[001083] Nucleic acid molecules utilized in accordance with the invention may be chemically modified. In certain embodiments the chemical modification is 2'Omethyl and/or 2'Fluoro. In some embodiments more than one chemical modification is present in the same molecule. In some embodiments chemical modification increases stability, increases evasion of immune regulation, and/or prevents off-target gene silencing. Chemical modification can be present on the passenger strand and/or the guide strand.

[001084] In some embodiments the single stranded region of at least eight phosphorothioate modified nucleotides is cleaved from the double stranded region of the nucleic acid molecule in a cell. In some embodiments the single stranded region of at least eight phosphorothioate modified nucleotides has complementarity to a mammalian gene. In certain embodiments the single stranded region of at least eight phosphorothioate modified nucleotides functions as an antisense molecule. The double stranded region may be at least 19 nucleotides long. In some embodiments the single stranded region is at least 12 nucleotides long.

[001085] In some embodiments, the invention relates to methods for inhibiting the expression of a target gene in a mammalian cell, comprising contacting the mammalian cell with an isolated double stranded nucleic acid molecule comprising a guide strand and a passenger strand, wherein the guide strand is connected through a cleavable linker to a single stranded region of at least eight phosphorothioate modified nucleotides. In some embodiments, the cleavable linker includes one or more unmodified nucleotides. In other embodiments, the cleavable linker is a phosphodiester bond. In certain embodiments, the cleavable linker is S-S. In some embodiments the cleavable linker is DNA or RNA.

[001086] In some embodiments, the invention relate to methods for inhibiting the expression of a target gene in a mammalian cell, including contacting the mammalian cell with a bifunctional nucleic acid molecule including a double stranded region that functions in RNA interference and a single stranded region that functions in antisense, wherein the double stranded region includes a guide strand and a passenger strand, and wherein the double stranded region and the single stranded region are connected through a cleavable linker. In other aspects a method for inhibiting the expression of a target gene in a mammalian cell is provided. The method involves contacting the mammalian cell with any of the isolated double stranded nucleic acid molecules described herein.

[001087] In some embodiments, isolated double stranded nucleic acid molecules utilized in accordance with the present invention include a chemical modification that increases stability. In some embodiments, isolated double stranded nucleic acid molecules include a chemical modification that increases evasion of immune regulation. In some embodiments, the isolated double stranded nucleic acid molecules include a chemical modification that prevents off-target gene silencing.

[001088] In some embodiments, as discussed herein, utilized oligonucleotides are single oliogonucleotide molecules capable of self-dimerizing into a partially complementaly duplex. In some embodiments, such single oligonucleotides are about 23-30 nucleotides in length, e.g., 23, 24, 25, 26, 27, 28, 29, and 30. In some embodiments, single oligonucloetides capable of self-dimerizing into a partially complementaly duplex contain about 14-18 nucleotide mRNA targeting region, e.g., 14, 15, 16, 17, and 18. In some embodiments, single oligonucloetides capable of self-dimerizing into a partially complementaly duplex contain an additional 7-11, e.g., 7, 8, 9, 10, and 11 nucleotides to enable self-dimerization into a partially complementary duplex. In some embodiments, single oligonucloetides capable of self-dimerizing into a partially complementaly duplex can efficiently enter and activate the RNA-induced silencing complex (RISC).

Ul adaptors

[001089] In some embodiments, provided compositions include one or more oligonucleotides that are or act as U1 adaptors.

[001090] Ul adaptors inhibit polyA sites and are bifunctional oligonucleotides with a target domain complementary to a site in the target gene's terminal exon and a 'Ul domain' that binds to the Ul smaller nuclear RNA component of the Ul snRNP. See for example, Int. Pat. App. Pub. No. W02008/121963 and Goraczniak, et al., 2008, Nature Biotechnology, 27(3), 257-263, each of which is incorporated by reference herein, in its entirety. Ul snRNP is a ribonucleoprotein complex that functions primarily to direct early steps in spliceosome formation by binding to the pre-mRNA exon-intron boundary, Brown and Simpson, 1998, Annu Rev Plant Physiol Plant Mol Biol 49:77-95.

[001091] In some embodiments, a utilized oligonucleotide is a Ul adaptor, wherein the oligonucleotide comprises at least one annealing domain (targeting domain) linked to at least one

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effector domain (U1 domain), wherein the annealing domain hybridizes to a target gene sequence and the effector domain hybridizes to the Ul snRNA of Ul snRNP. In some embodiments, the Ul adaptor comprises one annealing domain. In some embodiments, the Ul adaptor comprises one effector domain.

[001092] Without wishing to be bound by theory, the annealing domain will typically be from about 10 to about 50 nucleotides in length, rnore typically from about 10 to about 30 nucleotides or about 10 to about 20 nucleotides. In some embodiments, the annealing, domain is 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 21 nucleotides in length. The annealing domain may be at least 75%, at .least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least about 100% complementary to the target gene. In some embodiments, the annealing domain hybridizes with a target site within the *Y* terminal exon of a pre-MRNA, which includes the terminal coding region and the 3'UTR and polyalenylation signal sequences (e.g., through the polyadenyiation site). In some embodiments, the target sequence is within about 500 'basepair, about 250 basepair, about 100 basepair, or about 50 basepair of the poly (A) signal sequence of the, pre-mRNA. In some embodiments, the annealing domain comprises 1, 2, 3, or 4, mismatches with the target gene sequence.

[001093] In some embodiments, the effector domain is from about 8 nucleotides to about 30 nucleotides in length. In some embodiments, the effector domain is from about 10 nucleotides to about 20 nucleotides in length. In some embodiments, the effector domain is from about 10 nucleotides to about 15 nucleotides in length. The U1 domain can hybridize with UI snRNA, particularly the 5' end and more specifically nucleotides 2-11. In some embodiments, the Ul domain. is perfectly complementary to nucleotides 2-11 of endogenous U1 snRNA. In some embodiments, the U1 domain comprises a nucleotide sequence selected from the group consisting of SEQ .ID NO: 1 (5'- GCCAGGIL1AAGUAU-3'), SEQ .1D NO: 2 (5"-CCAGGLIAA.GUAII-3"). SEQ 11D NO: 4 (5"- CAGGUAAGUAU-3'), SEQ ID NO: 5 (5 ^{2}-CAGGLIAAGU-3'), SEQ ID NO: 6 (5'-- CM.16ITAAC1-3'), and. **SEQ ID NO:** 7 (5'-CAGG1IA2=',,3'). In some embodiments; the III domain comprises a nucleotide sequence, SEQ ID NO: 8 (5"-CAGGUAAGUA-3"). Without wishing to be bound by theory, increasing the length of the U1 domain to include basepairing into stem 1 and/or basepairing to position 1 of U1 snRNA improves th.e U1 adaptor's affinity to Ul snRNA.

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[001094] The annealing and effector domains of the Ul adaptor can be linked such that the effector domain is at the 5' end andlor 3' end of the annealing domain. The two domains can be linked by such that the 3' end of one domain is linked to 5' end of the other domain, or 3' end of one domain is linked to 3' end of the other domain, or 5' end of one domain is linked to 5' end of the other domain. The annealing and effector domains can be linked directly to each other or by a nucleotide based or non-nucleotide based linker. In some embodiments, a linker is nucleotide base and comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, up to 15, up to 20, or up to 25 nucleotides.

[001095] In some embodiments, a linker between an annealing domain and an effector dornain is multivalent, e.g., trivalent, tetravalent. pentavalent. Without wishing to be bound by theory, a multivalent linker can be used to .link togther a single annealing domain with a plurality of adaptor domains.

[001096] In some embodiments, a Ul adaptor comprises any oligonucleotide modification described herein. Exemplary such modifications include those that increase annealing affinity, specificity, bioavailability in the cell and organism, cellular and/or nuclear transport, stability, and/or resistance to degradation. In some embodiments, the U1 adaptor can be administered in combination with at least one other RNAi agent.

RNA activators

[001097] In some embodiments, provided compositions include one or more oligonucleotides that are or act as RNA activators.

[001098] Recent studies have found that dsRNA can also activate gene expression, a mechanism that has been termed "small RNA-induced gene activation" or RNAa. See for example Li, L.C. et al. *Proc Nati Auld Sci U S A.* (2006), 103(46):17337-42 and Li L.C. (2008). "Small RNAMediated Gene Activation". RNA and the Regulation of Gene Expression: A Hidden Layer of Complexity. Caister Academic Press. ISBN 978-1-904455-25-7. It has been shown that dsRNAs targeting gene promoters induce potent transcriptional activation of associated genes. Endogenous miRNA that cause RNAa has also been found in humans. Check E. Nature (2007).
448 (7156): 855-858.

[001099] Another observation is that gene activation by RNAa is long-lasting. Induction of gene expression has been seen to last for over ten days. The prolonged effect of RNAa could be attributed to epigenetic changes at dsRNA target sites.

[001100] In some embodiments, a provided oligonucleotide is an RNA activator, wherein a provided oligonucleotide increases the expression of a gene. In some embodiments, increased gene expression inhibits viability, growth development, and/or reproduction.

Non-coding RNAs (ncRNAs)

[001101] The present invention encompasses non-coding RNAs, such as long non-coding RNAs (lncRNAs) and short non-coding RNAs (sncRNAs).

[001102] Accordingly, provided oligonucleotide compositions of the present invention may be useful for modulating disease-associated long non-coding RNA. In some embodiments, oligonuclotides synthesized according to the methods provided herein are used to specifically block the binding of transcriptional repressor complexes to target lncRNA regions, thereby inducing the expression of the associated target gene. In some embodiments, the transcriptional repressor complex is a silencing factor. In some embodiments, the transcriptional repressor complex has Histone methyltransferase activity. In some embodiments, the transcriptional repressor complex methylates histone H3. In some embodiments, the transcriptional repressor complex is PRC2 (Polycomb Repressive Complex 2).

[001103] Certain PRC2-associated lncRNAs have been reported to be potential therapeutic targets and/or biomarkers (Zhao et al., 2010. "Genome-wide Identification of Polycomb-Associated RNAs by RIP-seq" Molelcular Cell 40: 939-53). Overexpression of PCR2 proteins have been linked to various types of cancer, including metastatic prostate and breast cancer, and cancers of the colon, breast, and liver. Pharmacological inhibition of PRC2-mediated gene repression was found to induce apoptosis in several cancer cell lines in vitro, but not in various types of normal cells. Induction of apoptosis in this system is dependent on reactivation of genes that had been repressed by PRC2. There is also evidence that PRC2-mediated gene repression may be linked to the maintenance of the stem-cell properties of cancer stem cells. These results suggest that at least in some cases, inhibition of PRC2-mediated gene repression–including via targeting lncRNAs that recruit PRC2 to critical genes–is a potential strategy for treating various types of cancer. Non-limiting sequences of nucleic acids, which can be prepared in accordance with the methods provided herein, can be found in, for example, International Patent Publication WO 2012/065143 entitiled "Polycomb-associated non-coding RNAs" (PCT/US2011/60493), the contents of which are incorporated by reference herein.

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[001104] Provided oligonucleotide compositions of the present invention may be or act as small non-coding RNAs, such as piwi-interacting RNAs (piRNAs). The piRNAs represent the largest class of small non-coding RNA molecules that is expressed in animal cells and are found in clusters throughout the genome. piRNAs are known to form RNA-protein complexes through interactions with piwi proteins. piRNA complexes have been implicated in both epigenetic and post-transcriptional gene silencing of retrotransposons and other genetic elements in germ cells, including spermatogenesis. Typically piRNAs are 26-31 nucleotides in length, and, as compared to typical miRNAs, they lack sequence conservation and exhibit higher complexity. In some embodiments, oligonucleotide compositions utilized in accordance with the present invention comprise 5' uridine. In some embodiments, oligonucleotide compositions utilized in accordance with the present invention comprise 5' monophosphoate and a 3' modification that acts to block either the 2' or 3' oxygen. In some embodiments, the 3' modification is a 2'-O-methylation.

Triplex forming oligonucleotides

[001105] In some embodiments, provided compositions include one or more oligonucleotides that are or act as triplex-forming oligonucleotides.

[001106] Recent studies have shown that triplex forming oligonucleotides (TFO) can be designed which can recognize and bind to polypurine/polypyrimidine regions in double-stranded helical DNA in a sequence-specific manner. These recognition rules are outline by Maher III, L.J., et al., *Science* (1989) vol. 245, pp 725-730; Moser, H. E., et al., *Science* (1987) vol. 238, pp 645- 630; Beal, P.A., et al., *Science* (1992) vol. 251, pp 1360-1363; Conney, M., et al., *Science* (1988) vol. 241, pp 456-459 and Hogan, M.E., et al., EP Publication 375408. Modification of the oligonucteotides, such as the introduction of intercalators and intersugar linkage substitutions, and optimization of binding conditions (pH and cation concentration) have aided in. overcoming inherent obstacles to TFO activity such as charge repulsion and instability, and it was recently shown that oligonucleotides can be targeted to specific sequences (for a recent review sec Seidman and Glazer, Clin Invest 2003 2.:487-94). In generaL the triplex-forming oligonucteotide has the sequence correspondence:

oligo 3'-A G G T

duplex 5'-A G C T

duplex 3'-T C G A

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[001107] However, it has been shown that the A-AT and G-GC triplets have the greatest triple helical stability (Reither and Jeltsch, BMC Biochem, 2002, Sept12, Epub). The same authors have demonstrated that TFOs designed according to the A-AT and G-GC rule do not form nonspecific triplexes, indicating that the triplex formation is indeed sequence specific. Thus for any given sequence a triplex forming sequence can be devised. In some embodiments, triplex-forming oligonucleotides are at least about 15, about 25, or about 30 or more nucleotides in length. In some embodiments, triplex-forming oligonucleotides are up to about 50 or about 100 nucleotides in length.

[001108] Formation of the triple helical structure with the target DNA induces steric and functional changes, blocking transcription initiation and elongation, allowing the introduction of desired sequence changes in the endogenous DNA and resulting in the specific down-regulation of gene expression. Examples of such suppression of gene expression in cells treated with TFOs include knockout of episomal supFGI and endogenous HPRT genes in mammalian cells (Vasquez et al., Nucl Acids Res. 1999;27: 1176-81, and Puri, et al, J Biol Chem, 2001;276:28991-98), and the sequence- and target specific downregulation of expression of the Ets2 transcription factor, important in prostate cancer etiology (Carbone, et al, Nucl Acid Res. 2003 ;31:833-43), and the pro-inflammatory ICAM-I gene (Besch et al, J Biol Chem, 2002;277:32473-79). In addition, Vuyisich and Beal have recently shown that sequence specific TFOs can bind to dsRNA, inhibiting activity dsRNA-dependent enzymes such as RNA- dependent kinases (Vuyisich and Beal, Nuc, Acids Res 2000;28:2369-74).

[001109] Additionally, TF0s designed according to the abovementioned principles can induce directed mutagenesis capable of effecting DNA repair, thus providing both down-regulation and up-regulation of expression of endogenous genes (Seidman and Glazer, JInvest 2003; 112:487-94). Detailed description of the design, synthesis and administration of effective TFOs can be found. S. Pat. App. Nos. 2003 017068 and 2003 0096980 to Froehier et at and 2002 012821/8 and 2002 0123476 to Emanude et aE, and U.S. Pat. No. 5,721,138 to Lawn, the contents of which are herein incorporated in their entireties.

Conjugates/linkers

[001110] The invention also contemplates that utilized oligonucleotides, in some embodiments, are optimized for cellular uptake. In any utilized oligonucleotides embraced by

the present invention, guide and/or passenger strands may be attached to a conjugate. In some embodiments the conjugate is hydrophobic. The hydrophobic conjugate can be a small molecule with a partition coefficient that is higher than 10. The conjugate can be a sterol-type molecule such as cholesterol, or a molecule with an increased length polycarbon chain attached to C17, and the presence of a conjugate can influence the ability of an RNA molecule to be taken into a cell with or without a lipid transfection reagent. The conjugate can be attached to the passenger or guide strand through a hydrophobic linker. In some embodiments, a hydrophobic linker is 5-12C in length, and/or is hydroxypyrrolidine-based. In some embodiments, a hydrophobic conjugate is attached to the passenger strand and the CU residues of either the passenger and/or guide strand are modified. In some embodiments, at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95% of the CU residues on the passenger strand and/or the guide strand are modified. In some aspects, molecules associated with the invention are self-delivering (sd). As used herein, "self-delivery" refers to the ability of a molecule to be delivered into a cell without the need for an additional delivery vehicle such as a transfection reagent. Aspects of the invention relate to selecting molecules for use in RNAi.

[001111] In any of the embodiments embraced herein, utilized oligonucleotides may be associated with a hydrophobic moiety for targeting and/or delivery of the molecule to a cell. In some embodiments, the hydrophobic moiety is associated with the nucleic acid molecule through a linker. In some embodiments, the association is through non-covalent interactions. In other some, the association is through a covalent bond. Any linker known in the art may be used to associate the nucleic acid with the hydrophobic moiety. Linkers known in the art are described in published international PCT applications, WO 92/03464, WO 95/23162, WO 2008/021157, WO 2009/021157, WO 2009/134487, WO 2009/126933, U.S. Patent Application Publication 2005/0107325, U.S. Patent 5,414,077, U.S. Patent 5,419,966, U.S. Patent 5,512,667, U.S. Patent 5,646, 126, and U.S. Patent 5,652,359, which are incorporated herein by reference. The linker may be as simple as a covalent bond to a multi-atom linker. The linker may be cyclic or acyclic. The linker may be optionally substituted. In some embodiments, the linker is capable of being cleaved from the nucleic acid. In certain embodiments, the linker is capable of being hydrolyzed under physiological conditions. In some embodiments, the linker is capable of being cleaved by an enzyme (e.g. , an esterase or phosphodiesterase). In some embodiments, the linker comprises a spacer element to separate the nucleic acid from the hydrophobic moiety. The spacer element

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may include one to thirty carbon or heteroatoms. In certain embodiments, the linker and/or spacer element comprises protonatable functional groups. Such protonatable functional groups may promote the endosomal escape of the nucleic acid molecule. The protonatable functional groups may also aid in the delivery of the nucleic acid to a cell, for example, neutralizing the overall charge of the molecule. In some embodiments, the linker and/or spacer element is biologically inert (that is, it does not impart biological activity or function to the resulting nucleic acid molecule).

[001112] The hydrophobic molecule may be connected to the polynucleotide by a linker moiety. Optionally the linker moiety is a non-nucleotidic linker moiety. Non-nucleotidic linkers are e.g. abasic residues (dSpacer), oligoethyleneglycol, such as triethyleneglycol (spacer 9) or hexaethylenegylcol (spacer 18), or alkane-diol, such as butanediol. The spacer units are preferably linked by phosphodiester or phosphorothioate bonds. The linker units may appear just once in the molecule or may be incorporated several times, e.g. via phosphodiester, phosphorothioate, methylphosphonate, or amide linkages.

[001113] Typical conjugation protocols involve the synthesis of polynucleotides bearing an aminolinker at one or more positions of the sequence, however, a linker is not required. The amino group is then reacted with the molecule being conjugated using appropriate coupling or activating reagents. The conjugation reaction may be performed either with the polynucleotide still bound to a solid support or following cleavage of the polynucleotide in solution phase. Purification of the modified polynucleotide by HPLC typically results in a pure material.

[001114] In some embodiments, a linking group can be attached to a nucleomonomer and the transporting peptide can be covalently attached to the linker. In some embodiments, a linker can function as both an attachment site for a transporting peptide and can provide stability against nucleases. Examples of suitable linkers include substituted or unsubstituted C_1-C_{20} alkyl chains, C_2-C_{20} alkenyl chains, C_2-C_{20} alkynyl chains, peptides, and heteroatoms (e.g. , S, O, NH, etc.). Other exemplary linkers include bifunctional crosslinking agents such as sulfosuccinimidyl-4-(maleimidophenyl)-butyrate (SMPB) (see, e.g. , Smith et al. Biochem J 1991.276: 417-2).

[001115] In some embodiments, provided oligonucleotides of the invention are synthesized as molecular conjugates which utilize receptor-mediated endocytotic mechanisms for delivering

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genes into cells (see, e.g. , Bunnell et al. 1992. Somatic Cell and Molecular Genetics. 18:559, and the references cited therein).

Targeting Agents

[001116] In some embodiments, provided compositions include one or more targeting agents, associated with the composition and/or with individual oligonucleotides therein. Exemplary such targeting agents are described above and further herein. The phrases "targeting agent" and "targeting moiety" are used herein interchangeably.

[001117] Delivery of oligonucleotides and/or compositions thereof can often be improved by targeting the oligonucleotides to a cellular receptor. The targeting moieties can be conjugated to the oligonucleotides or attached to a carrier group (i.e. , poly(L-lysine) or liposomes) linked to the oligonucleotides. This method is well suited to cells that display specific receptor- mediated endocytosis.

[001118] For instance, oligonucleotide conjugates to 6-phosphomannosylated proteins are internalized 20-fold more efficiently by cells expressing mannose 6-phosphate specific receptors than free oligonucleotides. The oligonucleotides may also be coupled to a ligand for a cellular receptor using a biodegradable linker. In another example, the delivery construct is mannosylated streptavidin which forms a tight complex with biotinylated oligonucleotides. Mannosylated streptavidin was found to increase 20-fold the internalization of biotinylated oligonucleotides. (Vlassov et al. 1994. Biochimica et Biophysica Acta 1197:95-108).

[001119] The field of RNA interference has revolutionized the study of biological processes. These tools include small inhibitory RNA (siRNA), small hairpin RNA (shRNA) and ribozymes. As RNA interference technology has grown, so has the list of validated RNA target sequences and reagents for use. Provided compositions and methods embraced by the present application can be readily applied to each of such target sequences. For a database of validated siRNA target sequences and links to kits and reagents that may be used for RNA interference experiments, see, for example, http://www.rnainterference.org/index.html. Exemplary siRNA target sequences useful for the present invention are provided in the accompanying **Appendix (A)**.

Immunomodulatory Oligonucleotides

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[001120] In some embodiments, provided compositions include one or more oligonucleotides that are or act as immunomodulatory oligonucleotides.

[001121] Oligonucleotides utilized in accordance with the invention can be immunomodulatory agents, i.e., agents that are capable of modulating or regulating an immune response when administered to a subject. Immune responses elicited by such an agent can be an innate and/or an adaptive immune response. The immune system is divided into a more innate immune system, and acquired adaptive immune system of vertebrates, the latter of which is further divided into humoral cellular components. In some embodiments, the immune response can be mucosal. Immunomodulatory motifs described herein can be used in the context of previously described classes of immunomodulatory oligonucleotides including ODN classes such as A class, B class, C class, E class, T class and P class.

[001122] In some embodiments of the invention immunomodulatory oligonucleotides utilized in accordance with the present invention include one or more immunostimulatory motifs. In some embodiments, oligonucleotides utilized in accordance with the present invention include one or more "CpG dinucleotides." A CpG dinucleotide can be methylated or unmethylated. An immunostimulatory oligonucleotide containing at least one unmethylated CpG dinucleotide is an oligonucleotide molecule which contains an unmethylated cytosine-guanine dinucleotide sequence (i.e., an unmethylated 5' cytidine followed by 3' guanosine and linked by a phosphate bond) and which activates the immune system; such an immunostimulatory oligonucleotide is a CpG oligonucleotide. CpG oligonucleotides have been described in a number of issued patents, published patent applications, and other publications, including: U.S. Patent Nos. 6,194,388; 6,207,646; 6,214,806; 6,218,371 ; 6,239,116; and 6,339,068, the contents of which are incorporated herein by reference. In some embodiments, utilized oligonucleotides are or act as an agonist for a Toll-like receptor. In some embodiments, utilized oligonucleotides are or act as an agonist for Toll-like receptor 9 (TLR9). In some embodiments, utilized oligonucleotides are or act as an antagonist for a Toll-like receptor. In some embodiments, utilized oligonucleotides are or act as an antagonist for Toll-like receptor 9 (TLR9). In some embodiments, stereo-defined oligonucleotides embraced by the invention exhibit greater affinity for respective receptor/target, as compared to stereo-random counterpart. In some embodiments, stereo-defined oligonucleotides embraced by the invention elicit one or more immune responses, when administered to subjects that meet certain clinical criteria, with

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less degree of variables amongst the population, as compared to stereo-random counterpart. In some embodiments, stereo-defined oligonucleotides embraced by the invention cause less degree of toxic side effects or fewer side effects, when administered to subjects that meet certain clinical criteria, as compared to stereo-random counterpart.

[001123] In some embodiments, immunostimulatory oligonucleotides utilized in accordance with the present invention are free of CpG dinucleotide motifs. These oligonucleotides which are free of CpG dinucleotides are referred to as non-CpG oligonucleotides, and they have non-CpG immunostimulatory motifs. In some embodiments, these are T-rich immunostimulatory oligonucleotides, such as oligonucleotides having at least 80% T.

[001124] In some embodiments, immunostimulatory oligonucleotides utilized in accordance with the present invention are B class immunomodulatory oligonucleotides.

[001125] "B class" ODN are potent at activating B cells but are relatively weak in inducing IFN-α and NK cell activation. The B class CpG oligonucleotides typically are fully stabilized and include an unmethylated CpG dinucleotide within certain preferred base contexts. See, e.g., U.S. Patent Nos. 6,194,388; 6,207,646; 6,214,806; 6,218,371 ; 6,239,116; and 6,339,068.

[001126] Another class is potent for inducing IFN-α and NK cell activation but is relatively weak at stimulating B cells; this class has been termed the "A class". In some embodiments, immunostimulatory oligonucleotides utilized in accordance with the present invention are A class immunomodulatory oligonucleotides. The A class CpG oligonucleotides typically have stabilized poly-G sequences at 5' and 3' ends and a palindromic phosphodiester CpG dinucleotide-containing sequence of at least 6 nucleotides. See, for example, published patent application PCT/US00/26527 (WO 01/22990).

[001127] Yet another class of CpG oligonucleotides activates B cells and NK cells and induces IFN-α; this class has been termed the C-class. In some embodiments, immunostimulatory oligonucleotides utilized in accordance with the present invention are C class immunomodulatory oligonucleotides. The "C class" immunostimulatory oligonucleotides contain at least two distinct motifs have unique and desirable stimulatory effects on cells of the immune system. Some of these ODN have both a traditional "stimulatory" CpG sequence and a "GC-rich" or "B-cell neutralizing" motif. These combination motif oligonucleotides have immune stimulating effects that fall somewhere between those effects associated with traditional

"class B" CpG ODN, which are strong inducers of B cell activation and dendritic cell (DC) activation, and those effects associated with a more recently described class of immune stimulatory oligonucleotides ("class A" CpG ODN) which are strong inducers of IFN-α and natural killer (NK) cell activation but relatively poor inducers of B-cell and DC activation. Krieg AM et al. (1995) Nature 374:546-9; Ballas ZK et al. (1996) J Immunol 157:1840-5; Yamamoto S et al. (1992) J Immunol 48:4072-6. While preferred class B CpG ODN often have phosphorothioate backbones and preferred class A CpG ODN have mixed or chimeric backbones, the C class of combination motif immune stimulatory oligonucleotides may have either stabilized, e.g., phosphorothioate, chimeric, or phosphodiester backbones, and in some preferred embodiments, they have semi-soft backbones. This class has been described in U.S. patent application USI 0/224,523 filed on August 19, 2002, the entire contents of which is incorporated herein by reference.

[001128] In some embodiments, immunostimulatory oligonucleotides utilized in accordance with the present invention are E class immunomodulatory oligonucleotides. The "E class" oligonucleotides have an enhanced ability to induce secretion of IFN-alpha. These ODN have a lipophilic substituted nucleotide analog 5' and/or 3' of a YGZ motif. The compound of the E class formula may be, for example, any of the following lipophilic substituted nucleotide analogs: a substituted pyrimidine, a substituted uracil, a hydrophobic T analog, a substituted toluene, a substituted imidazole or pyrazole, a substituted triazole, 5-chloro-uracil, 5-bromo-uracil, 5-iodo- uracil, 5-ethyl-uracil, 5-propyl-uracil, 5-propinyl-uracil, (E)-5-(2-bromovinyl)-uracil, or 2.4- difluoro-toluene. E class oligonucleotides are described at least in provisional patent application US 60/847,811.

[001129] In some embodiments, immunostimulatory oligonucleotides utilized in accordance with the present invention are T class immunomodulatory oligonucleotides. The "T class" oligonucleotides induce secretion of lower levels of IFN-alpha when not modified as in the ODNs of the invention and IFN-related cytokines and chemokines than B class or C class oligonucleotides, while retaining the ability to induce levels of IL-10 similar to B class oligonucleotides. T class oligonucleotides are described at least in US Patent Application No. 11/099,683, the entire contents of which are hereby incorporated by reference.

[001130] In some embodiments, immunostimulatory oligonucleotides utilized in accordance with the present invention are P class immunomodulatory oligonucleotides. The "P

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class" immunostimulatory oligonucleotides have several domains, including a 5TLR activation domain, 2 duplex forming regions and an optional spacer and 3' tail. This class of oligonucleotides has the ability in some instances to induce much higher levels of IFN-α secretion than the C-Class. The P-Class oligonucleotides have the ability to spontaneously self-assemble into concatamers either in vitro and/or in vivo. Without being bound by any particular theory for the method of action of these molecules, one potential hypothesis is that this property endows the P-Class oligonucleotides with the ability to more highly crosslink TLR9 inside certain immune cells, inducing a distinct pattern of immune activation compared to the previously described classes of CpG oligonucleotides. Cross-linking of TLR9 receptors may induce activation of stronger IFN-α secretion through the type I IFNR feedback loop in plasmacytoid dendritic cells. P class oligonucleotides are described at least in US Application Serial Number 11/706,561.

[001131] In some embodiments, immunostimulatory oligonucleotides utilized in accordance with the present invention are S class immunomodulatory oligonucleotides. The immunomodulatory oligonucleotides of the instant invention may be immunosuppressive oligonucleotides. The immunomodulatory motifs described above can be used in the context of previously described classes of immunosuppressive oligonucleotides including ODN classes such as the "S class". Inhibitory, or S class, ODN are useful whenever it is desirable to inhibit immunostimulation. Inhibitory ODN can be used for preventing and treating septic shock, inflammation, allergy, asthma, graft rejection, graft-versus host disease (GvHD), autoimmune diseases, Th1- or Th2- mediated diseases, bacterial infections, parasitic infections, spontaneous abortions, and tumors. The inhibitory ODN can be used generally to inhibit activation of all cells expressing the relevant TLRs, and more specifically to inhibit activation of antigen- presenting cells, B cells, plasmacytoid dendritic cells (pDCs), monocytes, monocyte- derived cells, eosinophils, and neutrophils. S class ODN are further described at least in US Application Serial No. 10/977,560.

[001132] According to the invention, immunomodulatory oligonucleotides may have a backbone of stabilized internucleotide linkages in addition to the stabilizing FANA purine nucleotide(s) or have a chimeric backbone of stabilized and phosphodiester nucleotide linkages. A "stabilized internucleotide linkage" shall mean an internucleotide linkage that is relatively resistant to in vivo degradation (e.g., via an exo- or endo-nuclease), compared to a

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phosphodiester internucleotide linkage. In some embodiments, stabilized internucleotide linkages include, without limitation, phosphorothioate, phosphorodithioate, methylphosphonate, methylphosphorothioate, phosphonoacetate, Rp-phosphorothioate, Sp- phosphorothioate, boranophosphate, or 3'-thioformacetal, or combinations thereof. Other stabilized oligonucleotides include: nonionic DNA analogs, such as alkyl- and aryl-phosphates (in which the charged phosphonate oxygen is replaced by an alkyl or aryl group), phosphodiester and alkylphosphotriesters, in which the charged oxygen moiety is alkylated. Oligonucleotides which contain diol, such as tetraethyleneglycol or hexaethyleneglycol, at either or both termini have also been shown to be substantially resistant to nuclease degradation.

[001133] As described in more detained herein, according to the invention, these and other modifications can be selectively introduced at a predetermined position(s)/pattern(s) within an oligonucleotide so as to obtain a stereospecific oligonucleotide molecule. Furthermore, according to the invention, a compositon comprising a plurality of such oligonucleotides at an extremely high degree of purity (e.g., at least 96%, at least 97%, at least 98%, at least 99%, essentially 100%) can be obtained. Thus, provided oligonucleotide compositions comprising oligonucleotides of one or more predetermined types are suitable for *in vivo* administration. It is contemplated that, due to high degree of structural purity of oligonucleotides in a composition (such that a composition comprises a single specified type of oligonucleotides), provided compositions can elicit improved biological activities, increased efficacy, reduced variables in response, and/or reduced unwanted side effects, when administered to subjects.

[001134] Accordingly, utilized oligonucleotides of the invention are particularly useful as an therapeutic agent when formulated into a pharmaceutical composition. In some embodiments, such therapeutic agent is an immunomodulatory agent. In some embodiments, provided immunomodualtory agents are immunostimulatory agents. In some embodiments, provided immunomodulatory agents are immunoinhibiotry (or immunosuppressive) agents. In some embodiments, provided immunomodulatory agents act as an adjuvant. In some embodiments, provided immunomodulatory agents can shift a Th2 type immune response to a Th1 type immune response by inducing Th1 type cytokines that can suppress inflammatory Th2 response. In some embodiments, provided oligonucleotide compositions are useful as an agent that regulates gene expression. For example, in some embodiments, provided oligonucleotide compositions are useful as an agent capable of silencing a gene of interest, e.g., genes associated

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with disease or disorder. In some embodiments, provided oligonucleotide compositions are useful as an agent to regulate RNA splicing.

[001135] For example, some embodiments of such oligonucleotides include those comprising at least one CpG dinucleotide motif. In some embodiments, such oligonucleotides comprise at least one unmethylated CpG dinucleotide motif. In some embodiments, CpG-containing oligonucleotides useful for the present invention are categorized as Class A (type D) oligonucleotides. In some embodiments, CpG-containing oligonucleotides useful for the present invention are categorized as Class B (type K) oligonucleotides. In some embodiments, CpG-containing oligonucleotides useful for the present invention are categorized as Class C oligonucleotides. In some embodiments, CpG-containing oligonucleotides useful for the present invention are categorized as Class P oligonucleotides. In some embodiments, CpG-containing oligonucleotides useful for the present invention contain at least one palindromic sequence. In some embodiments, CpG-containing oligonucleotides useful for the present invention can form a "dumbbell-like" structure. In some embodiments, CpG-containing oligonucleotides useful for the present invention can form a "Y" shaped structure, or multimers thereof. In some embodiments, CpG-containing oligonucleotides useful for the present invention can form a tetrahedral structure. For reviews, see, for example: Bode et al. (2011) Expert Rev. Vaccines 10(4): 499-511; Hanagata (2012) Int. J. Nanomedicine 7:2181-95.

[001136] In some embodiments, oligonucleotides utilized in accordance with the present invention elicit immunomodulatory effects on cells expressing Toll-like receptor(s). In some embodiments, target cells that respond to such oligonucleotides include, but are not limited to, antigen-presenting cells (APCs), antigen-specific T and B cells, cytotoxic T lymphocytes (CTLs), natural killer (NK) cells, and dendritic cells (DCs). In some embodiments, immunomodulatory effects are the direct effects elicited by cells expressing a receptor or receptors that recognize such an oligonucleotide. In some embodiments, such oligonucleotides elicit immunomodulatory effects on cells expressing Toll-like receptor 9 (TLR9) as ligands. For example, some embodiments of the invention include CpG oligonucleotides that act as agonists of one or more Toll-like receptors. Some embodiments of the invention include CpG oligonucleotides that act as antagonists of one or more Toll-like receptors. In some embodiments, immunomodulatory effects are indirect effects that occur downstream, involving cells that do not necessarily respond directly to such oligonucleotides or express such receptors.

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[001137] In some embodiments, CpG oligonucleotides useful for the present invention are characterized in that they directly activate human B cells and plasmacytoid dendritic cells via TLR-9. In some embodiments, CpG oligodeoxynucleotides useful for the present invention are characterized in that indirectly support the maturation and proliferation of natural killer cells, T cells and monocytes/macrophages.

[001138] In some embodiments, CpG oligonucleotides useful for the present invention trigger an immune response, which is characterized by the production of Th1-type and proinflammatory cytokines, chemokines and polyreactive IgM.

[001139] In some embodiments, CpG oligonucleotides useful for the present invention are characterized in that the immunogenicity of conventional protein antigens and peptide-based vaccines is enhanced by such oligonucleotides. Without wising to be bound by a particular theory, it is believed that such adjuvant effect is mediated through improved function of professional antigen-presenting cells and the resultant generation of humoral and cellular vaccine-specific immune responses.

[001140] In some embodiments, CpG oligonucleotides useful for the present invention are characterized in that CpG oligonucleotides increase the magnitude and accelerate the development of vaccineinduced responses. They also improve the induction of memory, thereby extending the duration of humoral and cellular immunity.

[001141] In some embodiments, CpG oligonucleotides useful for the present invention are characterized in that they boost immunity in groups of subjects (e.g., populations of patients) with reduced immune function, such as the elderly and those with suppressed immue system. They are effective when administered either systemically or mucosally. Preclinical and clinical trials using CpG oligonucleotides of mixed chirality (e.g., not chirally pure) demonstrate CpG oligonucleotides can boost the immunogenicity of vaccines targeting infectious diseases and cancer. Furthermore, clinical trials indicate that CpG oligonucleotides are reasonably safe when administered s vaccine adjuvants.

[001142] Immunomodulatory oligonucleotides prepared in accordance with the present disclosure are useful for a number of therapeutic applications. Therefore, utulized immunomodulatory oligonucleotides may be formulated into suitable pharmaceutical compositions. Such pharmaceutical compositons can be administered to a subject in an amount effective to treat a disease, disorder or condition, as described in further detail herein by suitable

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routes of administration. Accoring to the invention, an effective amount of an immunostimulatory oligonucleotides may be administered to a subject who is likely to benefit from boosting the immune system (e.g., enhanced immune response). In some embodiments, clinical benefits are conferred directly by an immunomodulatory oligonucleotide acting upon its target immune cells, e.g., via binding of the ligand to its Toll-like receptor proteins. In some embodiments, clinical benefits are achieved at least in part indirectly by overall boosting of the immune system of the subject.

[001143] As used herein, the terms "effective amount" and "effective dose" refer to any amount or dose of a compound or composition that is sufficient to fulfill its intended purpose(s), i.e., a desired biological or medicinal response in a tissue or subject at an acceptable benefit/risk ratio. The relevant intended purpose may be objective (i.e., measurable by some test or marker) or subjective (i.e., subject gives an indication of or feels an effect). In some embodiments, a therapeutically effective amount is an amount that, when administered to a population of subjects that meet certain clinical criteria for a disease or disorder (for example, as determined by symptoms manifested, disease progression/stage, genetic profile, etc.), a statistically significant therapeutic response is obtained among the population. A therapeutically effective amount is commonly administered in a dosing regimen that may comprise multiple unit doses. For any particular pharmaceutical agent, a therapeutically effective amount (and/or an appropriate unit dose within an effective dosing regimen) may vary, for example, depending on route of administration, on combination with other pharmaceutical agents. In some embodiments, the specific therapeutically effective amount (and/or unit dose) for any particular patient may depend upon a variety of factors including the disorder being treated and the severity of the disorder; the activity of the specific pharmaceutical agent employed; the specific composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, and/or rate of excretion or metabolism of the specific pharmaceutical agent employed; the duration of the treatment; and like factors as is well known in the medical arts. Those of ordinary skill in the art will appreciate that in some embodiments of the invention, a unit dosage may be considered to contain an effective amount if it contains an amount appropriate for administration in the context of a dosage regimen correlated with a positive outcome. In some embodiments, CpG oligodeoxynucleotides prepared according to the present invention therefore may exert improved efficacy and immunomodulatory effects due to their

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chiral purity. In some embodiments, an effective amount of provided immunomodulatory oligonucleotides is less than that of less-pure counterpart.

[001144] In some embodiments, an immunomodulatory oligonucleotide composition is administered to a healthy subject. In some embodiments, an immunostimulatory oligonucleotide composition is administered to a subject as part of a vaccine, in which the immunostimulatory oligonucleotide acts as an adjuvant. In some embodiments, an immunostimulatory oligonucleotide composition is administered to a subject with a suppressed (e.g., compromised) immune system. In some embodiments, a subject, whose immune system is suppressed and does not sufficiently respond to a conventional vaccine, responds to a vaccine comprising an immunostimulatory oligonucleotide embraced by the present invention. In some embodiments, a subject who may benefit from such a vaccine has a suppressed immune system associated with an infection, such as viral infection, e.g., HIV, HBV, HCV, etc.

[001145] The invention encompasses the use of immunomodulatory oligonucleotides contemplated herein for treatment of a subject having a condition that may be treated by stimulation or suppression of the immune response. Thus, provided immunomodulatory oligonucleotide compositions are useful for the treatment of diseases or conditions, including, without limitation, infection, cancer, allergy, asthma, an inflammatory condition, an autoimmune disease, and any combination thereof.

[001146] In some embodiments, provided immunomodulatory oligonucleotide compositions are useful in some aspects of the invention for the treatment of a subject at risk of developing a clinical condition. Non-limiting clinical conditions include allergy, asthma, an infection with an infectious organism, cancer, inflammation, and autoimmune disease. A subject at risk as used herein is a subject who has any risk of exposure to an infection causing pathogen or a cancer or an allergen or a risk of developing cancer. For instance, a subject at risk may be a subject who is planning to travel to an area where a particular type of infectious agent is found, or it may be a subject who through lifestyle or medical procedures is exposed to bodily fluids which may contain infectious organisms or directly to the organism, or even any subject living in an area where an infectious organism or an allergen has been identified. Subjects at risk of developing infection also include general populations to which a medical agency recommends vaccination with a particular infectious organism antigen. If the antigen is an allergen and the subject develops allergic responses to that particular antigen and the subject may be exposed to

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the antigen, i.e., during pollen season, then that subject is at risk of exposure to the antigen. A subject at risk of developing allergy or asthma includes those subjects that have been identified as having an allergy or asthma but that don't have the active disease during the immunomodulatory oligonucleotide treatment as well as subjects that are considered to be at risk of developing these diseases because of genetic or environmental factors. A subject at risk of developing a cancer is one who has a high probability of developing cancer. These subjects include, for instance, subjects having a genetic abnormality, the presence of which has been demonstrated to have a correlative relation to a higher likelihood of developing a cancer and subjects exposed to cancer causing agents such as tobacco, asbestos, or other chemical toxins, or a subject who has previously been treated for cancer and is in apparent remission. When a subject at risk of developing a cancer is treated with an antigen specific for the type of cancer to which the subject is at risk of developing and a CpG immunostimulatory oligonucleotide, the subject may be able to kill the cancer cells as they develop. If a tumor begins to form in the subject, the subject will develop a specific immune response against the tumor antigen.

[001147] In some embodiments, provided immunomodulatory oligonucleotide compositions are useful for treating a subject having an immune disease or disorder, including asthma, allergy, and related conditions.

[001148] A subject having an allergy is a subject that has or is at risk of developing an allergic reaction in response to an allergen. An allergy refers to acquired hypersensitivity to a substance (allergen). Allergic conditions include but are not limited to eczema, allergic rhinitis or coryza, hay fever, conjunctivitis, bronchial asthma, urticaria (hives) and food allergies, and other atopic conditions.

[001149] Allergies are generally caused by IgE antibody generation against harmless allergens. The cytokines that are induced by systemic or mucosal administration of immunomodulatory oligonucleotides are predominantly of a class called Th1 (examples are IL-12, IP-10, IFN-α and IFN-γ) and these induce both humoral and cellular immune responses. The other major type of immune response, which is associated with the production of IL-4 and IL-5 cytokines, is termed a Th2 immune response. In general, it appears that allergic diseases are mediated by Th2 type immune responses. Based on the ability of the immunomodulatory oligonucleotide to shift the immune response in a subject from a predominant Th2 (which is associated with production of IgE antibodies and allergy) to a balanced Th2/Th1 response (which

is protective against allergic reactions), an effective dose for inducing an immune response of a immunomodulatory oligonucleotide can be administered to a subject to treat or prevent asthma and allergy.

[001150] Thus, provided immunomodulatory oligonucleotide compositions have significant therapeutic utility in the treatment of allergic and non-allergic conditions such as asthma. Th2 cytokines, especially IL-4 and IL-5 are elevated in the airways of asthmatic subjects. These cytokines promote important aspects of the asthmatic inflammatory response, including IgE isotope switching, eosinophil chemotaxis and activation and mast cell growth. Th1 cytokines, especially IFN-γ and IL-12, can suppress the formation of Th2 clones and production of Th2 cytokines. Asthma refers to a disorder of the respiratory system characterized by inflammation, narrowing of the airways and increased reactivity of the airways to inhaled agents. Asthma is frequently, although not exclusively associated with atopic or allergic symptoms.

[001151] Provided immunomodulatory oligonucleotide compositions may also be administered in conjunction with an anti-allergy therapy. onventional methods for treating or preventing allergy have involved the use of allergy medicaments or desensitization therapies. Some evolving therapies for treating or preventing allergy include the use of neutralizing anti-IgE antibodies. Anti-histamines and other drugs which block the effects of chemical mediators of the allergic reaction help to regulate the severity of the allergic symptoms but do not prevent the allergic reaction and have no effect on subsequent allergic responses. Desensitization therapies are performed by giving small doses of an allergen, usually by injection under the skin, in order to induce an IgG-type response against the allergen. The presence of IgG antibody helps to neutralize the production of mediators resulting from the induction of IgE antibodies, it is believed. Initially, the subject is treated with a very low dose of the allergen to avoid inducing a severe reaction and the dose is slowly increased. This type of therapy is dangerous because the subject is actually administered the compounds which cause the allergic response and severe allergic reactions can result.

[001152] Anti-allergy medicaments include, but are not limited to, anti-histamines, corticosteroids, and prostaglandin inducers. Anti-histamines are compounds which counteract histamine released by mast cells or basophils. These compounds are well known in the art and commonly used for the treatment of allergy. Anti-histamines include, but are not limited to, acrivastine, astemizole, azatadine, azelastine, betatastine, brompheniramine, buclizine, cetirizine,

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cetirizine analogues, chlorpheniramine, clemastine, CS 560, cyproheptadine, desloratadine, dexchlorpheniramine, ebastine, epinastine, fexofenadine, HSR 609, hydroxyzine, levocabastine, loratidine, methscopolamine, mizolastine, norastemizole, phenindamine, promethazine, pyrilamine, terfenadine, and tranilast. Corticosteroids include, but are not limited to, methylprednisolone, prednisolone, prednisone, beclomethasone, budesonide, dexamethasone, flunisolide, fluticasone propionate, and triamcinolone. Although dexamethasone is a corticosteroid having antiinflammatory action, it is not regularly used for the treatment of allergy or asthma in an inhaled form because it is highly absorbed and it has long-term suppressive side effects at an effective dose. Dexamethasone, however, can be used according to the invention for treating allergy or asthma because when administered in combination with a composition of the invention it can be administered at a low dose to reduce the side effects. Some of the side effects associated with corticosteroid use include cough, dysphonia, oral thrush (candidiasis), and in higher doses, systemic effects, such as adrenal suppression, glucose intolerance, osteoporosis, aseptic necrosis of bone, cataract formation, growth suppression, hypertension, muscle weakness, skin thinning, and easy bruising. Barnes & Peterson (1993) Am Rev Respir Dis 148:S1-S26; and Kamada AK et al. (1996) Am J Respir Crit Care Med 153:1739-48.

[001153] Provided oligonucleotide compositions and methods in accordance with the invention can be used alone or in conjunction with other agents and methods useful for the treatment of asthma. In one aspect the invention provides a method of treating a subject having asthma. The method according to this aspect of the invention includes the step of administering to a subject having asthma an effective amount of a composition of the invention to treat the subject.

[001154] In some embodiments, the invention provides a method of treating a subject having asthma. The method according to this aspect of the invention includes the step of administering to a subject having asthma an effective amount of the composition of the invention and an anti-asthma therapy to treat the subject.

[001155] "Asthma" as used herein refers to a disorder of the respiratory system characterized by inflammation and narrowing of the airways, and increased reactivity of the airways to inhaled agents. Asthma is frequently, although not exclusively, associated with an atopic or allergic condition. Symptoms of asthma include recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, resulting from airflow obstruction. Airway

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inflammation associated with asthma can be detected through observation of a number of physiological changes, such as, denudation of airway epithelium, collagen deposition beneath basement membrane, edema, mast cell activation, inflammatory cell infiltration, including neutrophils, eosinophils, and lymphocytes. As a result of the airway inflammation, asthma patients often experience airway hyper-responsiveness, airflow limitation, respiratory symptoms, and disease chronicity. Airflow limitations include acute bronchoconstriction, airway edema, mucous plug formation, and airway remodeling, features which often lead to bronchial obstruction. In some cases of asthma, sub-basement membrane fibrosis may occur, leading to persistent abnormalities in lung function.

[001156] Research over the past several years has revealed that asthma likely results from complex interactions among inflammatory cells, mediators, and other cells and tissues resident in the airways. Mast cells, eosinophils, epithelial cells, macrophage, and activated T cells all play an important role in the inflammatory process associated with asthma. Djukanovic R et al. (1990) Am Rev Respir Dis 142:434-457. It is believed that these cells can influence airway function through secretion of preformed and newly synthesized mediators which can act directly or indirectly on the local tissue. It has also been recognized that subpopulations of T lymphocytes (Th2) play an important role in regulating allergic inflammation in the airway by releasing selective cytokines and establishing disease chronicity. Robinson DS et al. (1992) N Engl J Med 326:298-304.

[001157] Asthma is a complex disorder which arises at different stages in development and can be classified based on the degree of symptoms as acute, subacute, or chronic. An acute inflammatory response is associated with an early recruitment of cells into the airway. The subacute inflammatory response involves the recruitment of cells as well as the activation of resident cells causing a more persistent pattern of inflammation. Chronic inflammatory response is characterized by a persistent level of cell damage and an ongoing repair process, which may result in permanent abnormalities in the airway.

[001158] A "subject having asthma" is a subject that has a disorder of the respiratory system characterized by inflammation and narrowing of the airways and increased reactivity of the airways to inhaled agents. Factors associated with initiation of asthma include, but are not limited to, allergens, cold temperature, exercise, viral infections, and SO_2.

[001159] As mentioned above, asthma may be associated with a Th2-type of immune response, which is characterized at least in part by Th2 cytokines IL-4 and IL-5, as well as antibody isotype switching to IgE. Th1 and Th2 immune responses are mutually counter-regulatory, so that skewing of the immune response toward a Th1-type of immune response can prevent or ameliorate a Th2-type of immune response, including allergy. Provided immunomodulatory oligonucleotide compositions of the invention are therefore useful by themselves to treat a subject having asthma because the analogs can skew the immune response toward a Th1-type of immune response.

[001160] Provided immunomodulatory oligonucleotide compositions of the invention may also be administered in conjunction with an asthma therapy. Conventional methods for treating or preventing asthma have involved the use of anti-allergy therapies (described above) and a number of other agents, including inhaled agents.

[001161] Medications for the treatment of asthma are generally separated into two categories, quick-relief medications and long-term control medications. Asthma patients take the long-term control medications on a daily basis to achieve and maintain control of persistent asthma. Long-term control medications include anti-inflammatory agents such as corticosteroids, chromolyn sodium and nedocromil; long-acting bronchodilators, such as long-acting β_2-agonists and methylxanthines; and leukotriene modifiers. The quick-relief medications include short-acting β_2 agonists, anticholinergics, and systemic corticosteroids. There are many side effects associated with each of these drugs and none of the drugs alone or in combination is capable of preventing or completely treating asthma.

[001162] Anti-asthma medicaments include, but are not limited to, PDE-4 inhibitors, bronchodilator/beta-2 agonists, K+ channel openers, VLA-4 antagonists, neurokin antagonists, thromboxane A2 (TXA2) synthesis inhibitors, xanthines, arachidonic acid antagonists, 5 lipoxygenase inhibitors, TXA2 receptor antagonists, TXA2 antagonists, inhibitor of 5-lipox activation proteins, and protease inhibitors.

[001163] Bronchodilator/β_2 agonists are a class of compounds which cause bronchodilation or smooth muscle relaxation. Bronchodilator/β_2 agonists include, but are not limited to, salmeterol, salbutamol, albuterol, terbutaline, D2522/formoterol, fenoterol, bitolterol, pirbuerol methylxanthines and orciprenaline. Long-acting β_2 agonists and bronchodilators are compounds which are used for long-term prevention of symptoms in addition to the anti-inflammatory

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therapies. Long-acting β_2 agonists include, but are not limited to, salmeterol and albuterol. These compounds are usually used in combination with corticosteroids and generally are not used without any inflammatory therapy. They have been associated with side effects such as tachycardia, skeletal muscle tremor, hypokalemia, and prolongation of QTc interval in overdose.

[001164] Methylxanthines, including for instance theophylline, have been used for long-term control and prevention of symptoms. These compounds cause bronchodilation resulting from phosphodiesterase inhibition and likely adenosine antagonism. Dose-related acute toxicities are a particular problem with these types of compounds. As a result, routine serum concentration must be monitored in order to account for the toxicity and narrow therapeutic range arising from individual differences in metabolic clearance. Side effects include tachycardia, tachyarrhythmias, nausea and vomiting, central nervous system stimulation, headache, seizures, hematemesis, hyperglycemia and hypokalemia. Short-acting β_2 agonists include, but are not limited to, albuterol, bitolterol, pirbuterol, and terbutaline. Some of the adverse effects associated with the administration of short-acting β_2 agonists include tachycardia, skeletal muscle tremor, hypokalemia, increased lactic acid, headache, and hyperglycemia.

[001165] Chromolyn sodium and nedocromil are used as long-term control medications for preventing primarily asthma symptoms arising from exercise or allergic symptoms arising from allergens. These compounds are believed to block early and late reactions to allergens by interfering with chloride channel function. They also stabilize mast cell membranes and inhibit activation and release of mediators from inosineophils and epithelial cells. A four to six week period of administration is generally required to achieve a maximum benefit.

[001166] Anticholinergics are generally used for the relief of acute bronchospasm. These compounds are believed to function by competitive inhibition of muscarinic cholinergic receptors. Anticholinergics include, but are not limited to, ipratropium bromide. These compounds reverse only cholinerigically-mediated bronchospasm and do not modify any reaction to antigen. Side effects include drying of the mouth and respiratory secretions, increased wheezing in some individuals, and blurred vision if sprayed in the eyes.

[001167] In some embodiments, provided immunomodulatory oligonucleotide compositions may also be useful for treating airway remodeling. Airway remodeling results from smooth muscle cell proliferation and/or submucosal thickening in the airways, and ultimately causes narrowing of the airways leading to restricted airflow. The immunomodulatory

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oligonucleotides of the invention may prevent further remodeling and possibly even reduce tissue build-up resulting from the remodeling process.

[001168] In some embodiments, provided immunomodulatory oligonucleotide compositions are useful for treating a subject having an inflammatory disorder. As used herein, the term "inflammatory disorder" refers to a condition associated with an antigen-nonspecific reaction of the innate immune system that involves accumulation and activation of leukocytes and plasma proteins at a site of infection, toxin exposure, or cell injury. Cytokines that are characteristic of inflammation include tumor necrosis factor (TNF-α), interleukin 1 (IL-1), IL-6, IL-12, interferon alpha (IFN-α), interferon beta (IFN-β), and chemokines. Thus, certain types of asthma, allergy, and autoimmune disorders may have characteristics of an inflammatory disorder. Inflammatory disorders also include, for example cardiovascular disease, chronic obstructive pulmonary disease (COPD), bronchiectasis, chronic cholecystitis, tuberculosis, Hashimoto's thyroiditis, sepsis, sarcoidosis, silicosis and other pneumoconioses, and an implanted foreign body in a wound, but are not so limited. As used herein, the term "sepsis" refers to a well-recognized clinical syndrome associated with a host's systemic inflammatory response to microbial invasion. The term "sepsis" as used herein refers to a condition that is typically signaled by fever or hypothermia, tachycardia, and tachypnea, and in severe instances can progress to hypotension, organ dysfunction, and even death.

[001169] In some embodiments, provided immunomodulatory oligonucleotide compositions are useful for treating a subject having an infection. In some embodiments, provided immunomodulatory oligonucleotide compositions are useful for treating a subject who is susceptible to an infection, including those who may have exposure to a pathogen or pathogens. The immunomodulatory oligonucleotides utilized in accordance with the invention can in some aspects also be used to treat or prevent infections by viruses, bacteria, fungi, or parasites. A subject having an infection is a subject that has been exposed to an infectious pathogen and has acute or chronic detectable levels of the pathogen in the body. The immunomodulatory oligonucleotides can be used with or without an antigen to mount an antigen specific systemic or mucosal immune response that is capable of reducing the level of or eradicating the infectious pathogen. An infectious disease, as used herein, is a disease arising from the presence of a foreign microorganism in the body. It is particularly important to develop

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effective vaccine strategies and treatments to protect the body's mucosal surfaces, which are the primary site of pathogenic entry.

[001170] Viruses are small infectious agents which generally contain a nucleic acid core and a protein coat, but are not independently living organisms. Viruses can also take the form of infectious nucleic acids lacking a protein. A virus cannot survive in the absence of a living cell within which it can replicate. Viruses enter specific living cells either by endocytosis or direct injection of DNA (phage) and multiply, causing disease. The multiplied virus can then be released and infect additional cells. Some viruses are DNA-containing viruses and others are RNA-containing viruses. DNA viruses include Pox, Herpes, Adeno, Papova, Parvo, and Hepadna. RNA viruses include Picorna, Calici, Astro.Toga, Flavi, Corona, Paramyxo, Orthomyxo, Bunya, Arena, Rhabdo, FiIo, Borna, Reo, and Retro. In some aspects, the invention also intends to treat diseases in which prions are implicated in disease progression such as for example bovine spongiform encephalopathy (i.e., mad cow disease, BSE) or scrapie infection in animals, or Creutzfeldt-Jakob disease in humans.

[001171] Viruses include, but are not limited to, enteroviruses (including, but not limited to, viruses that the family picornaviridae, such as polio virus, Coxsackie virus, echo virus), rotaviruses, adenovirus, and hepatitis virus, such as hepatitis A, B, C D and E. Specific examples of viruses that have been found in humans include but are not limited to: Retroviridae (e.g., human immunodeficiency viruses, such as HIV-1 (also referred to as HTLV-III, LAV or HTLV-III/LAV, or HIV-III; and other isolates, such as HIV-LP; Picornaviridae (e.g., polio viruses, hepatitis A virus; enteroviruses, human Coxsackie viruses, rhinoviruses, echoviruses); Calciviridae (e.g., strains that cause gastroenteritis); Togaviridae (e.g., equine encephalitis viruses, rubella viruses); Flaviviridae (e.g., dengue viruses, encephalitis viruses, yellow fever viruses); Coronaviridae (e.g., coronavi ruses); Rhabdoviridae (e.g., vesicular stomatitis viruses, rabies viruses); Filoviridae (e.g., ebola viruses); Paramyxoviridae (e.g., parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); Orthomyxoviridae (e.g., influenza viruses); Bunyaviridae (e.g., Hantaan viruses, bunya viruses, phleboviruses and Nairo viruses); Arenaviridae (hemorrhagic fever viruses); Reoviridae (e.g., reoviruses, orbiviurses and rotaviruses); Birnaviridae; Hepadnaviridae (Hepatitis B virus); Parvoviridae (parvoviruses); Papovavihdae (papillomaviruses, polyoma viruses); Adenoviridae (most adenoviruses); Herpesviridae (herpes simplex virus (HSV) 1 and 2, varicella zoster virus, cytomegalovirus

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(CMV)); Poxviridae (variola viruses, vaccinia viruses, pox viruses); Iridoviridae (e.g., African swine fever virus); and other viruses acute laryngotracheobronchitis virus, Alphavirus, Kaposi's sarcoma-associated herpesvirus, Newcastle disease virus, Nipah virus, Norwalk virus, Papillomavirus, parainfluenza virus, avian influenza, SARs virus, West Nile virus. Viruses that infect plants include, for example, viruses of the genera and/or families of Alfamoviruses: Bromoviridae, Alphacryptoviruses: Partitiviridae, Badnaviruses, Betacryptoviruses: Partitiviridae, Bigeminiviruses: Geminiviridae, Bromoviruses: Bromoviridae, Bymoviruses: Potyviridae, Capilloviruses, Carlaviruses, Carmoviruses: Tombusviridae, Caulimoviruses, Closteroviruses, Comoviruses: Comoviridae, Cucumoviruses: Bromoviridae, Cytorhabdoviruses: Rhabdoviridae, Dianthoviruses, Enamoviruses, Fabaviruses: Comoviridae, Fijiviruses: Reoviridae, Furoviruses, Hordeiviruses, Hybrigeminiviruses: Geminiviridae, Idaeoviruses, Ilarviruses: Bromoviridae, Ipomoviruses: Potyviridae, Luteoviruses, Machlomoviruses, Macluraviruses, Marafiviruses, Monogeminiviruses: Geminiviridae, Nanaviruses, Necroviruses, Nepoviruses: Comoviridae, Nucleorhabdoviruses: Rhabdoviridae, Oryzaviruses: Reoviridae, Ourmiaviruses, Phytoreoviruses: Reoviridae, Potexviruses, Potyviruses: Potyviridae, Rymoviruses: Potyviridae, Satellite RNAs, Satelliviruses, Sequiviruses: Sequiviridae, Sobemoviruses, Tenuiviruses, Tobamoviruses, Tobraviruses, Tombusviruses: Tombusviridae, Tospoviruses: Bunyaviridae, Trichoviruses, Tymoviruses, Umbraviruses, Unassigned potyviruses: Potyviridae, Unassigned rhabdoviruses: Rhabdoviridae, Varicosaviruses, Waikaviruses: Sequiviridae, Ungrouped viruses, Bromoviridae Potyviridae and Tymoviridae; particular relevant plant viruses invclude, for example, Tobacco mosaic virus (TMV), Tomato spotted wilt virus, Tomato yellow leaf curl virus, Cucumber mosaic virus, Potato virus Y, Cauliflower mosaic virus, African cassava mosaic virus, Plum pox virus, Brome mosaic virus, Potato virus X, Citrus tristeza virus, Barley yellow dwarf virus, Potato leafroll virus and Tomato bushy stunt virus.

[001172] Viral hepatitis is an inflammation of the liver which may produce swelling, tenderness, and sometimes permanent damage to the liver. If the inflammation of the liver continues at least six months or longer, it is refered to as chronic hepatitis. There are at least five different viruses known to cause viral hepatitis, include hepatitis A $_1$ B, C D and E. Hepatitis A is genreally communicated through food or drinking water contaminated with human feces. Hepatitis B generally is spread through bodily fluids such as blood. For instance, it may be

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spread from mother to child at birth, through sexual contact, contaminated blood transfusions and needles. Hepatitis C is quite common and like Hepatitis B is often spread through blood transfusions and contaminated needles. Hepatitis D is found most often in IV drug users who are carriers of the hepatitis B virus with which it co-associates. Hepatitis E is similar to viral hepatitis A and is generally assoicated with poor sanitation.

[001173] Both gram negative and gram positive bacteria serve as antigens in vertebrate animals. Such gram positive bacteria include, but are not limited to, Pasteurella species, Staphylococci species, and Streptococcus species. Gram negative bacteria include, but are not limited to, Escherichia coli, Pseudomonas species, and Salmonella species. Specific examples of infectious bacteria include but are not limited to, Helicobacter pyloris, Borelia burgdorferi, Legionella pneumophilia, Mycobacteria sps (e.g. M. tuberculosis, M. avium, M. intracellular, M. kansaii, M. gordonae), Staphylococcus aureus, Neisseria gonorrhoeae, Neisseria meningitidis, Listeria monocytogenes, Streptococcus pyogenes (Group A Streptococcus), Streptococcus agalactiae (Group B Streptococcus), Streptococcus (viridans group), Streptococcus faecalis, Streptococcus bovis, Streptococcus (anaerobic sps.), Streptococcus pneumoniae, pathogenic Campylobacter sp., Enterococcus sp., Haemophilus influenzae, Bacillus antracis, corynebacterium diphtheriae, corynebacterium sp., Erysipelothrix rhusiopathiae, Clostridium pen 'ringers, Clostridium tetani, Enterobacter aerogenes, Klebsiella pneumoniae, Pasturella multocida, Bacteroides sp., Fusobacterium nucleatum,

[001174] Streptobacillus moniliformis, Treponema pallidium, Treponema pertenue, Leptospira, Rickettsia, and Actinomyces israelii.

[001175] Examples of fungi include Cryptococcus neoformans, Histoplasma capsulatum, Coccidioides immitis, Blastomyces dermatitidis, Chlamydia trachomatis, Candida albicans.

[001176] Other infectious organisms (i.e., protists) include Plasmodium spp. such as Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale, and Plasmodium vivax and Toxoplasma gondii. Blood-borne and/or tissues parasites include Plasmodium spp., Babesia microti, Babesia divergens, Leishmania tropica, Leishmania spp., Leishmania braziliensis, Leishmania donovani, Trypanosoma gambiense and Trypanosoma rhodesiense (African sleeping sickness), Trypanosoma cruzi (Chagas' disease), and Toxoplasma gondii.

[001177] Other medically relevant microorganisms have been described extensively in the literature, e.g., see C.G.A Thomas, Medical Microbiology, Bailliere Tindall, Great Britain 1983,

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the entire contents of which is hereby incorporated by reference. Agriculturally relevant microorganisms (e.g., those that infect crops and/or livestock) are also known, for example but not limited to those described in, *e.g.*, George N. Agrios, Plant Pathology, Elsevier Academic Press, 5th Edition, 2005; John Lucas, Plant Pathology and Plant Pathogens, Wiley-Blackwell; 3rd edition, 1998; Dwight C. Hirsh, N. James MacLachlan, Richard L. Walker, Veterinary Microbiology, Wiley-Blackwell; 2 edition, 2004; and P. J. Quinn, et al, Veterinary Microbiology and Microbial Disease, Wiley-Blackwell; 2 edition, 2011.

[001178] Provided oligonucleotide compositions of the invention may be administered to a subject (including, for example, a human subject or, in some embodiments an animal [e.g., livestock or pet animal] or plant [e.g., crop] subject) with an anti-microbial agent. An anti-microbial agent, as used herein, refers to a naturally- occurring or synthetic compound which is capable of killing or inhibiting infectious microorganisms. The type of anti-microbial agent useful according to the invention will depend upon the type of microorganism with which the subject is infected or at risk of becoming infected. Anti-microbial agents include but are not limited to anti-bacterial agents, anti-viral agents, anti-fungal agents and anti-parasitic agents. Phrases such as "anti-infective agent", "anti-bacterial agent", "anti-viral agent", "anti-fungal agent" , "antiparasitic agent" and "parasiticide" have well-established meanings to those of ordinary skill in the art and are defined in standard medical texts. Briefly, anti-bacterial agents kill or inhibit bacteria, and include antibiotics as well as other synthetic or natural compounds having similar functions. Antibiotics are low molecular weight molecules which are produced as secondary metabolites by cells, such as microorganisms. In general, antibiotics interfere with one or more bacterial functions or structures which are specific for the microorganism and which are not present in host cells. Anti-viral agents can be isolated from natural sources or synthesized and are useful for killing or inhibiting viruses. Anti-fungal agents are used to treat superficial fungal infections as well as opportunistic and primary systemic fungal infections. Anti-parasite agents kill or inhibit parasites.

[001179] Examples of anti-parasitic agents, also referred to as parasiticides useful for human administration include but are not limited to albendazole, amphotericin B, benznidazole, bithionol, chloroquine HCl, chloroquine phosphate, clindamycin, dehydroemetine, diethylcarbamazine, diloxanide furoate, eflornithine, furazolidaone, glucocorticoids, halofantrine, iodoquinol, ivermectin, mebendazole, mefloquine, meglumine antimoniate,

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melarsoprol, metrifonate, metronidazole, niclosamide, nifurtimox, oxamniquine, paromomycin, pentamidine isethionate, piperazine, praziquantel, primaquine phosphate, proguanil, pyrantel pamoate, pyrimethanmine- sulfonamides, pyrimethanmine-sulfadoxine, quinacrine HCI, quinine sulfate, quinidine gluconate, spiramycin, stibogluconate sodium (sodium antimony gluconate), suramin, tetracycline, doxycycline, thiabendazole, tinidazole, trimethroprim-sulfamethoxazole, and tryparsamide some of which are used alone or in combination with others.

[001180] Antibacterial agents kill or inhibit the growth or function of bacteria. A large class of antibacterial agents is antibiotics. Antibiotics, which are effective for killing or inhibiting a wide range of bacteria, are referred to as broad spectrum antibiotics. Other types of antibiotics are predominantly effective against the bacteria of the class gram- positive or gram-negative. These types of antibiotics are referred to as narrow spectrum antibiotics. Other antibiotics which are effective against a single organism or disease and not against other types of bacteria, are referred to as limited spectrum antibiotics. Antibacterial agents are sometimes classified based on their primary mode of action. In general, antibacterial agents are cell wall synthesis inhibitors, cell membrane inhibitors, protein synthesis inhibitors, nucleic acid synthesis or functional inhibitors, and competitive inhibitors.

[001181] Antiviral agents are compounds which prevent infection of cells by viruses or replication of the virus within the cell. There are many fewer antiviral drugs than antibacterial drugs because the process of viral replication is so closely related to DNA replication within the host cell, that non-specific antiviral agents would often be toxic to the host. There are several stages within the process of viral infection which can be blocked or inhibited by antiviral agents. These stages include, attachment of the virus to the host cell (immunoglobulin or binding peptides), uncoating of the virus (e.g. amantadine), synthesis or translation of viral mRNA (e.g. interferon), replication of viral RNA or DNA (e.g. nucleotide analogues), maturation of new virus proteins (e.g. protease inhibitors), and budding and release of the virus.

[001182] Nucleotide analogues are synthetic compounds which are similar to nucleotides, but which have an incomplete or abnormal deoxyribose or ribose group. Once the nucleotide analogues are in the cell, they are phosphorylated, producing the triphosphate formed which competes with normal nucleotides for incorporation into the viral DNA or RNA. Once the triphosphate form of the nucleotide analogue is incorporated into the growing nucleic acid chain, it causes irreversible association with the viral polymerase and thus chain termination.

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Nucleotide analogues include, but are not limited to, acyclovir (used for the treatment of herpes simplex virus and varicella- zoster virus), gancyclovir (useful for the treatment of cytomegalovirus), idoxuridine, ribavirin (useful for the treatment of respiratory syncitial virus), dideoxyinosine, dideoxycytidine, zidovudine (azidothymidine), imiquimod, and resimiquimod.

[001183] The interferons are cytokines which are secreted by virus-infected cells as well as immune cells. The interferons function by binding to specific receptors on cells adjacent to the infected cells, causing the change in the cell which protects it from infection by the virus, α and β-interferon also induce the expression of Class I and Class Il MHC molecules on the surface of infected cells, resulting in increased antigen presentation for host immune cell recognition, α and β-interferons are available as recombinant forms and have been used for the treatment of chronic hepatitis B and C infection. At the dosages which are effective for anti-viral therapy, interferons have severe side effects such as fever, malaise and weight loss.

[001184] Anti-viral agents useful in the invention include but are not limited to immunoglobulins, amantadine, interferons, nucleotide analogues, and protease inhibitors. Specific examples of anti-virals include but are not limited to Acemannan; Acyclovir; Acyclovir Sodium; Adefovir; Alovudine; Alvircept Sudotox; Amantadine Hydrochloride; Aranotin; Arildone; Atevirdine Mesylate; Avridine; Cidofovir; Cipamfylline; Cytarabine Hydrochloride; Delavirdine Mesylate; Desciclovir; Didanosine; Disoxaril; Edoxudine; Enviradene; Enviroxime; Famciclovir; Famotine Hydrochloride; Fiacitabine; Fialuridine; Fosarilate; Foscarnet Sodium; Fosfonet Sodium; Ganciclovir; Ganciclovir Sodium; Idoxuridine; Kethoxal; Lamivudine; Lobucavir; Memotine Hydrochloride; Methisazone; Nevirapine; Penciclovir; Pirodavir; Ribavirin; Rimantadine Hydrochloride; Saquinavir Mesylate; Somantadine Hydrochloride; Sorivudine; Statolon; Stavudine; Tilorone Hydrochloride; Trifluridine; Valacyclovir Hydrochloride; Vidarabine; Vidarabine Phosphate; Vidarabine Sodium Phosphate; Viroxime; Zalcitabine; Zidovudine; and Zinviroxime.

[001185] Anti-fungal agents are useful for the treatment and prevention of infective fungi. Anti-fungal agents are sometimes classified by their mechanism of action. Some antifungal agents function as cell wall inhibitors by inhibiting glucose synthase. These

[001186] include, but are not limited to, basiungin/ECB. Other anti-fungal agents function by destabilizing membrane integrity. These include, but are not limited to, immidazoles, such as clotrimazole, sertaconzole, fluconazole, itraconazole, ketoconazole, miconazole, and

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voriconacole, as well as FK 463, amphotericin B, BAY 38-9502, MK 991 , pradimicin, UK 292, butenafine, and terbinafine. Other anti-fungal agents function by breaking down chitin (e.g. chitinase) or immunosuppression (501 cream).

[001187] In some embodiments, provided immunomodulatory oligonucleotide compositions are useful for treating a subject having a cell proliferative disease, such as cancer. A subject having a cancer is a subject that has detectable cancerous cells. The cancer may be a malignant or non-malignant cancer. Cancers or tumors include but are not limited to biliary tract cancer; brain cancer; breast cancer; cervical cancer; choriocarcinoma; colon cancer; endometrial cancer; esophageal cancer; gastric cancer; intraepithelial neoplasms; lymphomas; liver cancer; lung cancer (e.g. small cell and non-small cell); melanoma; neuroblastomas; oral cancer; ovarian cancer; pancreas cancer; prostate cancer; rectal cancer; sarcomas; skin cancer; testicular cancer; thyroid cancer; and renal cancer, as well as other carcinomas and sarcomas. In one embodiment the cancer is hairy cell leukemia, chronic myelogenous leukemia, cutaneous T-cell leukemia, multiple myeloma, follicular lymphoma, malignant melanoma, squamous cell carcinoma, renal cell carcinoma, prostate carcinoma, bladder cell carcinoma, or colon carcinoma.

[001188] Provided immunomodulatory oligonucleotide compositions may be administered alone or in conjunction with an anti-cancer therapy. Anti-cancer therapies include but are not limited to radiation therapy, chemotherapy, immunotherapy, a cancer vaccine, hormone therapy, a biological response modifier, and surgical procedures. A cancer medicament refers to an agent which is administered to a subject for the purpose of treating a cancer. As used herein, "treating cancer" includes preventing the development of a cancer, reducing the symptoms of cancer, and/or inhibiting the growth of an established cancer. In other aspects, the cancer medicament is administered to a subject at risk of developing a cancer for the purpose of reducing the risk of developing the cancer. Various types of medicaments for the treatment of cancer are described herein. For the purpose of this specification, cancer medicaments are classified as chemotherapeutic agents, immunotherapeutic agents, cancer vaccines, hormone therapy, and biological response modifiers.

[001189] Additionally, the methods of the invention are intended to embrace the use of more than one cancer medicament along with provided immunomodulatory oligonucleotide compositions. As an example, where appropriate, provided immunomodulatory oligonucleotide compositions may be administered with both a chemotherapeutic agent and an

immunotherapeutic agent. Alternatively, the cancer medicament may embrace an immunotherapeutic agent and a cancer vaccine, or a chemotherapeutic agent and a cancer vaccine, or a chemotherapeutic agent, an immunotherapeutic agent and a cancer vaccine all administered to one subject for the purpose of treating a subject having a cancer or at risk of developing a cancer.

[001190] Chemotherapeutic agent may be selected from the group consisting of methotrexate, vincristine, adriamycin, cisplatin, non-sugar containing chloroethylnitrosoureas, 5-fluorouracil, mitomycin C, bleomycin, doxorubicin, dacarbazine, taxol, fragyline, Meglamine GLA, valrubicin, carmustaine and poliferposan, MMI270, BAY 12-9566, RAS famesyl transferase inhibitor, famesyl transferase inhibitor, MMP, MTA/LY231514, LY264618/Lometexol, Glamolec, CI-994, TNP-470, Hycamtin/Topotecan, PKC412, Valspodar/PSC833, Novantrone/Mitroxantrone, Metaret/Suramin, Batimastat, E7070, BCH-4556, CS-682, 9-AC, AG3340, AG3433, lncel/VX-710, VX-853, ZD0101 , ISI641 , ODN 698, TA 2516/Marmistat, BB2516/Marmistat, CDP 845, D2163, PD183805, DX8951f, Lemonal DP 2202, FK 317, Picibanil/OK-432, AD 32/Valrubicin, Metastron/strontium derivative, Temodal/Temozolomide, Evacet/liposomal doxorubicin, Yewtaxan/Paclitaxel, Taxol/Paclitaxel, Xeload/Capecitabine, Furtulon/Doxifluridine, Cyclopax/oral paclitaxel, Oral Taxoid, SPU-077/Cisplatin, HMR 1275/Flavopiridol, CP-358 (774)/EGFR, CP-609 (754)/RAS oncogene inhibitor, BMS-182751/oral platinum, UFT(Tegafur/Uracil), Ergamisol/Levamisole, Eniluracil/776C85/5FU enhancer, Campto/Levamisole, Camptosar/lrinotecan, Tumodex/Ralitrexed, Leustatin/Cladribine, Paxex/Paclitaxel, Doxil/liposomal doxorubicin, Caelyx/liposomal doxorubicin, Fludara/Fludarabine, Pharmarubicin/Epirubicin, DepoCyt, ZD1839, LU 79553/Bis-Naphtalimide, LU 103793/Dolastain, Caetyx/liposomal doxorubicin, Gemzar/Gemcitabine, ZD 0473/Anormed, YM 116, Iodine seeds, CDK4 and CDK2 inhibitors, PARP inhibitors, D4809/Dexifosamide, Ifes/Mesnex/lfosamide, Vumon/Teniposide, Paraplatin/Carboplatin, Plantinol/cisplatin, Vepeside/Etoposide, ZD 9331 , Taxotere/Docetaxel, prodrug of guanine arabinoside, Taxane Analog, nitrosoureas, alkylating agents such as melphelan and cyclophosphamide, Aminoglutethimide, Asparaginase, Busulfan, Carboplatin, Chlorambucil, Cytarabine HCI, Dactinomycin, Daunorubicin HCI, Estramustine phosphate sodium, Etoposide (VP16-213), Floxuridine, Fluorouracil (5-FU), Flutamide, Hydroxyurea (hydroxycarbamide), Ifosfamide, Interferon Alfa-2a, Alfa-2b, Leuprolide acetate (LHRH-

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releasing factor analogue), Lomustine (CCNU), Mechlorethamine HCI (nitrogen mustard), Mercaptopurine, Mesna, Mitotane (o.p ' -DDD), Mitoxantrone HCI, Octreotide, Plicamycin, Procarbazine HCI, Streptozocin,

[001191] Tamoxifen citrate, Thioguanine, Thiotepa, Vinblastine sulfate, Amsacrine (m-AMSA), Azacitidine, Erthropoietin, Hexamethylmelamine (HMM), lnterleukin 2, Mitoguazone (methyl-GAG; methyl glyoxal bis-guanylhydrazone; MGBG), Pentostatin (2'deoxycoformycin), Semustine (methyl-CCNU), Teniposide (VM-26) and Vindesine sulfate, but it is not so limited.

[001192] Immunotherapeutic agent may be selected from the group consisting of Ributaxin, Herceptin, Quadramet, Panorex, IDEC-Y2B8, BEC2, C225, Oncolym, SMART M195, ATRAGEN, Ovarex, Bexxar, LDP-03, ior tβ, MDX-210, MDX-11 , MDX- 22, OV103, 3622W94, anti-VEGF, Zenapax, MDX-220, MDX-447, MELIMMUNE-2, MELIMMUNE-1 , CEACIDE, Pretarget, NovoMAb-G2, TNT, Gliomab-H, GNI-250, EMD- 72000, LymphoCide, CMA 676, Monopharm-C, 4B5, ior egf.r3, ior c5, BABS, anti-FLK- 2, MDX-260, ANA Ab, SMART 1 D10 Ab, SMART ABL 364 Ab and ImmuRAIT-CEA, but it is not so limited.

[001193] Cancer vaccine may be selected from the group consisting of EGF, Anti- idiotypic cancer vaccines, Gp75 antigen, GMK melanoma vaccine, MGV ganglioside conjugate vaccine, Her2/neu, Ovarex, M-Vax, O-Vax, L-Vax, STn-KHL theratope, BLP25 (MUC-1), liposomal idiotypic vaccine, Melacine, peptide antigen vaccines, toxin/antigen vaccines, MVA-based vaccine, PACIS, BCG vacine, TA-HPV, TA-CIN, DISC-virus and ImmuCyst/TheraCys, but it is not so limited.

[001194] As used herein, the terms "cancer antigen" and "tumor antigen" are used interchangeably to refer to antigens which are differentially expressed by cancer cells and can thereby be exploited in order to target cancer cells. Cancer antigens are antigens which can potentially stimulate apparently tumor-specific immune responses. Some of these antigens are encoded, although not necessarily expressed, by normal cells. These antigens can be characterized as those which are normally silent (i.e., not expressed) in normal cells, those that are expressed only at certain stages of differentiation and those that are temporally expressed such as embryonic and fetal antigens. Other cancer antigens are encoded by mutant cellular genes, such as oncogenes (e.g., activated ras oncogene), suppressor genes (e.g., mutant p53), fusion proteins resulting from internal deletions or chromosomal translocations. Still other cancer antigens can be encoded by viral genes such as those carried on RNA and DNA tumor viruses.

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[001195] In some embodiments, provided immunomodulatory oligonucleotide compositions may also be useful for treating and preventing autoimmune disease. Autoimmune disease is a class of diseases in which a subject's own antibodies react with host tissue or in which immune effector T cells are autoreactive to endogenous self peptides and cause destruction of tissue. Thus an immune response is mounted against a subject's own antigens, referred to as self antigens. Autoimmune diseases include but are not limited to alopecia areata, acquired hemophilia, ankylosing spondylitis, antiphospholipid syndrome, autoimmune-associated infertility, autoimmune encephalomyelitis, autoimmune hepatitis, autoimmune hemolytic anemia, autoimmune diabetes mellitus, autoimmune thrombocytopenic purpura, Behget's syndrome, bullous pemphigoid, cardiomyopathy, chronic fatigue immune dysfunction syndrome (CFIDS), chronic inflammatory demyelinating polyneuropathy, Churg-Strauss syndrome, cicatricial pemphigoid, CREST syndrome, cold agglutinin disease, dermatomyositis, discoid lupus, essential mixed cryoglobulinemia, fibromyalgia, fibromyositis, Guillain-Barre syndrome, Hashimoto's thyroiditis, glomerulonephritis (e.g., crescentic glomerulonephritis, proliferative glomerulonephritis), Grave's disease, graft versus host disease, Goodpasture's syndrome, pemphigus (e.g., pemphigus vulgaris), idiopathic pulmonary fibrosis, idiopathic thrombocytopenic purpura, insulin resistance, idiopathic Addison's disease, IgA nephropathy, inflammatory bowel disease (including Crohn's disease and ulcerative colitis), juvenile arthritis, lichen planus, myasthenia gravis, multiple sclerosis, mixed connective tissue disease, polymyositis, pernicious anemia, polyarteritis nodosa, polychondritis, polyglandular syndromes, polymyalgia rheumatica, primary agammaglobulinemia, primary biliary cirrhosis, psoriasis, Raynaud's phenomena, Reite^s syndrome, juvenile and adult rheumatoid arthritis, Sjorgen's syndrome, scleroderma with anti-collagen antibodies, sarcoidosis, stiff-man syndrome, systemic lupus erythematosus (SLE), Takayasu arthritis, transplanted organ rejection, temporal arteritis/giant cell arteritis, uveitis, ulcerative colitis, vasculitis, and vitiligo.

[001196] A "self-antigen" as used herein refers to an antigen of a normal host tissue. Normal host tissue does not include cancer cells. Thus an immune response mounted against a self-antigen, in the context of an autoimmune disease, is an undesirable immune response and contributes to destruction and damage of normal tissue, whereas an immune response mounted against a cancer antigen is a desirable immune response and contributes to the destruction of the tumor or cancer. Thus, in some aspects of the invention aimed at treating autoimmune disorders

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it is not recommended that the immunomodulatory oligonucleotides be administered with self antigens, particularly those that are the targets of the autoimmune disorder.

[001197] In other instances, immunomodulatory oligonucleotides used in according to the present invention may be delivered with low doses of self-antigens. A number of animal studies have demonstrated that mucosal administration of low doses of antigen can result in a state of immune hyporesponsiveness or "tolerance." The active mechanism appears to be a cytokine-mediated immune deviation away from a Th1 towards a predominantly Th2 and Th3 (i.e., TGF-β dominated) response. The active suppression with low dose antigen delivery can also suppress an unrelated immune response (bystander suppression) which is of considerable interest in the therapy of autoimmune diseases, for example, rheumatoid arthritis and SLE. Bystander suppression involves the secretion of Th1 - counter-regulatory, suppressor cytokines in the local environment where proinflammatory and Th1 cytokines are released in either an antigen-specific or antigen- nonspecific manner. 'Tolerance" as used herein is used to refer to this phenomenon. Indeed, oral tolerance has been effective in the treatment of a number of autoimmune diseases in animals including: experimental autoimmune encephalomyelitis (EAE), experimental autoimmune myasthenia gravis, collagen-induced arthritis (CIA), and insulin-dependent diabetes mellitus. In these models, the prevention and suppression of autoimmune disease is associated with a shift in antigen-specific humoral and cellular responses from a Th1 to Th2/Th3 response

[001198] Non-limiting examples of useful immunomodulatory oligonucleotides that can be utilized in accordance with the present invention are provided in the accompanying **Appendix (B).**

RIPtides

[001199] In some embodiments, oligonucleotides utilized in accordance with the present invention are or act as RNA-interacting polynucleotides ("RIPtides"). RIPtides are a class of therapeutics intended to open up the space of "undruggable" targets by inactivating structural RNAs implicated in disease. RIPtides are typically small nucleotide stretches (around 8 nucleotides) that bind and disrupt the action of structured RNA. Due to their size, these molecules can readily pass through the cell membrane. See, for example, U.S. Patent 6,080,585.

[001200] RIPtides may be useful for a number of clinical applications. In the context of cancer therapy, at least one target is telomerase, which is present in about 90% of cancer cells.

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Therefore, in some embodiments, stereo-defined oligonucloetides of the invention can be used to target telomerase for the treatment of various cancer. In addition, RIPtides may be useful for the treatment of infectious disease. Viruses, such as HIV and Hepatitis C, must be able to take over a host cell in order to replicate; therefore, structural RNA can play an important role in the maturation and replication of many viruses. Accordingly, in some embodiments, stereo-defined oligonucloetides of the invention can be used to target portions of DNA or RNA involved in viral maturation and/or replication.

[001201] Thus, the invention is useful for treating various viral infections. Viruses include, but are not limited to, enteroviruses (including, but not limited to, viruses that the family picornaviridae, such as polio virus, Coxsackie virus, echo virus), rotaviruses, adenovirus, and hepatitis virus, such as hepatitis A, B, C D and E. Specific examples of viruses that have been found in humans include but are not limited to: Retroviridae (e.g., human immunodeficiency viruses, such as HIV-1 (also referred to as HTLV-III, LAV or HTLV-III/LAV, or HIV-III; and other isolates, such as HIV-LP; Picornaviridae (e.g., polio viruses, hepatitis A virus; enteroviruses, human Coxsackie viruses, rhinoviruses, echoviruses); Calciviridae (e.g., strains that cause gastroenteritis); Togaviridae (e.g., equine encephalitis viruses, rubella viruses); Flaviviridae (e.g., dengue viruses, encephalitis viruses, yellow fever viruses); Coronaviridae (e.g., coronavi ruses); Rhabdoviridae (e.g., vesicular stomatitis viruses, rabies viruses); Filoviridae (e.g., ebola viruses); Paramyxoviridae (e.g., parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); Orthomyxoviridae (e.g., influenza viruses); Bunyaviridae (e.g., Hantaan viruses, bunya viruses, phleboviruses and Nairo viruses); Arenaviridae (hemorrhagic fever viruses); Reoviridae (e.g., reoviruses, orbiviurses and rotaviruses); Birnaviridae; Hepadnaviridae (Hepatitis B virus); Parvoviridae (parvoviruses); Papovavihdae (papillomaviruses, polyoma viruses); Adenoviridae (most adenoviruses); Herpesviridae (herpes simplex virus (HSV) 1 and 2, varicella zoster virus, cytomegalovirus (CMV)); Poxviridae (variola viruses, vaccinia viruses, pox viruses); Iridoviridae (e.g., African swine fever virus); and other viruses acute laryngotracheobronchitis virus, Alphavirus, Kaposi's sarcoma-associated herpesvirus, Newcastle disease virus, Nipah virus, Norwalk virus, Papillomavirus, parainfluenza virus, avian influenza, SARs virus, West Nile virus.

Antisense drugs

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[001202] The present invention also encompasses a variety of antisense-based therapeutics. Typically, antisense drugs are small (12-21 nucleotides) DNA- or RNA-like compounds that are chemically modified to engineer favorable drug properties. Some of the antisense agents available in the art include phosphorothioate or deoxyoligonucleotides, and in this situation a sulfur group is exchanged for a nonbridging oxygen on the phosphate backbone. This increases the resistance to RNase degradation and improves the pharmacologic stability. Yet in other agents, so-called "the next generation of antisense molecules," the oligonucleotides the 2'-O-methoxyethyl modification to the backbone, which can increase the affinity for these oligonucleotides for the target RNA. However, in some cases, many of these agents are associated with side effects, such as unwanted immune responses. At least in some situations, such undesirable properties of these agents are attributable at least in part to their stereorandom nature. The present invention provides stereodefined counterpart having preferred properties, such as improved lipophilicity and increased RNA binding.

[001203] A number of antisense-based therapeutics have been developed or in development. Non-limiting examples of antisense therapies are provided below. Stereo-defined (e.g., chirally pure) oligonucleotides as described herein can be prepared for achieving improved efficacy, increased affinity to target, reduced side effects, improved pharmacokinetics, enhanced stability and/or increased bioavailability, relative to the antisense drugs currently available in the art. In some embodiments, antisense therapeutics inclide Morpholino drugs. See, for example: Morcos, PA (2007). "Achieving targeted and quantifiable alteration of mRNA splicing with Morpholino oligos". Biochem Biophys Res Commun 358 (2): 521–7.

[001204] *Cytomegalovirus retinitis*: Fomivirsen (marketed as Vitravene), was approved by the U.S. FDA in August 1998 as a treatment for cytomegalovirus retinitis.

[001205] *Hemorrhagic fever viruses*: In early 2006, scientists studying the Ebola hemorrhagic fever virus at USAMRIID announced a 75% recovery rate after infecting four rhesus monkeys and then treating them with an antisense Morpholino drug developed by AVI BioPharma, a U.S. biotechnology firm.[U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland. News Release: Gene-Specific Ebola Therapies Protect Nonhuman Primates from Lethal Disease. January 13, 2006] The usual mortality rate for monkeys infected with Ebola virus is 100%. In late 2008, AVI BioPharma successfully filed Investigational New Drug (IND) applications with the FDA for its two lead products for

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Marburg and Ebola viruses. These drugs, AVI-6002 (Lu, X; Yu, Q; Binder, GK; Chen, Z; Slepushkina, T; Rossi, J; Dropulic, B (2004). "Antisense-Mediated Inhibition of Human Immunodeficiency Virus (HIV) Replication by Use of an HIV Type 1-Based Vector Results in Severely Attenuated Mutants Incapable of Developing Resistance". Journal of Virology 78 (13): 7079–88) and AVI-6003 are novel analogs based on AVI's PMO antisense chemistry in which anti-viral potency is enhanced by the addition of positively-charged components to the morpholino oligomer chain. Preclinical results of AVI-6002 and AVI-6003 demonstrated reproducible and high rates of survival in non-human primates challenged with a lethal infection of the Ebola and Marburg viruses, respectively (Medical News Today. AVI BioPharma Announces FDA Clears IND Applications For Clinical Trials Of RNA Therapeutic Agents For Treatment Of Ebola And Marburg Viruses. 30 Dec 2008).

[001206] *Cancer*: Also in 2006, German physicians reported on a dose-escalation study for the compound AP 12009 (a phosphorothioate antisense oligodeoxynucleotide specific for the mRNA of human transforming growth factor TGF-beta2) in patients with high grade gliomas. At the time of the report, the median overall survival had not been obtained and the authors hinted at a potential cure (Results of G004, a phase IIb actively controlled clinical trial with the TGF-b2 targeted compound AP 12009 for recurrent anaplastic astrocytoma - Hau et al. 24 (18 Supplement): 1566 - ASCO Meeting Abstracts).

[001207] For example, trabedersen (AP 12009-P001) is developed as monotherapy for the treatment of patients with advanced pancreatic cancer, malignant melanoma, brain tumors, anaplastic astrocytoma, and colorectal carcinoma. AP 12009-P001 has been evaluated in an open-label, multicenter, Phase I/II dose escalation trial to evaluate safety and tolerability of i.v. administration of trabedersen in 61 patients with advanced solid tumors known to overproduce TGF-b2, who were either not or no longer amenable to established forms of therapies.

[001208] Other examples of antisense drugs that target proteins overexpressed in cancers include: Clusterin (OGX-011/TV01011), which regulates epithelial-mesenchymal transition induced by the growth factor TGF beta and the transcription factor TWIST1 in prostate cancer cells; ATL1101 (a second generation antisense inhibitor of the insulin-like growth factor-I receptor (IGF-IR)) in prostate cancer treatment; oblimersen (trade name: Genasense) for treating chronic lymphocytic leukemia; G4460 and LR3001 (inhibitors of c-myb) for the treatment of CML, melanoma, neuroblastoma, and cancers of the breast, pancreas and colon; MG98 (inhibitor

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of DNA methyltransferase I) for the treatment of renal cell carcinoma; ISIS 5132 (c-Raf antisense); LY900003 (Protein kinase C-alpha antisense); G3139 (Bcl-2 antisense), etc.

[001209] *HIV/AIDS*: Starting in 2004, researchers in the US have been conducting research on using antisense technology to combat HIV. It is known that antisense-mediated inhibition of human immunodeficiency virus (HIV) replication by use of an HIV type 1-based vector results in severely attenuated mutants incapable of developing resistance. In February 2010 researchers reported success in reducing HIV viral load using patient T-cells which had been harvested, modified with an RNA antisense strand to the HIV viral envelope protein, and re-infused into the patient during a planned lapse in retroviral drug therapy.

[001210] *High cholesterol*: In 2010 mipomersen (previously ISIS 301012, trade name Kynamro) successfully completed phase 3 trials for some types of high cholesterol. Mipomersen is a cholesterol-reducing drug candidate. It is an antisense therapeutic that targets the messenger RNA for apolipoprotein B. See: Merki E, Graham MJ, Mullick AE, et al. (August 2008). "Antisense oligonucleotide directed to human apolipoprotein B-100 reduces lipoprotein(a) levels and oxidized phospholipids on human apolipoprotein B-100 particles in lipoprotein(a) transgenic mice". Circulation 118 (7): 743–53; El Harchaoui K, Akdim F, Stroes ES, Trip MD, Kastelein JJ (2008). "Current and future pharmacologic options for the management of patients unable to achieve low-density lipoprotein-cholesterol goals with statins". Am J Cardiovasc Drugs 8 (4): 233–42; Athyros VG, Kakafika AI, Tziomalos K, Karagiannis A, Mikhailidis DP (July 2008). "Antisense technology for the prevention or the treatment of cardiovascular disease: the next blockbuster?". Expert Opin Investig Drugs 17 (7): 969–72. It is administered as a weekly injection. The compound is a 'second-generation' antisense oligonucleotide; the nucleotides are linked with phosphorothioate linkages rather than the phosphodiester linkages of RNA and DNA, and the sugar parts are deoxyribose in the middle part of the molecule and 2'-O-methoxyethyl-modified ribose at the two ends. These modifications make the drug resistant to degradation by nucleases, allowing it to be administered weekly. The drug accumulates in the liver, which is convenient since apolipoprotein B predominantly acts there. The complete sequence is

G*-C*-C*-U*-C*-dA-dG-dT-dC-dT-dG-dmC-dT-dT-dmC-G*-C*-A*-C*-C* [d = 2'-deoxy, * = 2'-O-(2-methoxyethyl)] with 3'→5' phosphorothioate linkages. The Phase 3 trials were in patients with familial hypercholesterolemia (FH), both homozygous (ho) and heterozygous (he).

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FH is a genetic disorder that causes exceptionally high levels of low-density lipoprotein cholesterol. Both trials showed exceptional performance with the highest efficacy seen so far in those two patient populations, and with relatively low drop-out rates compared to other injectable drugs.

[001211] *Duchenne muscular dystrophy*: In Duchenne muscular dystrophy (DMD), the patient's muscle cells break down and are lost, leading to progressive muscle weakness and death. AVI-4658 is a targeted antisense therapy to restore expression of dystrophin, a key protein which patients with Duchenne muscular dystrophy lack. Reesults from an open-label, phase 2, dose-escalation study with 19 patients showed that when treatment was completed, a muscle biopsy was taken from each patient. The team discovered that the patients' ability to produce functional mRNA through "exon skipping' was repaired with the use of AVI-4658, eventually allowing them to manufacture functional dystrophin protein.

[001212] *Myotonic dystrophy*: Myotonic dystrophy is the most common muscular disease in adults, affecting mainly the skeletal muscles, heart and central nervous system. It occurs because of a mutation that causes numerous repeats of three letters of the genetic code (CTG) in a gene called DMPK. The messenger RNA that is produced from the mutated gene also contains the abnormal long repeats that cause the RNA to accumulate in the cell's nucleus. There it sequesters and blocks the function of a protein called Muscleblind-like 1 and activates another protein called CELF1. These proteins antagonize one another and the result is abnormal expression of proteins from many other genes in adult tissues, resulting in disease. To counteract this, Cooper and his colleagues created antisense oligonucleotides that are simply strands of genetic material that seek out portions of the abnormal RNA repeats and target RNase H to the toxic RNA causing its degradation. It was also reported that combining the antisense oligonucleotides with other antisense oligonucleotides that help released the sequestered Muscleblind-like1 can enhance the effect.

[001213] *Diabetes*: SGLT2 (ISIS 388626) is an antisense drug to achieve reduction in sodium dependent glucose co-transporter type 2 (SGLT2) levels that resulted in a significant reduction in blood glucose levels in diabetic patients.

[001214] *Hepatitis*: Persistent hepatitis C vius (HCV) infection is a leading cause of liver disease. Antisense oligonucloetides represent a promising class of antiviral agents. Such

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drugs under development include ISIS 14803, which is a 20-unit phosphorothioate oligodeoxynucleotide that inhibits HCV replication and protein expression.

Methods of Treatment

[001215] Provided oligonucleotides and compositions thereof, described herein, are useful as therapeutic agents against various disease states, including use as antiviral agents. In some embodiments, provided oligonucleotides can be used as agents for treatment of diseases through modulation of DNA and/or RNA activity. In some embodiments, provided oligonucloetides can be used for inhibiting specific gene expression. For example, a provided oligonucloetide can be complementary to a specific target messenger RNA (mRNA) sequence. It can be used to inhibit viral replication of myriad viruses. Exemplary virus families include orthomyxoviruses, pox viruses, herpes viruses, papilloma viruses, picornaviruses, flaviviruses, retroviruses, hepatitis viruses, paramyxoviruses, reoviruses, parvoviruses, filoviruses, coronaviruses, arenaviruses, rhabdoviruses and adenoviruses. Additional virus families are known and are also contemplated herein. Other examples include uses as antisense compounds against HIV RNA or other retroviral RNA or for hybridizing to HIV mRNA encoding the tat protein, or to the TAR region of HIV mRNA. In some embodiments, the nucleic acids mimic the secondary structure of the TAR region of HIV mRNA, and by doing so bind the tat protein. In some embodiments, a provided oligonucloetide is used to inhibit expression of a target protein by contacting a cell with a provided oligonucleotide, wherein the expression of other proteins in the cell are not inhibited or are minimally inhibited. In some embodiment, target protein inhibition occurs *in vivo* in a mammal. In some embodiments, a therapeutically effective amount of a provided oligonucleotide is administered for inhibiting the expression of a target protein.

[001216] Other examples of proteins where expression can be modulated include Jun N-terminal kinase (JNK) proteins, diacylglycerol acyltransferase I, apolipoprotein B, glucagon receptor, Aurora B, acyl CoA cholesterol acyltransferase-2, c-reactive protein, STAT (signal transducers and activators of transcription) family of proteins, and MDR P-glycoprotein. In some embodiments, a provided oligonucleotide can be used to inhibit protein phosphatase 1B (PTP1B) expression, RNA-dependent RNA viral polymerase. In some embodiments, a provided oligonucleotide can be used to induce events such as apoptosis in cancer cells or to make a cell more susceptible to apoptosis. In some embodiments, a provided oligonucleotide can be used to

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modulate activities of proteins. In some embodiments, a provided oligonucleotide can help modulate RNase H activity targeting multidrug resistance (MDR) RNA molecules.

[001217] In some embodiments, the present invention provides methods of treating a disease mediated by undesired gene expression in a subject (e.g., mammals, such as humans) in need of such treatment. By "diseases" is meant diseases, or disease symptoms. Methods include, e.g., administering to the subject an effective amount of a provided oligonucleotide.

[001218] Examples of diseases mediated by undesired gene expression include cancer (e.g., leukemia, tumors, and metastases), allergy, asthma, obesity, inflammation (e.g., inflammatory diseases such as inflammatory airways disease), hypercholesterolemia, hematological disorders, severe acute respiratory syndrome (SARS), obstructive airways disease, asthma, autoimmune diseases, retroviral diseases such as AIDS or HIV, other viral infections, intrauterine infections, metabolic diseases, infection (e.g., bacterial, viral, yeast, fungal), CNS diseases, brain tumors, degenerative neural diseases, cardiovascular diseases, and diseases associated with angiogenesis, neovascularization, and vasculogenesis.

[001219] In some embodiments, provided oligonucleotides are useful for treating cancer, including pancreatic cancer, and other diseases or disorders involving abnormal cell proliferation.

[001220] Located in the upper abdomen (in the retroperitoneum), the pancreas is a dual-function gland of the digestive and endocrine system. In certain instances, the pancreas functions as an endocrine gland (e.g., producing several important hormones). In certain instances, the pancreas functions as an exocrine gland (e.g., secreting fluids containing digestive enzymes that pass to the small intestine).

[001221] Pancreatic cancer is the fourth most common cause of cancer death in the US (after lung, colon and breast), comprising 6% of all cancer-related deaths. In 2008, an estimated 37,680 new cases of pancreatic cancer will have been diagnosed in the US, with 34,290 deaths. Incidence of the disease, rises linearly after age 50, with the only definitive risk factor being cigarette smoking (smokers are four times more likely to develop the disease than non-smokers). Invasive pancreatic cancer is almost always fatal. The collective median survival time of all patients is 4-6 months. Relative 1-year survival is 24%; the overall 5-year survival rate < 5%.

[001222] Pancreatic cancer is asymptomatic in its early stage and often remains undiagnosed for several months (less than one third of patients being diagnosed within 2 months

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of the onset symptoms). In certain instances, the delayed diagnosis results in (either partially or fully) metastasis of the cancerous cells to the liver or lymph nodes.

[001223] Currently, surgery (resectioning of the pancreas) is the primary and only curative therapy for pancreatic cancer. However, only 15-25% of tumors are resectable at the time of diagnosis and only 10-20% of patients undergoing surgery survive more than two years. Once tumor infiltration occurs and other tissues have been affected, surgery is no longer possible.

[001224] In certain instances, diabetes mellitus or pancreatitis predisposes an individual to develop a proliferative disorder of a plurality of pancreatic cells. In certain instances, individuals are at an increased risk of developing a proliferative disorder of a plurality of pancreatic cells due to a hereditary syndrome selected from the group consisting of hereditary nonpolyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP). In certain instances, individuals are at an increased risk of developing a proliferative disorder of a plurality of pancreatic cells due to a mutation in a gene selected from the group consisting of MSH2, MSH6, MLH1, and APC.

[001225] Ideally, effective treatment of pancreatic cancer should (i) control the primary tumor mass, both initially and subsequently, and (ii) treat the metastatic tumor cells. Chemoprevention (the administration of agents such as drugs, biologics, nutrients and the like) slows the progression of, reverses, or inhibits carcinogenesis, thereby lowering the risk of developing invasive or clinically significant disease.

[001226] Disclosed herein, in certain embodiments, are methods of treating pancreatic cancer. As used herein, "pancreatic cancer" includes forms of cancer of the pancreas. In some embodiments, a pancreatic cancer is metastatic pancreatic cancer. In some embodiments, a pancreatic cancer is a carcinoma, sarcoma, cancer, or combinations thereof. In some embodiments, a pancreatic cancer to be treated includes sporadic and hereditary pancreatic cancers. In some embodiments, a pancreatic cancer is duct cell carcinoma, acinar cell carcinoma, papillary mucinous carcinoma, signet ring carcinoma, adenosquamous carcinoma, undifferentiated carcinoma, mucinous carcinoma, giant cell carcinoma, small cell carcinoma, cystcancer, serous cystcancer, mucinous cystcancer, unclassified pancreatic cancer, pancreatoblastoma, or combinations thereof.

[001227] In some embodiments, an individual in need of treatment for pancreatic cancer presents with a localized tumor of the pancreas. In some embodiments, an individual in need of

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treatment for pancreatic cancer presents with a negative regional lymph node biopsy. In some embodiments, an individual in need of treatment for pancreatic cancer presents with a positive regional lymph node biopsy. In some embodiments, an individual in need of treatment for pancreatic cancer presents with a nodal negative pancreatic tumor (e.g., node-negative). In some embodiments, an individual in need of treatment for pancreatic cancer presents with a nodal positive tumor (e.g., node-positive).

[001228] In some embodiments, a pancreatic cancer in an individual in need of treatment for pancreatic cancer has metastasized to other locations in the body. In some embodiments, a pancreatic cancer has metastasized to a location selected from the group consisting of lymph node, stomach, bile duct, liver, bone, ovary, peritoneum and brain.

[001229] In some embodiments, cancer cells or precancerous cells are identified by histological typing or grading of a tissue sample (e.g., a biopsy sample). In some embodiments, cancer cells or precancerous cells are identified through the use of appropriate molecular markers.

[001230] In some embodiments, a pancreatic cancer in an individual in need of treatment for pancreatic cancer is staged according to the American Joint Committee on Cancer (AJCC) TNM classification system, where the tumor (T) has been assigned a stage of Tx, T1, T2, T3, T4; and where the regional lymph nodes (N) have been assigned a stage of NX, N0, N1; and where distant metastasis (M) has been assigned a stage of MX, M0, or M1. In some embodiments, a pancreatic cancer in an individual in need of treatment for pancreatic cancer is staged as Stage 0, I, IA, IB, II, IIA, IIB, III, and IV pancreatic cancer. In some embodiments, a pancreatic cancer in an individual in need of treatment for pancreatic cancer is staged as Grade GX (e.g., grade cannot be assessed), Grade 1, Grade 2, Grade 3 or Grade 4.

[001231] More specific examples of cancers treated with provided oligonucleotides include breast cancer, lung cancer, melanoma, colorectal cancer, bladder cancer, ovarian cancer, prostate cancer, renal cancer, squamous cell cancer, glioblastoma, Kaposi's sarcoma, multiple myeloma, and leukemia.

Evaluation and Treatment of Cancer

[001232] The term "tumor cell antigen" is defined herein as an antigen that is present in higher quantities on a tumor cell or in body fluids than unrelated tumor cells, normal cells, or in

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normal body fluid. The antigen presence may be tested by any number of assays known to those skilled in the art and include without limitation negative and/or positive selection with antibodies, such as an ELISA assay, a radioimmunoassay, or by Western Blot.

[001233] "Apoptosis inducing agent" is defined herein to induce apoptosis/programmed cell death, and include, for example, anticancer agents and treatments wherein cells (e.g., tumor cells) are induced to undergo programmed cell death. Exemplary apoptosis inducing agents are described in more detail below.

[001234] The terms "apoptosis" or "programmed cell death," refers to the physiological process by which unwanted or useless cells are eliminated during development and other normal biological processes. Apoptosis is a mode of cell death that occurs under normal physiological conditions and the cell is an active participant in its own demise ("cellular suicide"). It is most often found during normal cell turnover and tissue homeostasis, embryogenesis, induction and maintenance of immune tolerance, development of the nervous system and endocrine-dependent tissue atrophy. Cells undergoing apoptosis show characteristic morphological and biochemical features. These features include chromatin aggregation, nuclear and cytoplasmic condensation, partition of cytoplasm and nucleus into membrane bound vesicles (apoptotic bodies), which contain ribosomes, morphologically intact mitochondria and nuclear material. *In vivo*, these apoptotic bodies are rapidly recognized and phagocytized by macrophages, dendritic cells or adjacent epithelial cells. Due to this efficient mechanism for the removal of apoptotic cells *in vivo* no inflammatory response is elicited. *In vitro*, the apoptotic bodies as well as the remaining cell fragments ultimately swell and finally lyse. This terminal phase of *in vitro* cell death has been termed "secondary necrosis." Apoptosis can be measured by methods known to those skilled in the art like DNA fragmentation, exposure of Annexin V, activation of caspases, release of cytochrome c, etc. A cell that has been induced to die is termed herein as an "apoptotic cell." Apoptosis can also be tested using a standard Annexin V Apoptosis Assay: NIH:OVCAR-3 cells are grown in 6-well plates (NUNC) and irradiated or treated with an antagonist (or in combination with another anti-cancer drug) for 4-48 hours, washed and stained with Annexin V-FITC (BD-Pharmingen) for 1 hour. Cells are analyzed by flow cytometry (Becton-Dickinson, CellQuest), counterstained with Propidium Iodide and analyzed again in the flow cytometer.

[001235] Patients can be assessed with respect to symptoms at one or more multiple time points including prior to, during, and after treatment regimens. Treatment can result in

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improving the subject's condition and can be assessed by determining if one or more of the following factors has occurred: decreased tumor size, decreased cell proliferation, decreased numbers of cells, decreased neovascularization, increased apoptosis, or decreased survival of at least a portion of the tumor cells. One or more of these occurrences may, in some cases, result in partial or total elimination of the cancer and prolongation of survival of the patient. Alternatively, for terminal stage cancers, treatment may result in stasis of disease, better quality of life and/or prolongation of survival.

Methods of Assaying Cell Migration

[001236] Assays for cell migration have been described in the literature, e.g., by Brooks, et al., J. Clin. Invest 1997, 99:1390-1398 and methods for measuring cell migration are known to those of skill in the art. In one method for measuring cell migration described herein, membranes from transwell migration chambers are coated with substrate, the transwells washed, and non-specific binding sites blocked with BSA. Tumor cells from sub-confluent cultures are harvested, washed, and resuspended in migration buffer in the presence or absence of assay antibodies. After the tumor cells are allowed to migrate to the underside of the coated transwell membranes, the cells remaining on the top-side of the membrane are removed and cells that migrate to the under-side are stained with crystal violet. Cell migration is then quantified by direct cell counts per microscopic field.

Methods of Assaying Tumor Growth

[001237] Tumor growth can be assayed by methods known to those of skill in the art, e.g., the SCID mouse model, the nude mouse model, and BALB/c mice with syngeneic tumors. SCID mouse models for tumor growth are carried out as follows: subconfluent human M21 melanoma cells (or any desired tumor cell type) are harvested, washed, and resuspended in sterile PBS (20 x 106 per mL). SCID mice are injected subcutaneously with 100 μL of M21 human melanoma cell (2 x 106) suspension. Three days after tumor cell injection, mice are either untreated or treated intraperitoneally with an antagonist in the desired dose ranges. The mice are treated daily for 24 days. Tumor size is measured with calipers and the volume estimated using the formula V = (L x W2)/2, where V is equal to the volume, L is equal to the length, and W is equal to the width.

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[001238] Alternatively, nude mouse models, SCID mouse models and/or BALB/c syngeneic mouse models can also be utilized to assess tumor growth and inhibition thereof by the humanized anti-endoglin antibodies or antigen-binding fragments described herein.

Methods of Assaying Cell Proliferation

[001239] Cell proliferation can be assayed by methods known to those of skill in the art. As described herein, subconfluent human endothelial cells (HUVECs) can be resuspended in proliferation buffer containing low (5.0%) serum in the presence or absence of CM (25 μL) from ECV or ECVL cells, and endothelial cells allowed to proliferate for 24 hours. Proliferation can be quantified by measuring mitochondrial dehydrogenase activity using a commercially available WST-1 assay kit (Chemicon). Also, as described herein, proliferation can be quantified by measuring 3H incorporation using standard methods. (She et al., Int. J. Cancer, 108: 251-257 (2004)).

[001240] Other methods of assessing cell proliferation are known in the art and are contemplated herein. Further non-limiting examples are described in more detail in the examples.

[001241] One would understand that classification and staging systems described herein represent one means to assess treatment of cancers described herein; additionally, other staging schemes are known in the art and may be used in connection with the methods described herein. By way of example only, the TNM classification of malignant tumors may be used as a cancer staging system to describe the extent of cancer in a patient's body. T describes the size of the tumor and whether it has invaded nearby tissue, N describes regional lymph nodes that are involved, and M describes distant metastasis. TNM is maintained by the International Union Against Cancer (UICC) and is used by the American Joint Committee on Cancer (AJCC) and the International Federation of Gynecology and Obstetrics (FIGO). One would understand that not all tumors have TNM classifications such as, for example, brain tumors. Generally, T (a,is,(0), 1-4) is measured as the size or direct extent of the primary tumor. N (0-3) refers to the degree of spread to regional lymph nodes: N0 means that tumor cells are absent from regional lymph nodes, N1 means that tumor cells spread to the closest or small numbers of regional lymph nodes, N2 means that tumor cells spread to an extent between N1 and N3; N3 means that tumor cells spread to most distant or numerous regional lymph nodes. M (0/1) refers to the presence of

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metastasis: M0 means that no distant metastasis are present; M1 means that metastasis has occurred to distant organs (beyond regional lymph nodes). Other parameters may also be assessed. G (1-4) refers to the grade of cancer cells (i.e., they are low grade if they appear similar to normal cells, and high grade if they appear poorly differentiated). R (0/1/2) refers to the completeness of an operation (i.e., resection-boundaries free of cancer cells or not). L (0/1) refers to invasion into lymphatic vessels. V (0/1) refers to invasion into vein. C (1-4) refers to a modifier of the certainty (quality) of V.

[001242] Provided herein are methods for degrading, inhibiting the growth of or killing cancer cells comprising contacting the cells with an amount of a compound described herein effective to degrade, inhibit the growth of or kill cancer cells.

[001243] Provided herein are methods of inhibiting tumor size increase, reducing the size of a tumor, reducing tumor proliferation or preventing tumor proliferation in an individual comprising administering to said individual an effective amount of a compound described herein to inhibit tumor size increase, reduce the size of a tumor, reduce tumor proliferation or prevent tumor proliferation. Treatment of tumors in some cases includes stasis of symptoms, that is, by treating the patient, the cancer does not worsen and survival of the patient is prolonged.

[001244] Patients may be assessed with respect to symptoms at one or more multiple time points including prior to, during, and after treatment regimens. Treatment can result in improving the subject's condition and can be assessed by determining if one or more of the following events has occurred: decreased tumor size, decreased tumor cell proliferation, decreased numbers of cells, decreased neovascularization and/or increased apoptosis. One or more of these occurrences may, in some cases, result in partial or total elimination of the cancer and prolongation of survival of the patient. Alternatively, for terminal stage cancers, treatment may result in stasis of disease, better quality of life and/or prolongation of survival.

[001245] Other methods of assessing treatment are known in the art and contemplated herein. In an exemplary embodiment, the pro-oligonucleotide compounds of the invention are administered to a subject such as a mammal (e.g., a human), suffering from a medical disorder, e.g., a cancer, or non-malignant conditions characterized by the presence of a class of unwanted cells.

[001246] Primary outcome measures may be assessed for patients treated using the methods described herein and include, for example, progression-free survival. In one embodiment, an

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increase in progression free survival is observed in an amount of by about 2-fold, 5-fold, 10-fold, 20 fold, 50 fold or more compared to lack of treatment. In another embodiment, an increase in progression free survival is increased survival by about 3 months, about 6 months, about 9 months, about 12 months, about 18 months, about 2 years, about 3 years, about 4 years, about 5 years or more compared to lack of treatment.

[001247] Secondary outcome measures may also be assessed and include duration of response, time to tumor progression, overall survival, serious and non-serious adverse events. For example, a treatment may prevent progression of the disease (i.e., stasis) or may result in an improvement. Alternately, or in addition, other goals can be measured with respect to one or more of the following: decreased tumor burden, decreased neovascularization, reduced side effects, decreased adverse reactions, and/or increased patient compliance.

[001248] Other specific examples of diseases or disorders for which treatment by the compounds or compositions of the invention are useful for treatment or prevention include, but are not limited to transplant rejection (e.g., kidney, liver, heart, lung, islet cells, pancreas, bone marrow, cornea, small bowel, skin allografts or xenografts and other transplants), graft vs. host disease, osteoarthritis, rheumatoid arthritis, multiple sclerosis, diabetes, diabetic retinopathy, inflammatory bowel disease (e.g., Crohn's disease, ulcerative colitis, and other bowel diseases), renal disease, cachexia, septic shock, lupus, myasthenia gravis, psoriasis, dermatitis, eczema, seborrhea, Alzheimer's disease, Parkinson's disease, stem cell protection during chemotherapy, ex vivo selection or ex vivo purging for autologous or allogeneic bone marrow transplantation, ocular disease, retinopathies (e.g., macular degeneration, diabetic retinopathy, and other retinopathies), corneal disease, glaucoma, infections (e.g., bacterial, viral, or fungal), heart disease, including, but not limited to, restenosis.

Activation of RNAse L

[001249] The 2'-5' oligoadenylate (2-5A)/RNase L pathway is one of the enzymatic pathways induced by interferon. Rnase L is activated after binding to 5'-phosphoroylated fragments of 2'-5' adenylic acid. These fragments of 2'-5' adenylic acid (2-5A) are produced under the control of 2'-5' oligo(A) synthetase. This pathway is part of the innate immune system and has an important role in preventing viral infection. 2-5A-Induced cleavage of single-stranded RNA results in apoptosis. Biostable phosphorothioate analogs of 2-5A have been

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shown to be potent activators of Rnase L (Xianh et al., Cancer Research (2003), 63:6795-6801). In this study, the 2-5A analogs induced Rnase L activity and caused apoptosis in cultures of late-stage, metastatic human prostate cancer cell lines DU145, PC3 and LNCaP.

[001250] Sustained activation of RNase L triggers a mitochondrial pathway of apoptosis that eliminates virus-infected cells as well as cancerous/tumor cells. RNase L can inhibit fibrosarcoma growth, prostate cancer growth, colorectal cancer growth and pancreatic cancer growth. Given the common role of RNase L in different cancers, it is contemplated that the invention described herein can be use for the treatment of any type of cancer. Silverman, RH, Cytokine Growth Factor Rev, 18(5-6): 381-388 (2007); Bisbal, C. and Silverman, RH, *Biochimie*. 89(6-7): 789–798 (2007). By way of example, downregulation of RNase L refers to any reduction in expression levels of the gene or genes encoding RNase L, silencing of the gene or genes encoding RNase L, reduction in the levels of expression/translation of the proteins comprising RNase L, redudction in the amount of RNase L present within a cell, and/or any reduction in activity of RNase L as compared to a predetermined level of RNase L in an exemplary healthy population. Alternatively any reduction in RNase L levels as described herein can be indicative of downregulation of RNase L.

[001251] In some embodiments, provided oligonucleotides are useful for the treatment of diseases having downregulated RNase L. In some embodiment, a disease associated with downregulated RNase L is cancer. In some embodiments, the cancer is pancreatic cancer, prostate cancer, or colorectal cancer. Alternatively, provided oligonucleotides described herein are useful for the treatment of disease having upregulated RNase L. In some embodiments, the disease having upregulated RNase L is chronic fatigue syndrome. Additional diseases having upregulated RNase L are known in the art and contemplated herein.

[001252] When used as therapeutics, a provided oligonucleotide is administered as a pharmaceutical composition. In some embodiments, the pharmaceutical composition comprises a therapeutically effective amount of a provided oligonucleotide comprising, or a pharmaceutically acceptable salt thereof, and at least one pharmaceutically acceptable inactive ingredient selected from pharmaceutically acceptable diluents, pharmaceutically acceptable excipients, and pharmaceutically acceptable carriers. In another embodiment, the pharmaceutical composition is formulated for intravenous injection, oral administration, buccal administration, inhalation, nasal administration, topical administration, ophthalmic

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administration or otic administration. In further embodiments, the pharmaceutical composition is a tablet, a pill, a capsule, a liquid, an inhalant, a nasal spray solution, a suppository, a suspension, a gel, a colloid, a dispersion, a suspension, a solution, an emulsion, an ointment, a lotion, an eye drop or an ear drop.

Pharmaceutical compositions

[001253] When used as therapeutics, a provided oligonucleotide or oligonucleotide composition described herein is administered as a pharmaceutical composition. In some embodiments, the pharmaceutical composition comprises a therapeutically effective amount of a provided oligonucleotides, or a pharmaceutically acceptable salt thereof, and at least one pharmaceutically acceptable inactive ingredient selected from pharmaceutically acceptable diluents, pharmaceutically acceptable excipients, and pharmaceutically acceptable carriers. In some embodiments, the pharmaceutical composition is formulated for intravenous injection, oral administration, buccal administration, inhalation, nasal administration, topical administration, ophthalmic administration or otic administration. In some embodiments, the pharmaceutical composition is a tablet, a pill, a capsule, a liquid, an inhalant, a nasal spray solution, a suppository, a suspension, a gel, a colloid, a dispersion, a suspension, a solution, an emulsion, an ointment, a lotion, an eye drop or an ear drop.

[001254] In some embodiments, the present invention provides a pharmaceutical composition comprising chirally controlled oligonucleotide, or composition thereof, in admixture with a pharmaceutically acceptable excipient. One of skill in the art will recognize that the pharmaceutical compositions include the pharmaceutically acceptable salts of the chirally controlled oligonucleotide, or composition thereof, described above.

Compounds for enhancing and targeting delivery

[001255] A provided oligonucleotide as described herein can be delivered using a variety of delivery strategies, including conjugates of nucleic acids with various ligands, as well as use of nanocarrier approaches. Any nucleic acid delivery strategies are contemplated for use with provided oligonucleotides described herein. The choice between exemplary delivery strategies, including but not limited to, chemical conjugates, cationic lipid/liposomal transfer vessicles and

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supramolecular nanocarriers depends on the therapeutic context, and methods for determining the optimal delivery modality are known in the art and further contemplated herein.

Cell Penetrating Compounds ("CPCs")

[001256] Numerous compounds are known to act as carriers for cargo such as nucleic acids and facilitate entry of the nucleic acid in a cell in an *in vivo* setting. Exemplary carriers are described in Dietz et al., Molecular & Cellular Neuroscience, 27(2): 85-131 (2004) which is incorporated herein by reference. The prototypical CPCs derived from the Tat and antennepedia transcriptional regulators have been joined by a large number of new moieties. As an example, CPCs that are peptides can be relatively short (9–30 amino acids) polycationic peptides rich in argine and lysine, or membrane-interactive hydrophobic sequences. CPCs can be linked by recombinant DNA techniques or chemically coupled to peptides, oligonucleotides or nanocarriers, which then comprise the 'cargo' for the CPC.

Cell Targeting Ligands ("CTLs")

[001257] Another strategy is to deliver oligonucleotides by use of a CTL that binds with high affinity to a cell surface receptor that is capable of undergoing efficient internalization. Potential ligands include antibodies, polypeptides derived from phage display libraries, and small organic molecules. Additional cell-targeting ligands are known in the art, or will be developed, and are contemplated for use with the invention described herein (for ASGPR – GalNAc conjugated siRNA and oligonucleotides, e.g., WO2012037254A1). Because various receptors are often preferentially expressed on particular cell types, this approach offers the possibility of improved selectivity for the oligonucleotide reagents. Exemplary receptor targets include, but are not limited to, lipoprotein receptors (such as those in the liver), integrins, receptor tyrosine kinases, and the G-protein coupled receptor (GPCR) superfamily.

Nanocarriers

[001258] A variety of supramolecular nanocarriers can be used to deliver nucleic acids. Exemplary nanocarriers include, but are not limited to liposomes, cationic polymer complexes and various polymeric. Complexation of nucleic acids with various polycations is another approach for intracellular delivery; this includes use of PEGlyated polycations,

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polyethyleneamine (PEI) complexes, cationic block co-polymers, and dendrimers. Several cationic nanocarriers, including PEI and polyamidoamine dendrimers help to release contents from endosomes. Other approaches include use of polymeric nanoparticles, polymer micelles, quantum dots and lipoplexes.

[001259] Additional nucleic acid delivery strategies are known in addition to the exemplary delivery strategies described herein.

[001260] In therapeutic and/or diagnostic applications, the compounds of the invention can be formulated for a variety of modes of administration, including systemic and topical or localized administration. Techniques and formulations generally may be found in Remington, The Science and Practice of Pharmacy, (20th ed. 2000).

[001261] Provided oligonucleotides, and compositions thereof, are effective over a wide dosage range. For example, in the treatment of adult humans, dosages from about 0.01 to about 1000 mg, from about 0.5 to about 100 mg, from about 1 to about 50 mg per day, and from about 5 to about 100 mg per day are examples of dosages that may be used. The exact dosage will depend upon the route of administration, the form in which the compound is administered, the subject to be treated, the body weight of the subject to be treated, and the preference and experience of the attending physician.

[001262] Pharmaceutically acceptable salts are generally well known to those of ordinary skill in the art, and may include, by way of example but not limitation, acetate, benzenesulfonate, besylate, benzoate, bicarbonate, bitartrate, bromide, calcium edetate, carnsylate, carbonate, citrate, edetate, edisylate, estolate, esylate, fumarate, gluceptate, gluconate, glutamate, glycollylarsanilate, hexylresorcinate, hydrabamine, hydrobromide, hydrochloride, hydroxynaphthoate, iodide, isethionate, lactate, lactobionate, malate, maleate, mandelate, mesylate, mucate, napsylate, nitrate, pamoate (embonate), pantothenate, phosphate/diphosphate, polygalacturonate, salicylate, stearate, subacetate, succinate, sulfate, tannate, tartrate, or teoclate. Other pharmaceutically acceptable salts may be found in, for example, Remington, The Science and Practice of Pharmacy (20th ed. 2000). Preferred pharmaceutically acceptable salts include, for example, acetate, benzoate, bromide, carbonate, citrate, gluconate, hydrobromide, hydrochloride, maleate, mesylate, napsylate, pamoate (embonate), phosphate, salicylate, succinate, sulfate, or tartrate.

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[001263] Depending on the specific conditions being treated, such agents may be formulated into liquid or solid dosage forms and administered systemically or locally. The agents may be delivered, for example, in a timed- or sustained- low release form as is known to those skilled in the art. Techniques for formulation and administration may be found in Remington, The Science and Practice of Pharmacy (20th ed. 2000). Suitable routes may include oral, buccal, by inhalation spray, sublingual, rectal, transdermal, vaginal, transmucosal, nasal or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intra-articullar, intra-sternal, intra-synovial, intra-hepatic, intralesional, intracranial, intraperitoneal, intranasal, or intraocular injections or other modes of delivery.

[001264] For injection, the agents of the invention may be formulated and diluted in aqueous solutions, such as in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological saline buffer. For such transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

[001265] Use of pharmaceutically acceptable inert carriers to formulate the compounds herein disclosed for the practice of the invention into dosages suitable for systemic administration is within the scope of the invention. With proper choice of carrier and suitable manufacturing practice, the compositions of the present invention, in particular, those formulated as solutions, may be administered parenterally, such as by intravenous injection.

[001266] The compounds can be formulated readily using pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration. Such carriers enable the compounds of the invention to be formulated as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a subject (e.g., patient) to be treated.

[001267] For nasal or inhalation delivery, the agents of the invention may also be formulated by methods known to those of skill in the art, and may include, for example, but not limited to, examples of solubilizing, diluting, or dispersing substances such as, saline, preservatives, such as benzyl alcohol, absorption promoters, and fluorocarbons.

[001268] Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its

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intended purpose. Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

[001269] In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. The preparations formulated for oral administration may be in the form of tablets, dragees, capsules, or solutions.

[001270] Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipients, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethyl-cellulose (CMC), and/or polyvinylpyrrolidone (PVP: povidone). If desired, disintegrating agents may be added, such as the cross-linked polyvinylpyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

[001271] Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinylpyrrolidone, carbopol gel, polyethylene glycol (PEG), and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dye-stuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

[001272] Pharmaceutical preparations that can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin, and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols (PEGs). In addition, stabilizers may be added.

[001273] Depending upon the particular condition, or disease state, to be treated or prevented, additional therapeutic agents, which are normally administered to treat or prevent that

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condition, may be administered together with the inhibitors of this invention. For example, chemotherapeutic agents or other anti-proliferative agents may be combined with the inhibitors of this invention to treat proliferative diseases and cancer. Examples of known chemotherapeutic agents include, but are not limited to, adriamycin, dexamethasone, vincristine, cyclophosphamide, fluorouracil, topotecan, taxol, interferons, and platinum derivatives.

[001274] Other examples of agents the non-racemic pro-oligonucleotide of this invention may also be combined with include, without limitation, anti-inflammatory agents such as corticosteroids, TNF blockers, IL-1 RA, azathioprine, cyclophosphamide, and sulfasalazine; immunomodulatory and immunosuppressive agents such as cyclosporin, tacrolimus, rapamycin, mycophenolate mofetil, interferons, corticosteroids, cyclophophamide, azathioprine, and sulfasalazine; neurotrophic factors such as acetylcholinesterase inhibitors, MAO inhibitors, interferons, anti-convulsants, ion channel blockers, riluzole, and anti-Parkinsonian agents; agents for treating cardiovascular disease such as beta-blockers, ACE inhibitors, diuretics, nitrates, calcium channel blockers, and statins; agents for treating liver disease such as corticosteroids, cholestyramine, interferons, and anti-viral agents; agents for treating blood disorders such as corticosteroids, anti-leukemic agents, and growth factors; agents for treating diabetes such as insulin, insulin analogues, alpha glucosidase inhibitors, biguanides, and insulin sensitizers; and agents for treating immunodeficiency disorders such as gamma globulin.

[001275] These additional agents may be administered separately, as part of a multiple dosage regimen, from provided chirally controlled oligonucleotides, and composition thereof. Alternatively, these agents may be part of a single dosage form, mixed together with the provided chirally controlled oligonucleotides, and composition thereof, in a single composition.

[001276] The examples and preparations provided below further illustrate and exemplify provided oligonucleotides and compositions thereof, and methods of preparing the same. It is to be understood that the scope of the present invention is not limited in any way by the scope of the following examples and preparations.

[001277] The function and advantage of these and other embodiments of the present invention will be more fully understood from the examples described below. The following examples are intended to illustrate the benefits of the present invention, but do not exemplify the full scope of the invention.

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Method of Analyzing Oligonucleotide Compositions

[0001] In some embodiments, the present invention provides methods for analyzing oligonucleotide compositions. In some embodiments, a provided method detects, determines, and/or quantifies, for example, the identity, purity, quantity and/or quality of one or more oligonucleotides. In some embodiments, the present invention provides a method comprising steps of:

a) performing a first analysis of a first composition, which first composition comprises a plurality of different types of an oligonucleotide; and

b) comparing the performed first analysis with a second analysis, under comparable conditions as the first analysis, of a second composition, which second composition is a chirally controlled composition of the oligonucleotide, where differences between the first and second analyses reflect differences in presence or level of at least one type of the oligonucleotide in the first as compared with the second composition.

[0002] In some embodiments, a second composition contains only a single type of the oligonucleotide. In some embodiments, a second composition contains more than one types of the oligonucleotide. In some embodiments, a provided method is used for quality control of oligonucleotide compositions, for example, as illustrated in Examples 17 and 48. As will be appreciated by those skilled in the art, the presented data in Example 48 confirm that the depicted analyses comparing the compositions show that the random oligonucleotide composition (the first composition), as prepared by non-chiral controlled oligonucleotide synthesis, comprises extremely low level of certain oligonucleotide types, such as the full *R*p or *S*p type of the chirally controlled composition (the second composition).

Exemplification

[0003] The foregoing has been a description of certain non–limiting embodiments of the invention. Accordingly, it is to be understood that the embodiments of the invention herein described are merely illustrative of the application of the principles of the invention. Reference herein to details of the illustrated embodiments is not intended to limit the scope of the claims.

[0004] **General Description of Oligonucleotide Synthesis**

[0005] As described above and herein, in some embodiments, the present invention provides oligonucleotides and the methods of preparing the same. In some embodiments, the

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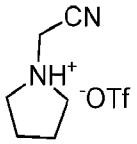
synthesis of oligonucleotide is performed using solid support. In some embodiments, the synthesis of oligonucleotide is performed in solution. In some embodiments, the synthesis of oligonucleotide comprises steps using solid support and steps in solution phase. In some embodiments, the steps using solid support are performed on an oligonucleotide synthesizer.

[0006] Preparation of the Solid Support: The support (Highly Cross-linked Polystyrene (HCP) or Controlled Pore Glass (CPG)) was used to load the first 5′–*O*-DMTr protected nucleoside contained in the oligonucleotide sequence. In some embodiments, an oxalyl group was used to link the 3′–OH group of the first nucleoside to an amine group on the solid support, as described in Alul *et al.*, Oxalyl-CPG: a labile support for synthesis of sensitive oligonucleotide derivatives, *Nucleic Acids Research* **1991**, *19(7)*, 1527. In some embodiments, the standard succinyl group was used as the linker.

[0007] Detritylation of Nucleotide on the Solid Support: A solution of 3% TCA (trichloroacetic acid) in dichloromethane (DCM, CH_2Cl_2) was delivered to the column containing the support which was installed on a synthesizer to de-block the 5′–*O*-DMTr.

[0008] CMPT-Mediated Chiral Phosphoramidite Coupling: After washing of the solid support with anhydrous acetonitrile (ACN) and drying by reverse flush with dry argon, the free 5′–OH was coupled with the next nucleotide in the oligonucleotide sequence, growing from the 3′ to the 5′ end. This entailed co-delivery to the solid support of a solution of the chiral phosphoramidite together with CMPT (below), which resulted in highly efficient coupling with excellent diastereomeric excess (typically >99%) to give a chiral phosphite diester product, as described by Wada *et al. J. Am. Chem. Soc.*, **2008**, *130*, 16031–1603; *Angew. Chem. Intern. Ed.* **2009**, 48, 496–499. The solvent was normally acetonitrile (ACN), but other solvents could also be used.



CMPT

Chiral Phosphoramidites Employed in These Studies:

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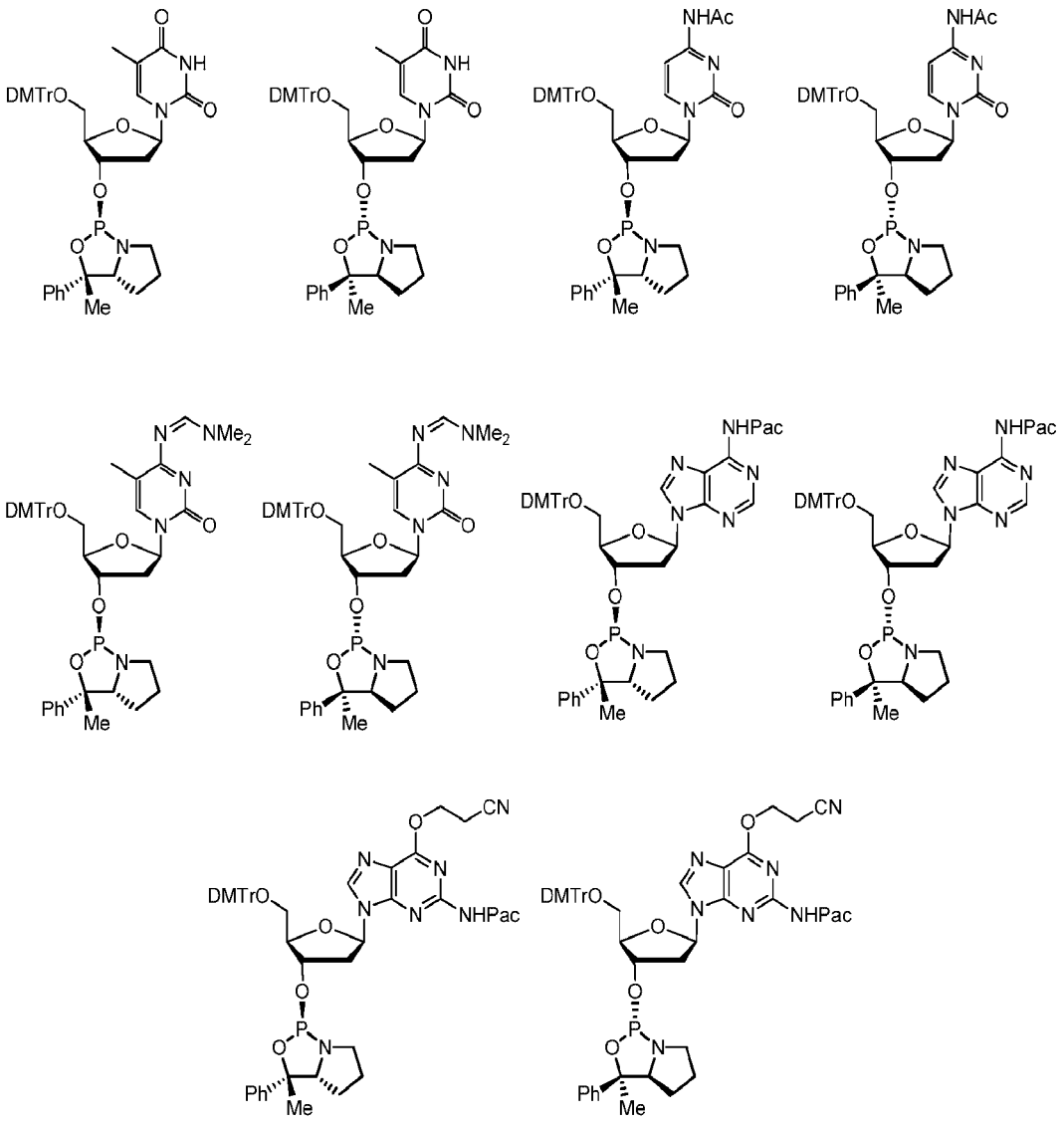
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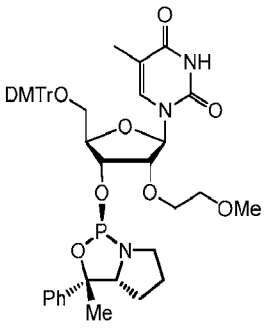
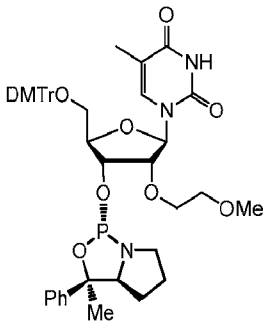
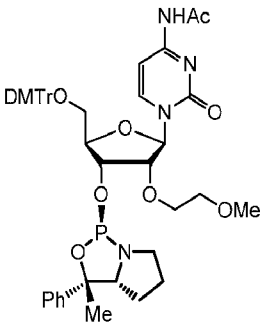
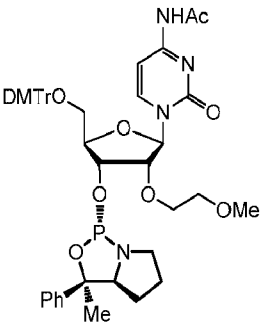
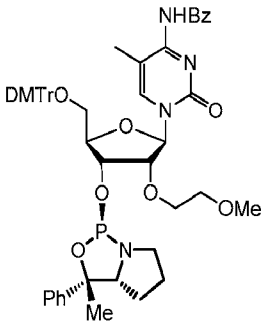
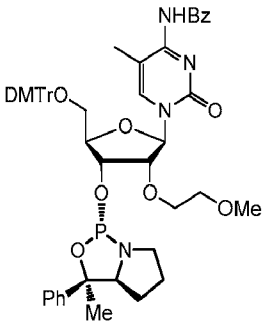
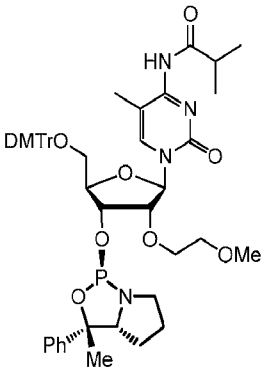
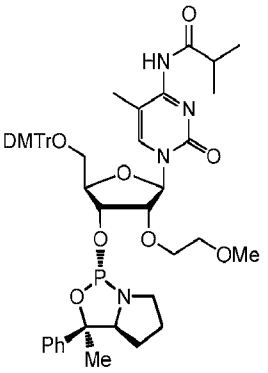
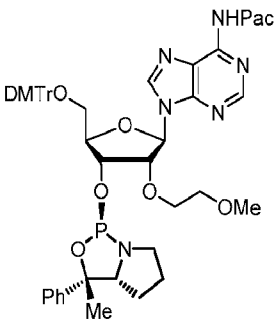
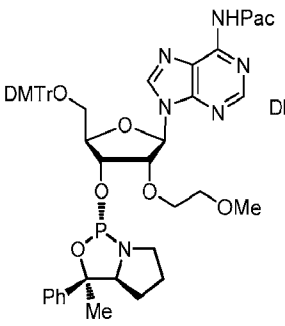
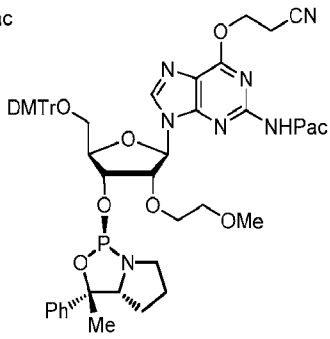
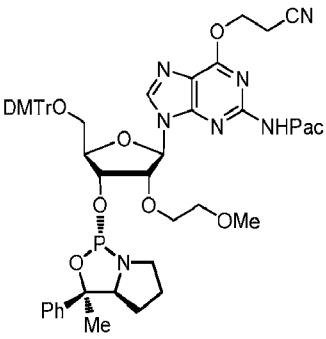
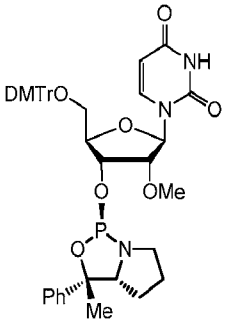
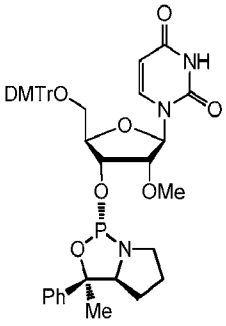
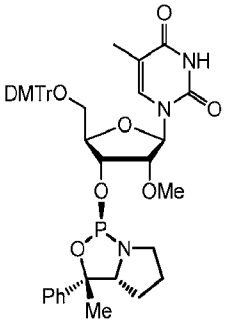
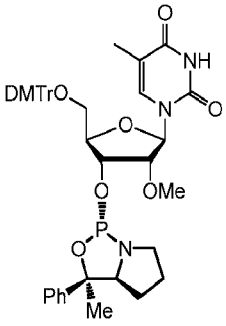
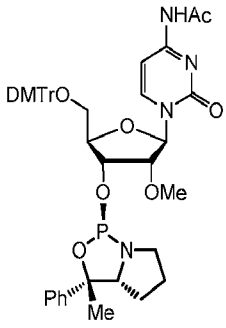
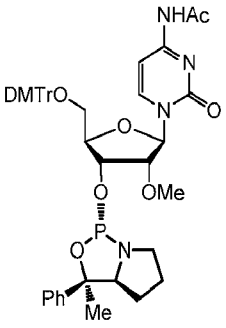
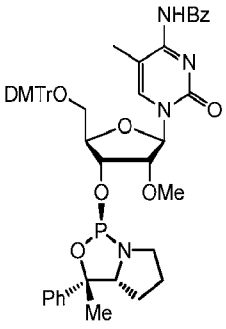
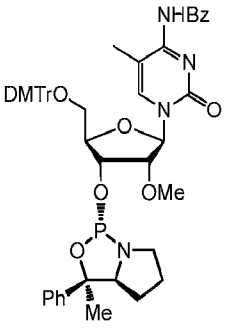
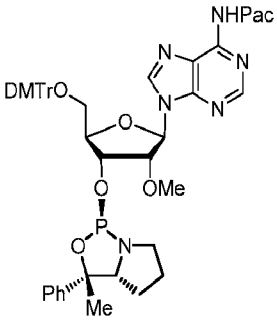
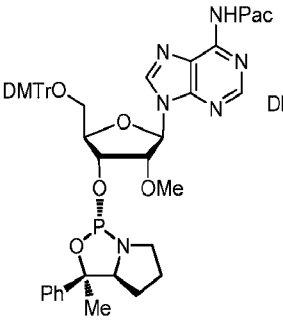
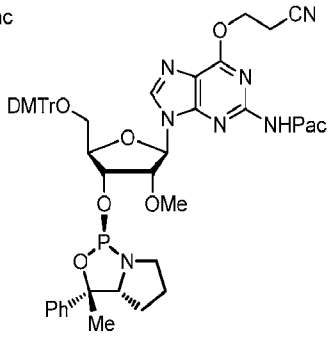
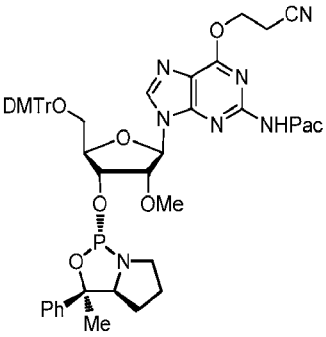
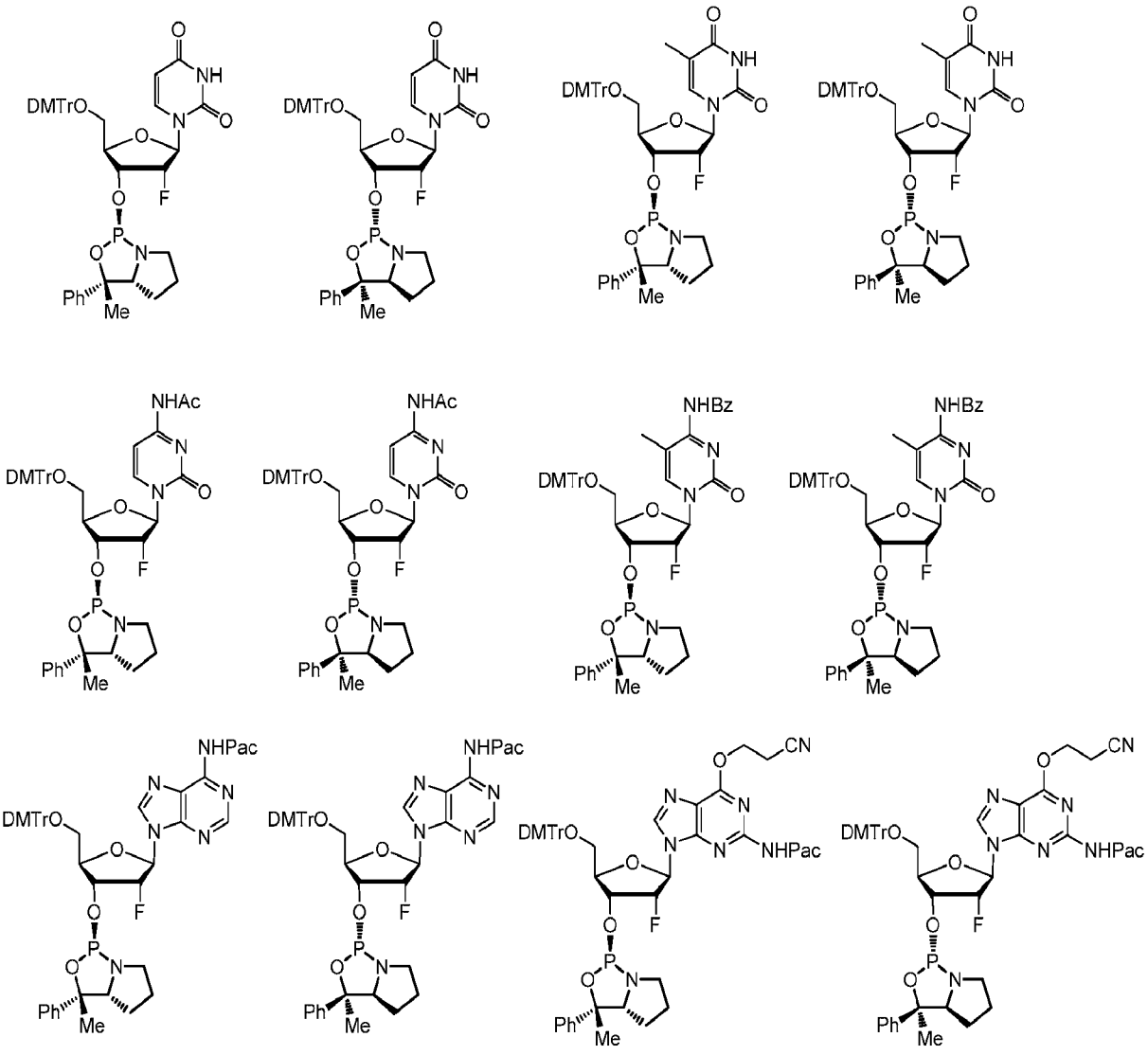
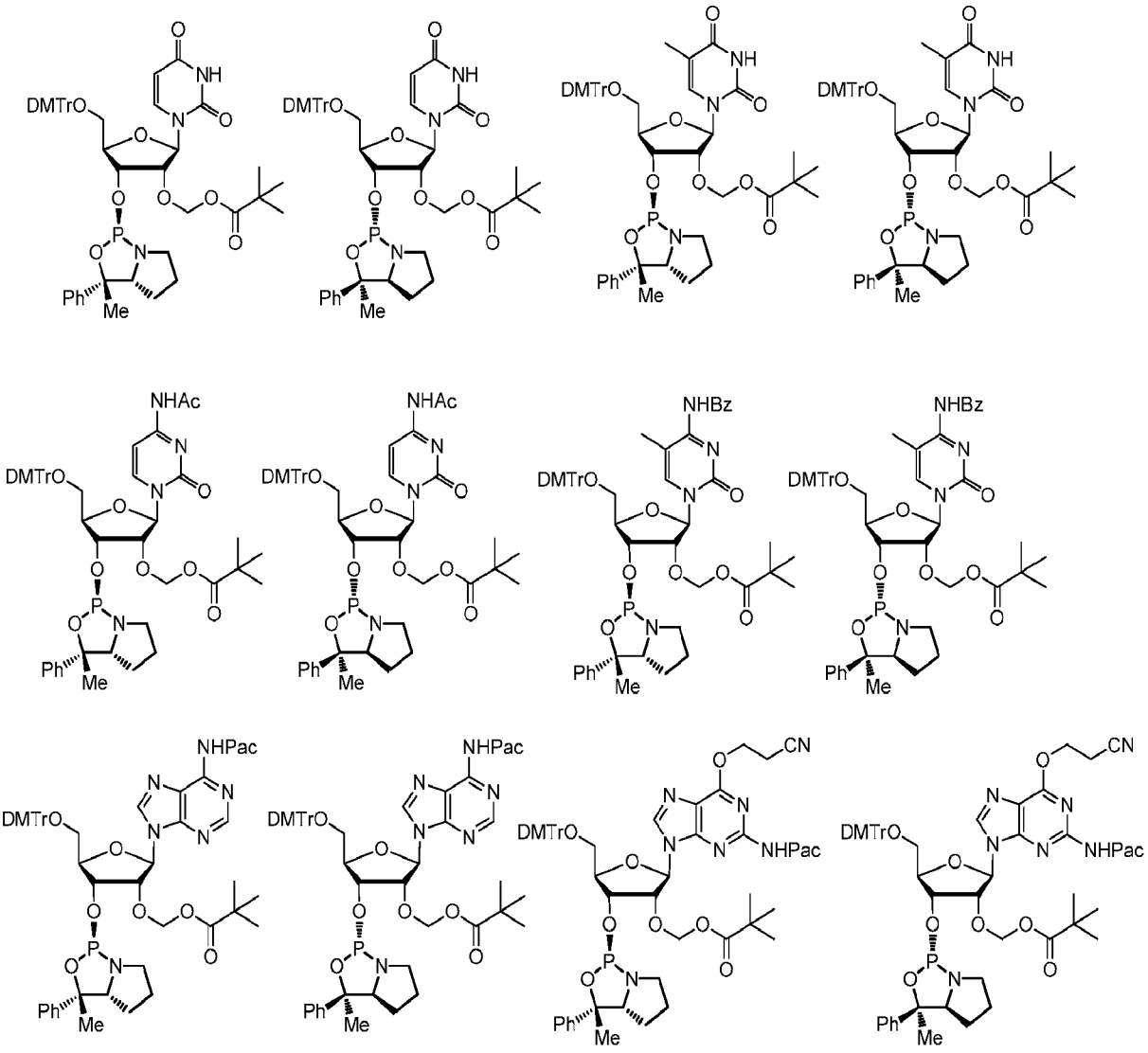















Additional Phosphoramidites: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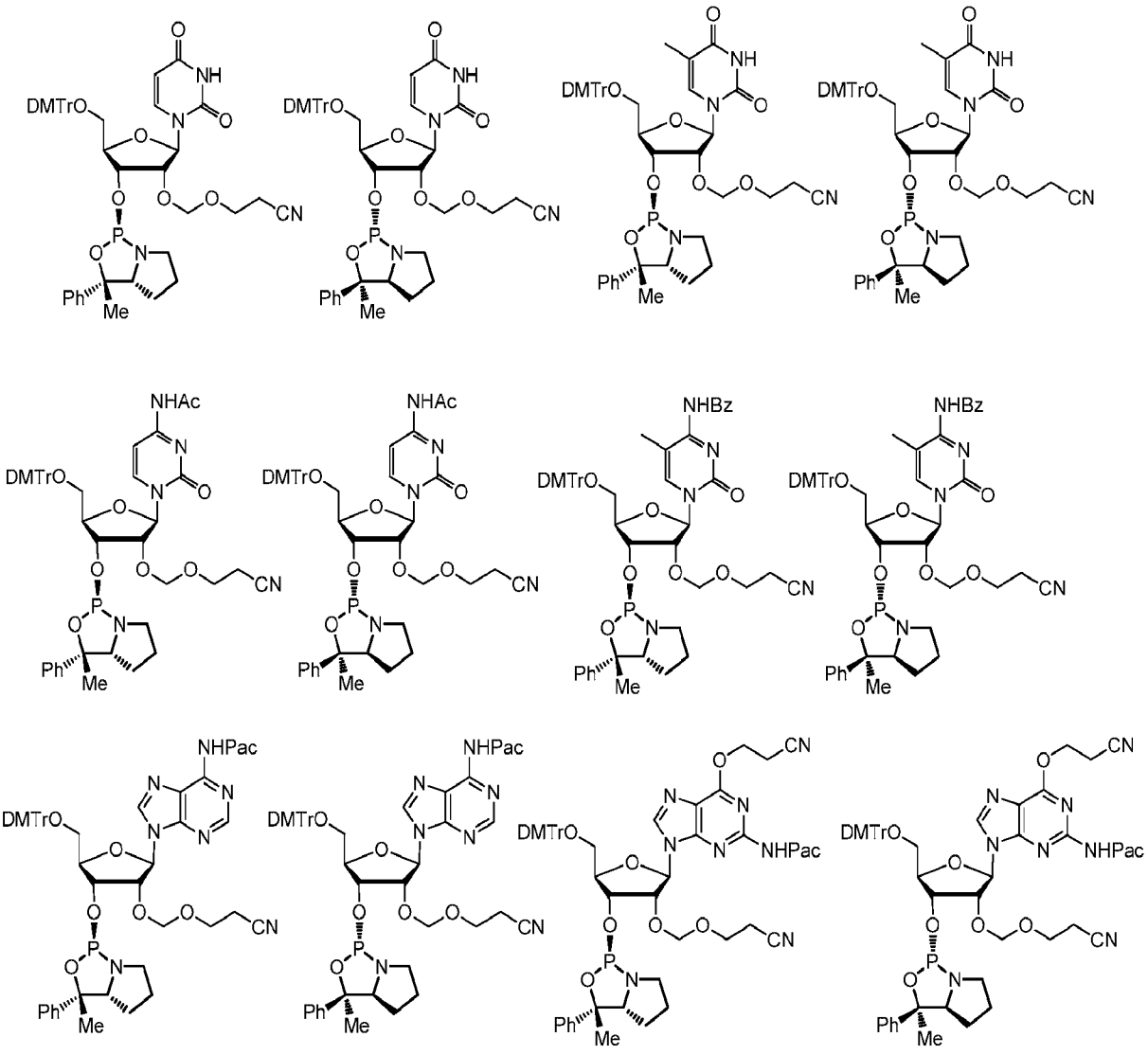

[0009] **Capping**: In some embodiments, the capping step was carried out in two steps. In some embodiments, the above-mentioned two capping steps were combined into one and performed in one-pot. Unless otherwise specified, the two-step capping was used in the synthesis of the examples described herein.

[0010] For the two-step capping, after washing of the solid support with anhydrous ACN and drying by reverse flush with dry argon, the 'Cap A' reagent, typically comprising 5% Pac_2O solution in 2,6-lutidine/THF – 1:4 (v/v) was first introduced to the solid support. Without the intention to be limited by theory, Cap A was sufficiently reactive to effectively cap (acylate) the free amine of the chiral auxiliary. In some embodiments, such protection prevents rearrangement/decomposition of the intermediate phosphite. Immediately following this treatment, a 1:1 mixture of 'Cap A' and 'Cap B' reagents was sent to the column containing the

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solid support. 'Cap B' was a solution of 16% *N*-methylimidazole (NMI) in THF which, when mixed with 'Cap A' effected capping of, for example, the unreacted 5′–OH oligonucleotide bound to the solid support. Not wishing to be limited by theory, the capping step was extremely important in that, without capping of the amino group of the chiral auxiliary the following sulfurization step within the cycle induced the cleavage of the internucleotidic linkage and decomposition of the oligonucleotide, for instance, due to an undesired intermolecular nucleophilic attack of the free amine of the auxiliary on the phosphorous atom. Moreover, capping of the 5′–OH groups of the run-off uncoupled solid-supported oligonucleotide shortmers is crucial in regards of obtaining high purity of the crude full-length oligonucleotide, avoiding low yields due to accumulation of extended failed sequences and tedious and difficult, if not impossible final purifications.

[0011] **Sulfurization:** After washing of the solid support with anhydrous ACN and drying by reverse flush with dry argon, the resulting capped phosphite triester intermediate was subject to oxidative sulfurization. A solution of sulfurizing reagent was prepared by mixing the sulfurizing reagent, for example, an alkyl thiosulfonate derivative (300 mM), with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (100 mM) in ACN. This was then delivered to the column containing the solid support and allowed to react for a certain amount of time (usually 5 – 60 min). In some embodiments, the sulfurizing thiosulfonate reagent was used as a 300 mM solution in anhydrous ACN, without addition of BSTFA. In some embodiments, the reagent solutions with and without BSTFA produced similar results.

[0012] **Oxidation**: In some embodiments, oxidation to phosphodiester was performed instead of the sulfurization step. Oxidation was achieved by delivering a solution of 0.02 M I_2 in a THF/pyridine/water (70:20:10 – v/v/v) co-solvent system, forming a non-chiral phosphodiester linkage.

[0013] **5′-Deblocking of the DMTr group:** DMTr removal was effected using 3% TCA in DCM. After de-blocking, the cycle can then be carried forward for further iterative rounds of coupling, capping, sulfurization/oxidation (either sulfurization with the same sulfurizing agent, or sulfurization with a different sulfurizing agent, or oxidation instead of sulfurization, for each of the next cycles) and de-blocking. Alternatively, if the pre-designed length was reached, the oligonucleotide was subject to cycle exit and, optionally, post-synthetic treatment. In some

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embodiments, the oligonucleotide was removed before de-blocking of the terminal 5′-*O*-DMTr group.

[0014] **Spontaneous Removal of Chiral Auxiliary:** The spontaneous removal of chiral auxiliary was achieved during sulfurization, oxidation, de-blocking of the DMTr group, or the combination thereof. No specific step was needed to remove the chiral auxiliary.

[0015] **Cleavage and Deprotection:** The desired length oligonucleotide was removed from its solid support by cleaving the corresponding linker (oxalyl or succinyl linked HCP or CPG) whilst at the same time, the protecting groups on the oligonucleotide were deprotected. In some embodiments, the solid-supported oligonucleotide was first treated with an anhydrous 1 M solution of 1,5-diazabicyclo(4.3.0)non-5-ene (DBN) or 1,8-diazabicycloundec-7-ene (DBU) in dry ACN-trimethylsilyl chloride – 16:1 (v/v) for 10 min at r.t. and then washed with dry ACN, as described by Reese and Yan, *J. Chem. Soc., Perkin Trans. 1*, **2002**, 2619. In some embodiments, the material was treated with solutions of dry propylamine in dry pyridine (typically in a 1:4 ratio) for a period of 18 h at r.t. or at 60 °C for 2 h. In some embodiments, either condition afforded the crude compound with similar quality. In some embodiments, the crude oligonucleotides were cleaved from the support and deprotected by treatment with 28% aqueous ammonia for a period of 18 h at r.t. or at 60 °C for 5 h. In some embodiments, either condition afforded the crude compound with similar quality. The solvents were then evaporated and the residue was treated with ~pH 1.5 aqueous solution (the pH may be altered if desired) with 0-50% DMSO, and the crude product was subject to analysis by a combination of HPLC and UPLC/MS. The product was purified by one of or a combination of reverse phase HPLC (RP-HPLC), normal phase HPLC, ion exchange HPLC (IE-HPLC) or size exclusion chromatography. In some embodiments, the oligonucleotide was removed from the solid support, deprotected and purified before de-blocking of the DMTr group.

[0016] **Example 1: Synthesis of Sulfurizing Reagents and Synthesis of Phosphoramidites**

[0017] In some embodiments, the present invention provides sulfurizing reagents, and methods of making the same. In some embodiments, the provided sulfurizing reagents were used in the synthesis of oligonucleotides described in the present application. Exemplary sulfurizing reagents and their synthesis are illustrated in Scheme E-1

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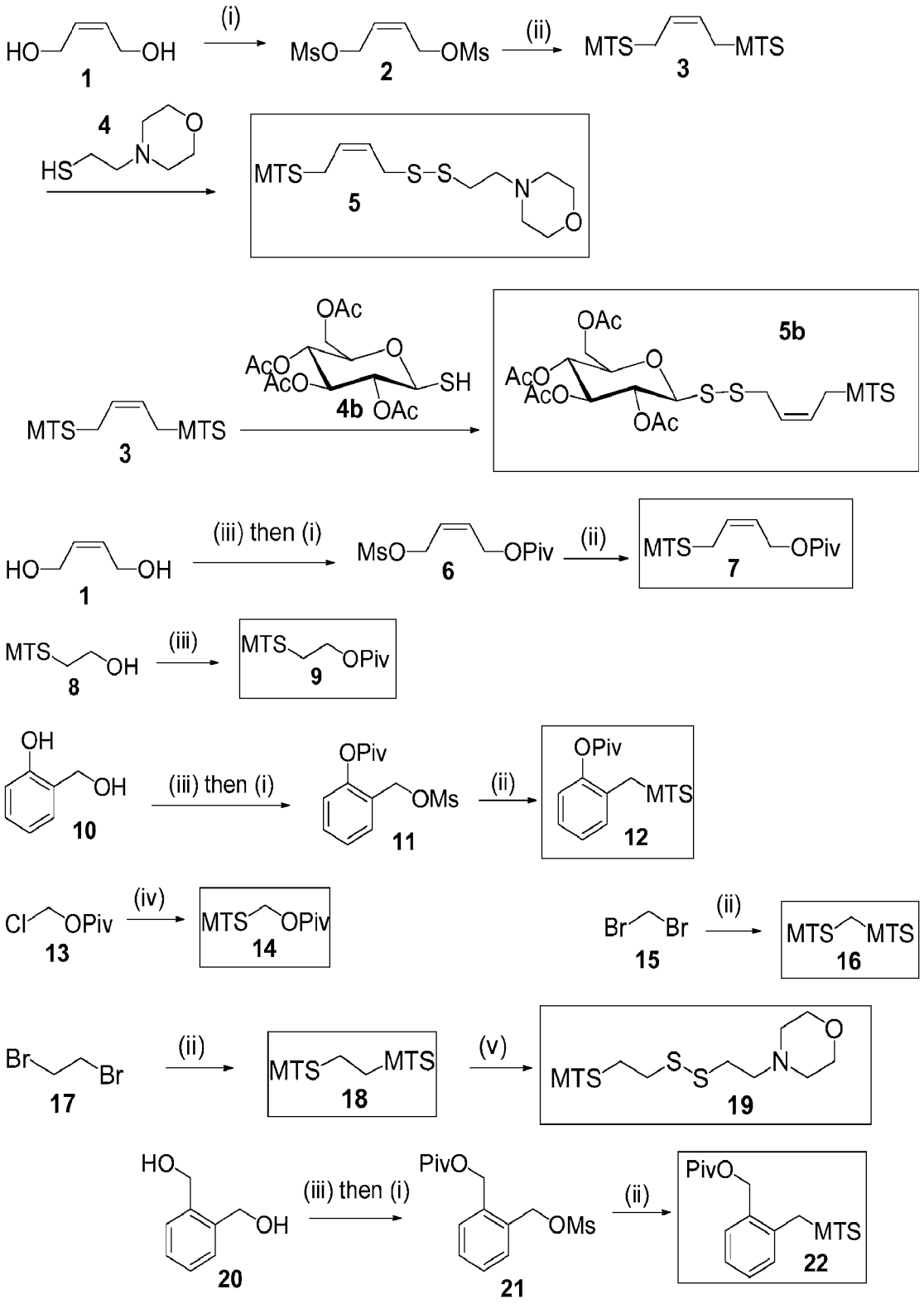
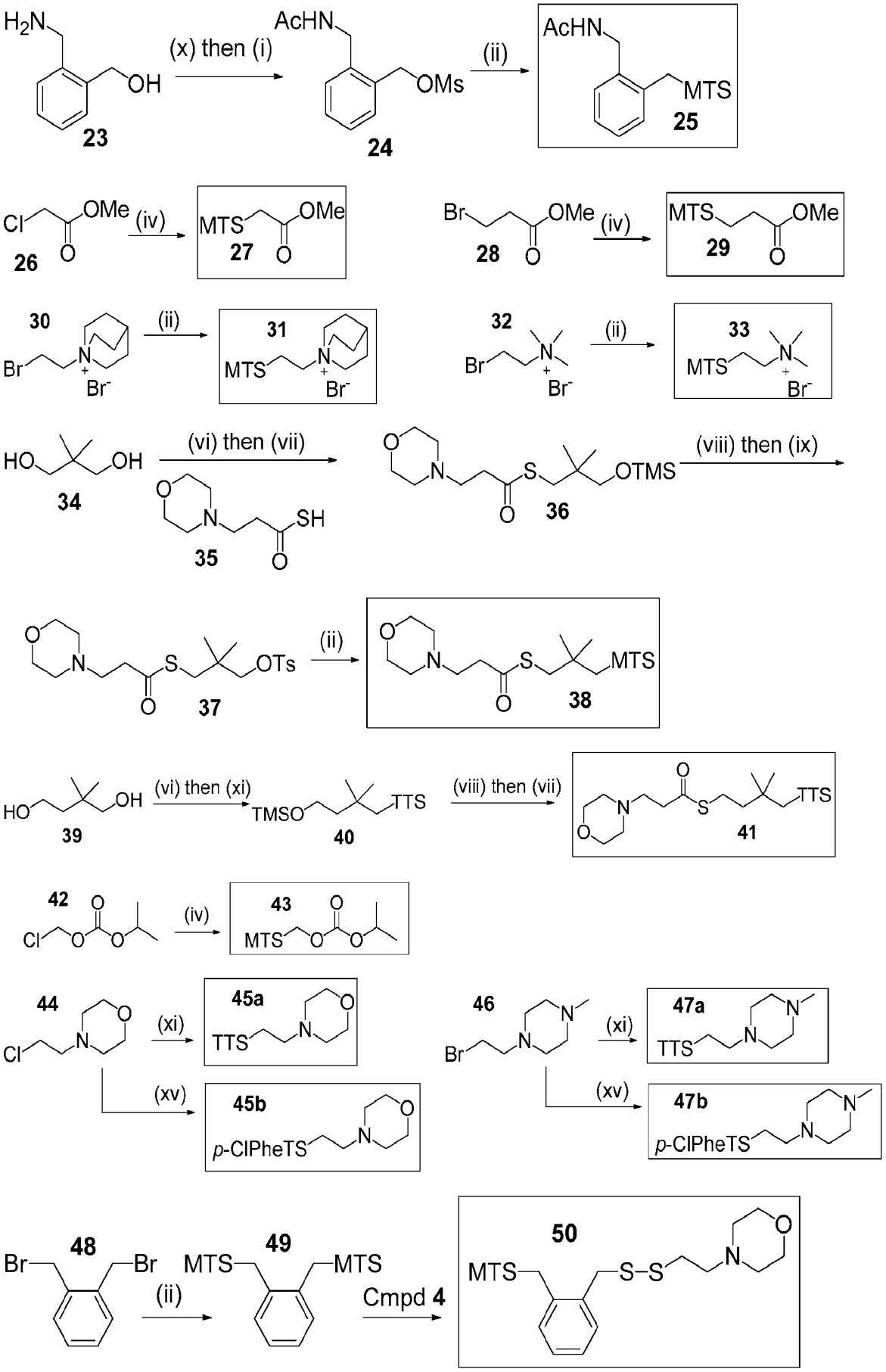
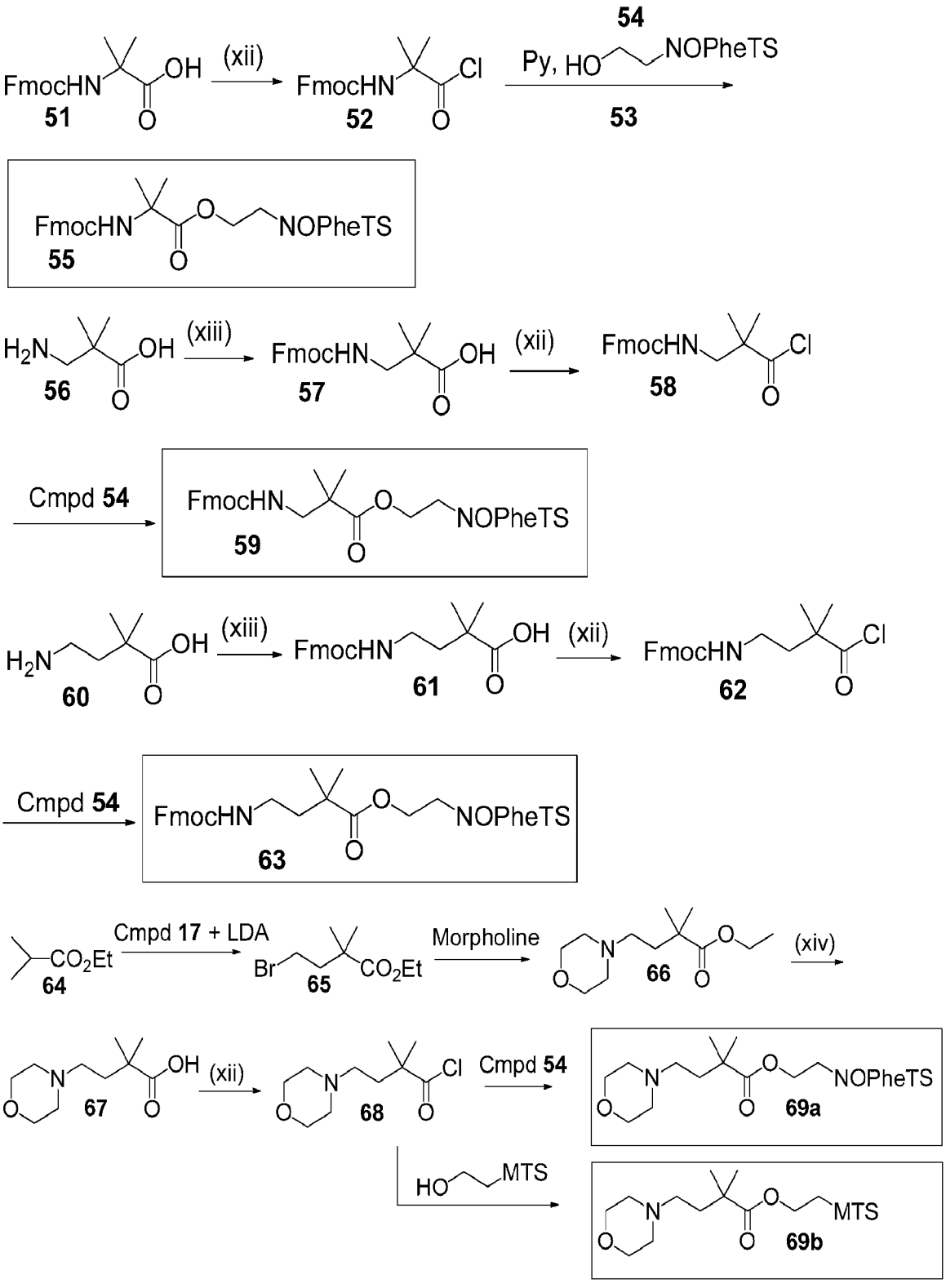
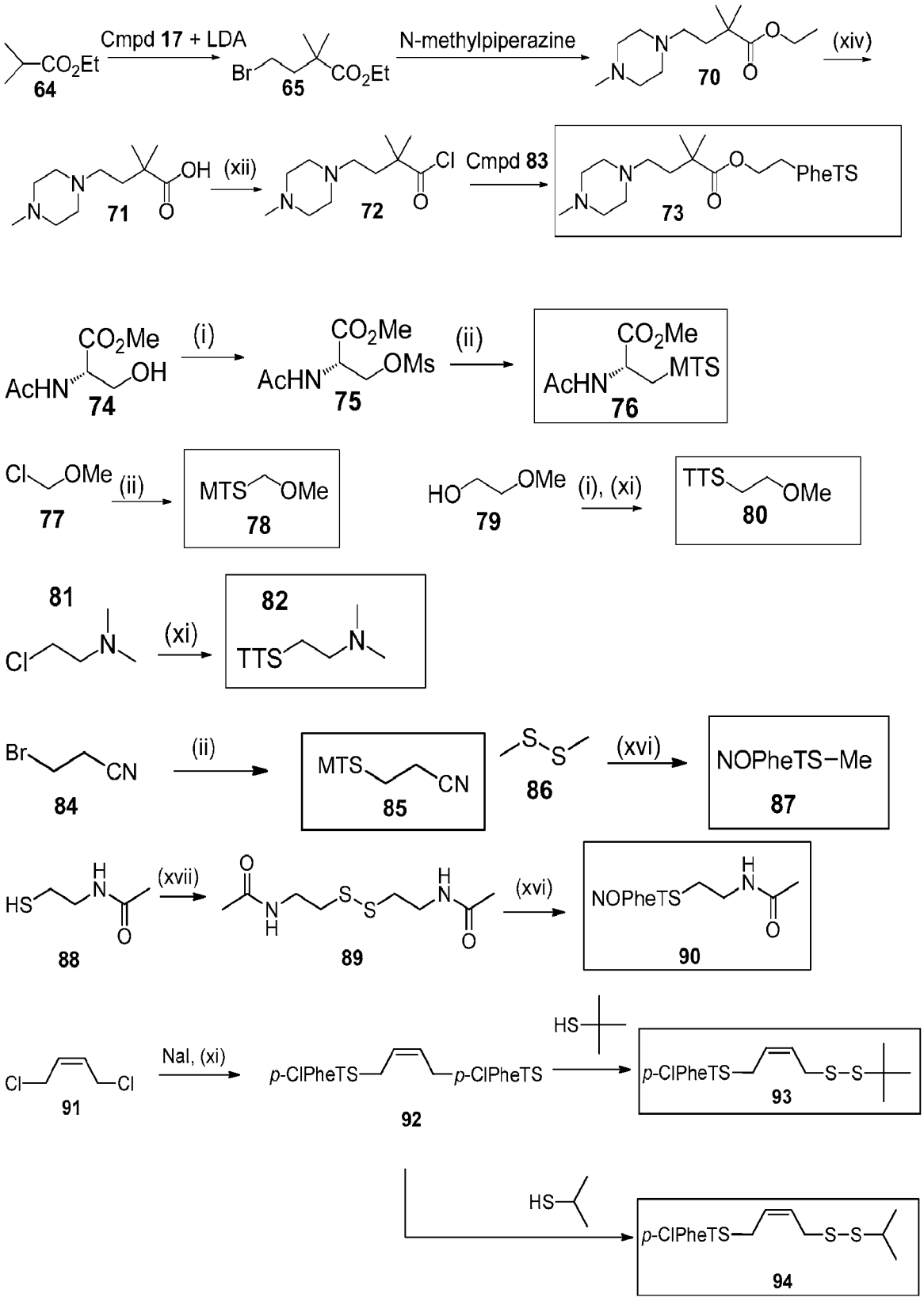
Scheme E-1. Synthesis of sulfurizing reagents.




H2N
OH
23
(x) then (i)
AcHN
OMs
24
(ii)
AcHN
MTS
25
Cl
OMe
O
26
(iv)
MTS
OMe
27
Br
28
29
30
31
32
33
(ii)
Br-
HO
34
(vi) then (vii)
SH
35
OTMS
36
(viii) then (ix)
OTs
37
38
39
(vi) then (xi)
TMSO
TTS
40
(viii) then (vii)
41
42
43
44
45a
45b
46
47a
47b
(xi)
(xv)
TTS
p-ClPheTS
48
49
50
Cmpd 4


FmocHN
OH
51
(xii)
FmocHN
Cl
52
54
Py, HO
NOPheTS
53
FmocHN
O
NOPheTS
55
H2N
OH
56
(xiii)
FmocHN
OH
57
(xii)
FmocHN
Cl
58
Cmpd 54
FmocHN
O
NOPheTS
59
H2N
OH
60
(xiii)
FmocHN
OH
61
(xii)
FmocHN
Cl
62
Cmpd 54
FmocHN
O
NOPheTS
63
CO2Et
64
Cmpd 17 + LDA
Br
CO2Et
65
Morpholine
N
O
66
(xiv)
N
O
OH
67
(xii)
N
O
Cl
68
Cmpd 54
N
O
O
NOPheTS
69a
HO
MTS
N
O
O
MTS
69b


Cmpd 17 + LDA
N-methylpiperazine
(xiv)
(xii)
Cmpd 83
PheTS
(i)
(ii)
(i), (xi)
(xi)
(xvi)
(xvii)
NaI, (xi)
64
65
70
71
72
73
74
75
76
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78
79
80
81
82
84
85
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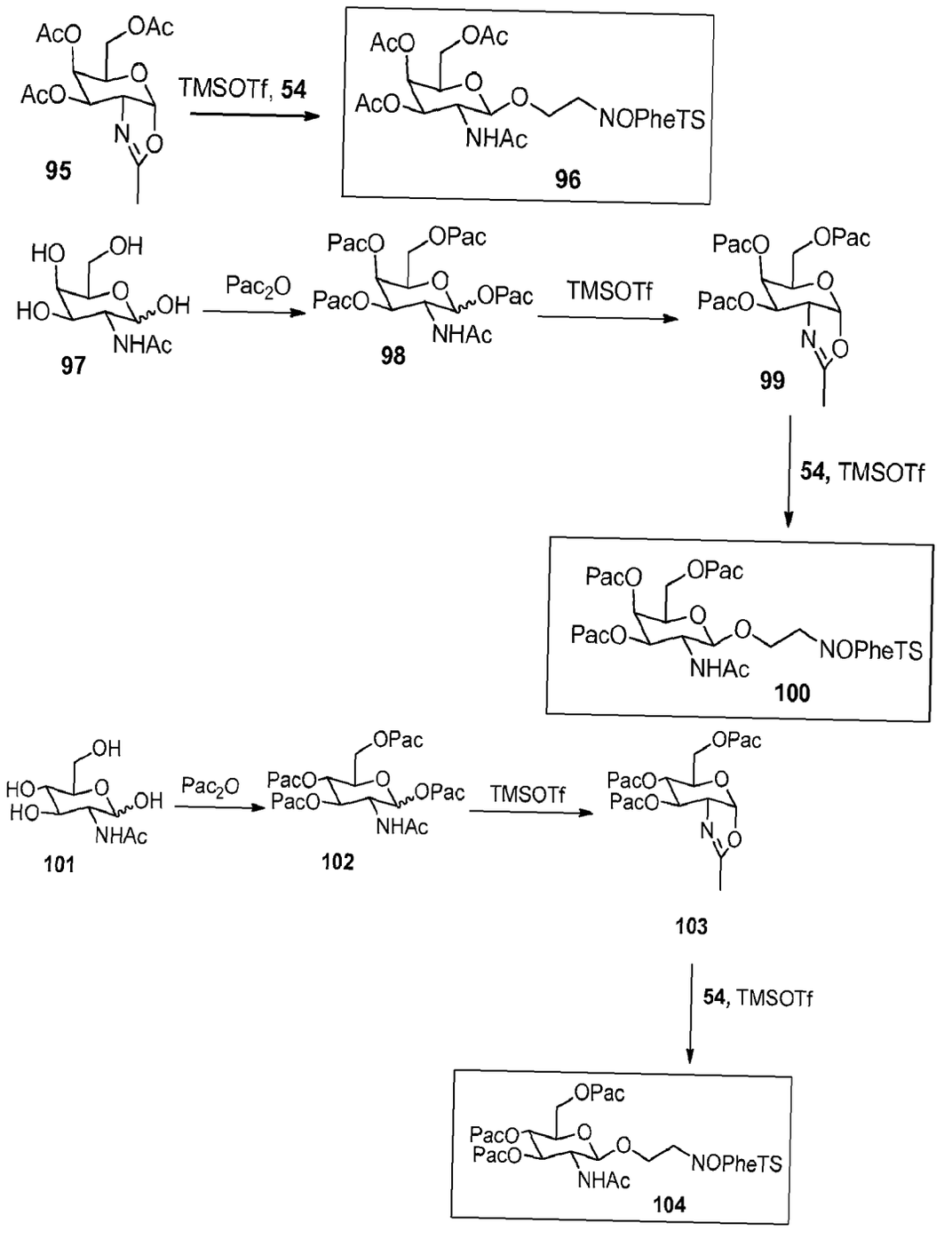
89
90
91
92
93
94


AcO
OAc
AcO
O
N
O
95
TMSOTf, 54
AcO
OAc
AcO
O
O
NOPheTS
NHAc
96
HO
OH
HO
O
OH
NHAc
97
Pac2O
PacO
OPac
PacO
O
OPac
NHAc
98
TMSOTf
PacO
OPac
PacO
O
N
O
99
54, TMSOTf
PacO
OPac
PacO
O
O
NOPheTS
NHAc
100
OH
HO
O
OH
HO
NHAc
101
Pac2O
OPac
PacO
O
PacO
OPac
NHAc
102
TMSOTf
OPac
PacO
O
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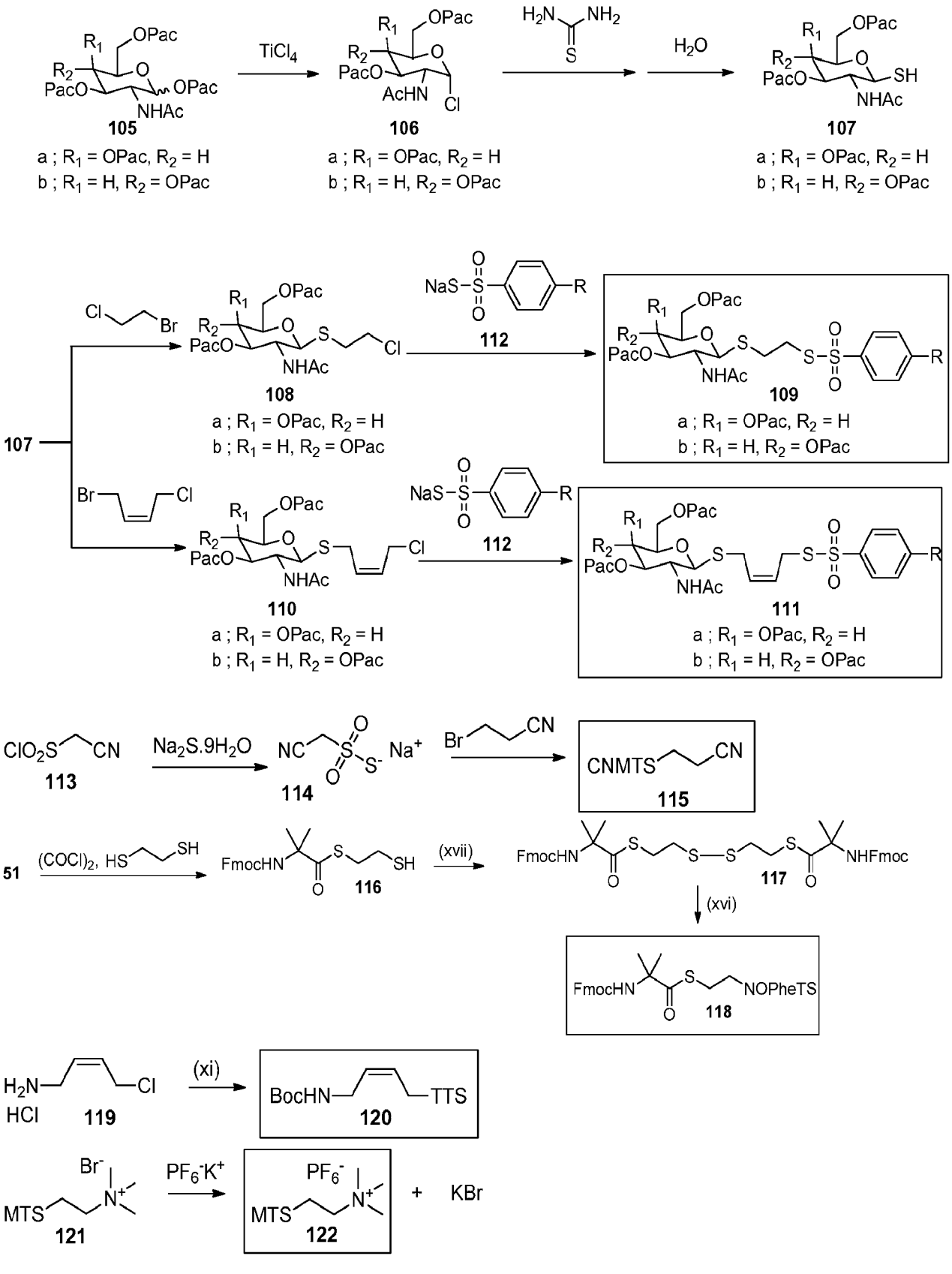
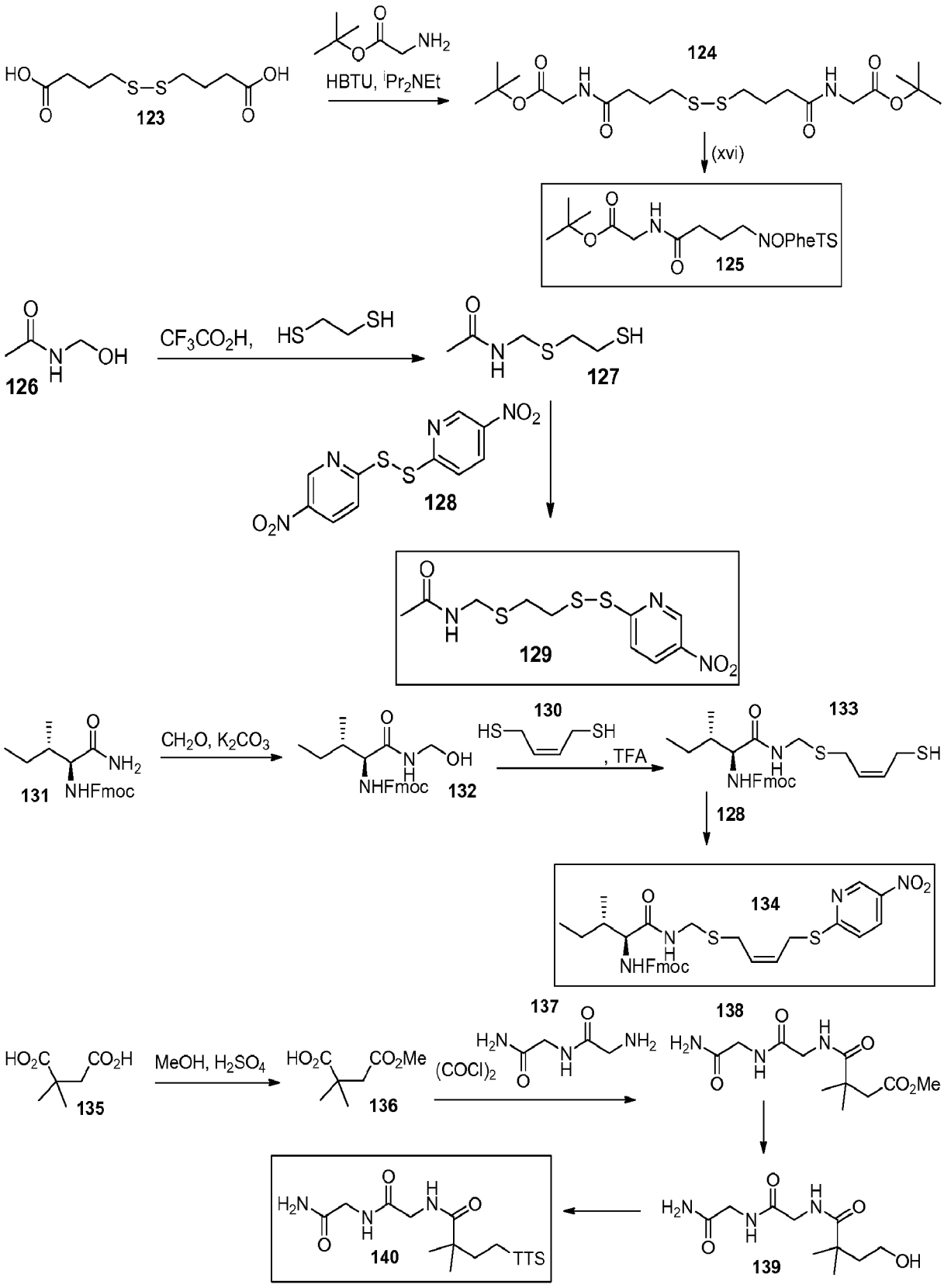
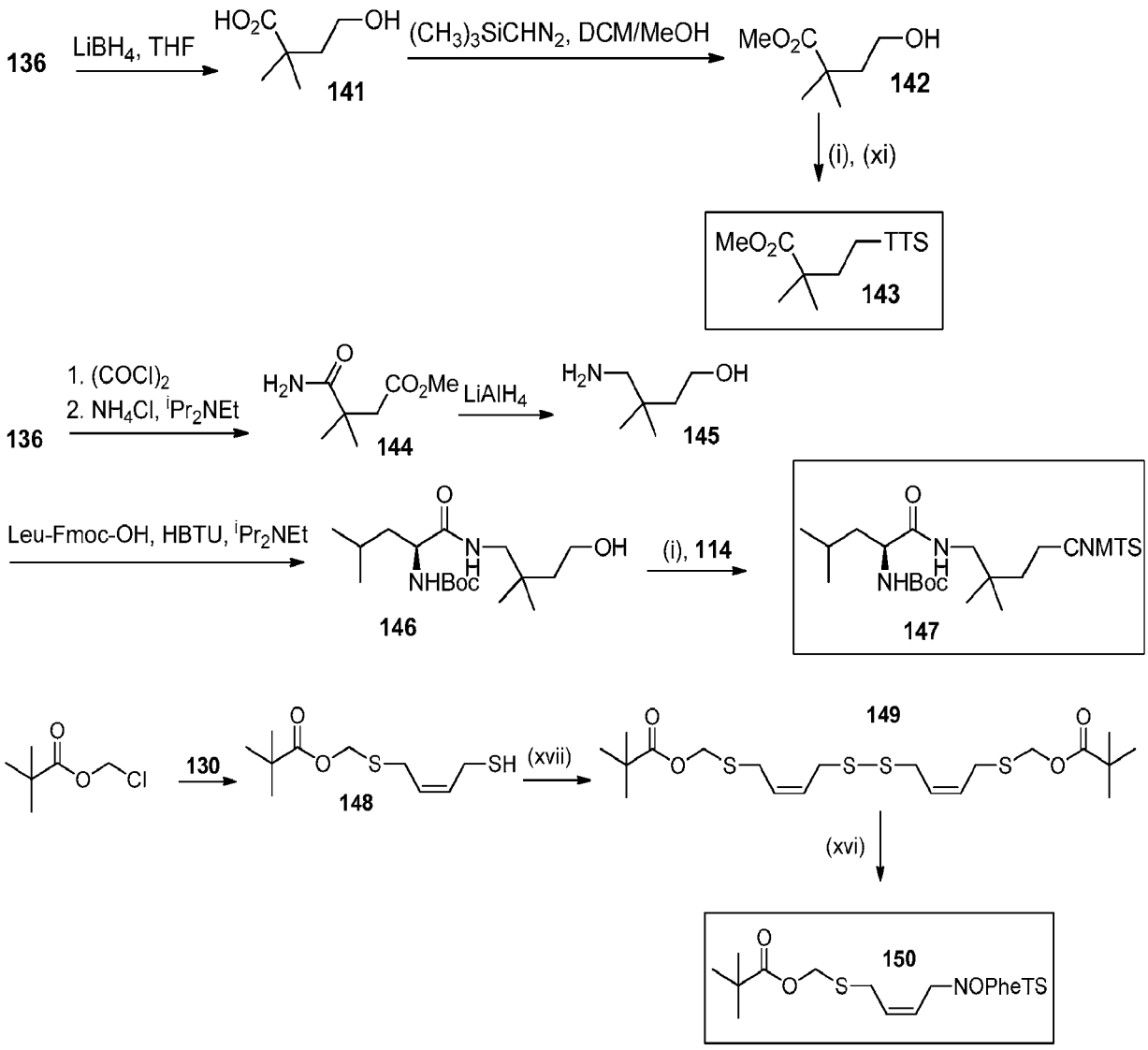
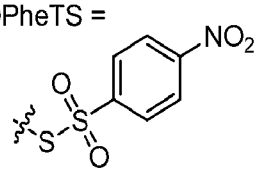
N
O
103
54, TMSOTf
OPac
PacO
O
PacO
O
NOPheTS
NHAc
104


105
a ; R1 = OPac, R2 = H
b ; R1 = H, R2 = OPac
TiCl4
106
a ; R1 = OPac, R2 = H
b ; R1 = H, R2 = OPac
H2O
107
a ; R1 = OPac, R2 = H
b ; R1 = H, R2 = OPac
108
a ; R1 = OPac, R2 = H
b ; R1 = H, R2 = OPac
112
109
a ; R1 = OPac, R2 = H
b ; R1 = H, R2 = OPac
110
a ; R1 = OPac, R2 = H
b ; R1 = H, R2 = OPac
111
a ; R1 = OPac, R2 = H
b ; R1 = H, R2 = OPac
113
Na2S.9H2O
114
115
CNMTS
51
(COCl)2, HS SH
116
(xvii)
117
(xvi)
118
119
(xi)
120
BocHN TTS
121
PF6-K+
122
KBr

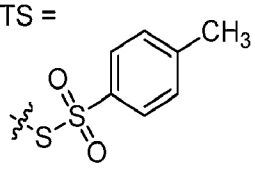

123
HBTU, iPr2NEt
124
(xvi)
125
NOPheTS
126
CF3CO2H,
127
128
129
131
CH2O, K2CO3
132
130
, TFA
133
128
134
137
138
(COCl)2
135
MeOH, H2SO4
136
139
140
TTS



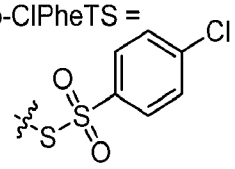
NOPheTS =



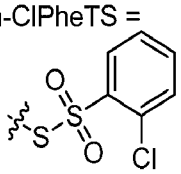
TTS =



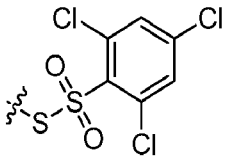
p-ClPheTS =



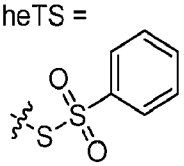
m-ClPheTS =



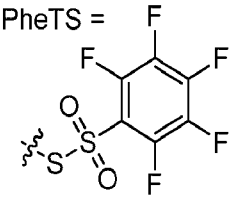
2,4,6-TriClPheTS =



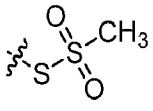
PheTS =



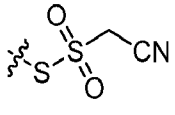
PFPheTS =



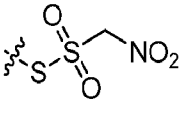
MTS =



α–CNMTS =

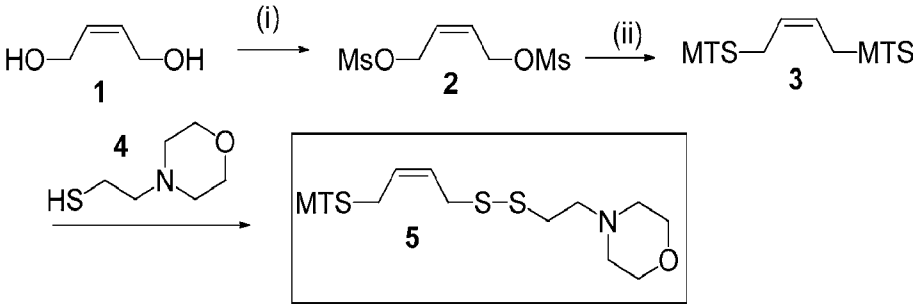


α–NO2MTS =



(i) MsCl, NEt_3; (ii) NaMTS; (iii) PivCl, NEt_3; (iv) NaMTS, NaI; (v) compound 4; (vi) TMSCl, NEt_3; (vii) compound 35, DEAD, PPh_3; (viii) TBAF; (ix) TsCl, Pyridine; (x) Ac_2O, Pyridine; (xi) KTTS; (xii) $(COCl)_2$; (xiii) Fmoc-OSu, Py; (xiv) NaOH (aq); (xv) Na-*p*-ClPheTS; sodium 4-nitrobenzenesulfinate, Br_2; (xvii) H_2O_2, NaI

Synthesis of Compound 5



[0018] **Compound 2:** A solution of (*Z*)-but-2-ene-1,4-diol (0.93 ml, 11.3 mmol) and triethylamine (3.3 ml, 24 mmol) in dichloromethane (DCM, 50 mL) was added in a dropwise fashion to a stirring ice cold solution of methanesulfonyl chloride (1.9 ml, 24 mmol) in DCM (50 mL). After stirring for 0.5h at r.t. the mixture was poured onto ice and extracted. The organic layer was collected, dried ($MgSO_4$) and filtered. After removal of solvent, 2.66 g compound **2** was obtained (96%), which was judged by NMR to be sufficiently pure for direct use in the next step of the reaction. ^{1}H NMR (399 MHz, $CDCl_3$) δ 5.94 (ddd, J = 5.4, 4.1, 1.3 Hz, 2H), 4.83 (dd, J = 4.1, 1.3 Hz, 4H), 3.04 (s, 6H); ^{13}C NMR 128.34, 64.38, 38.27; MS (ESI +ve): calc $(M+NH_4)^+$: 262.04, found: 262.05; R_f = 0.3 (1:1 EtOAc/hexane).

[0019] **Compound 3:** A solution of sodium methanesulfonothioate (1.51 g, 11.3 mmol) in MeOH (20 ml) was treated with neat (*Z*)-but-2-ene-1,4-diyl dimethanesulfonate (1.25 g, 5.12 mmol) at room temperature. After 5 min, precipitation was observed to occur. After 36 h, the mixture was partitioned between water and DCM. The organic layer was separated, dried ($MgSO_4$), and filtered. Removal of solvent afforded colorless oil. Column chromatography (ISCO) gave pure compound **3** (0.89 g, 63%) as a colorless oil. ^{1}H NMR (399 MHz, $CDCl_3$) δ 5.84 (ddd, J = 6.6, 5.1, 1.5 Hz, 2H), 3.92 (dd, J = 5.1, 1.5 HZ, 4H), 3.33 (s, 6H); ^{13}C NMR 128.1, 51.47, 33.13; MS (ESI +ve): calc $(M+NH_4)^+$: 294.00, found: 294.04; R_f = 0.4 (1:1 EtOAc/hexane).

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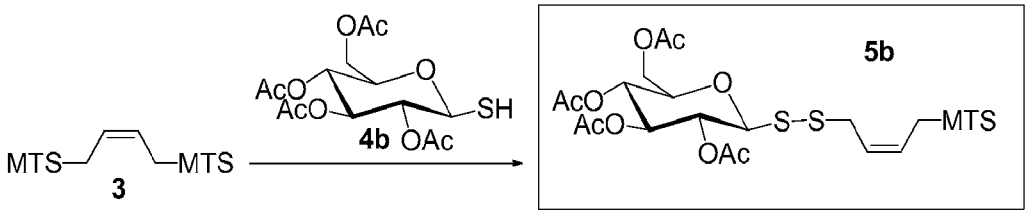
[0020] **Compound 4:** Under argon atmosphere, morpholine (10 g, 115 mmol) was added to ethylene sulfide (15 g, 250 mmol) in a round bottom flask. The reaction was stirred for 7 hrs and was directly loaded on to a silica gel column. The column was washed with DCM first and then 2% MeOH/DCM was used to obtain compound **4** (15.3 g, 91%) as colorless oil. ^{1}H NMR (399MHz, $CDCl_3$) δ 3.67-3.59 (m, 4H), 2.63-2.52 (m, 2H), 2.51-2.45 (m, 2H), 2.44-2.34 (m, 4H); MS (ESI +ve): calc $(M+H)^+$ = 148.07, found: 148.1.

[0021] **Compound 5:** A DCM solution (1 mL) of 2-morpholinoethanethiol (0.21 g, 1.44 mmol) was added dropwise via syringe to a stirring solution compound **3** (0.40 g, 1.44 mmol) in DCM (10 mL) at room temperature. Immediately after addition, the reaction was checked by TLC, showing rapid formation of the product and some dimer. After 0.5 h, the mixture was partitioned by addition of water. Upon extraction, the organic layer was separated, dried ($MgSO_4$) and filtered. After removal of solvent *in vacuo*, column chromatography gave compound **5** (0.29 g, 58%) as colorless oil. ^{1}H NMR (399 MHz, $CDCl_3$) δ 5.78 (m, 2H), 3.92 (d, J = 7.3 Hz, 2H), 3.70 (t, J = 4.7 Hz, 4H), 3.46 (d, J = 5.5 Hz, 2H), 3.31 (s, 3H), 2.84 (dd, J = 7.8, 6.7 Hz, 2H), 2.66 (dd, J = 7.8, 6.7, 2H), 2.48 (t, J = 4.6 Hz, 4H); ^{13}C NMR 130.35, 126.27, 66.97, 58.20, 53.67, 51.52, 36.22, 35.16, 33.67; MS (ESI +ve): calc $(M+H)^+$: 344.05, found: 344.06; R_f = 0.3 (EtOAc).



[0022] **Compound 5b:** A DCM solution (1 mL) of compound **4b** (395 mg, 1.085 mmol) was added dropwise via syringe to a stirring DCM (15 mL) solution compound **3** (300 mg, 1.085 mmol) at r.t. After 1h, the resulting solution was partitioned by addition of water. Upon extraction, the organic layer was separated, dried ($MgSO_4$) and filtered. After removal of the solvent *in vacuo*, column chromatography gave compound **5b** as a colorless oil (0.35 g, 58%). ^{1}H NMR (399 MHz, $CDCl_3$) δ 5.83 – 5.70 (m, 2H), 5.35 – 5.21 (dt, J = 26.0, 9.3 Hz, 2H), 5.16 – 5.07 (m, 1H), 4.59 – 4.54 (d, J = 9.5 Hz, 1H), 4.29 – 4.23 (m, 1H), 4.23 – 4.18 (m, 1H), 3.99 – 3.88 (dd, J = 6.7, 1.2 Hz, 2H), 3.80 – 3.72 (ddd, J = 10.1, 4.6, 2.6 Hz, 1H), 3.64 – 3.56 (m, 1H), 3.50 – 3.43 (m, 1H), 3.31 (s, 3H), 2.09 (s, 3H), 2.03 (s, 6H), 2.00 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.68, 170.30, 169.51, 169.30, 129.43, 127.14, 87.73, 76.49, 73.89, 69.16, 67.99,

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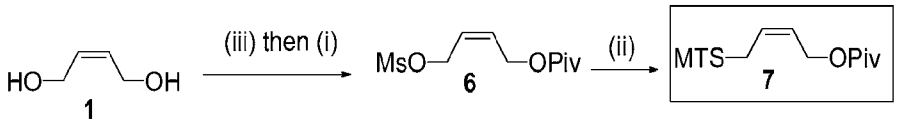
61.99, 51.64, 35.89, 33.58, 20.95, 20.80, 20.74, 20.71; MS (ESI +ve): calc $(M+NH_4)^+$: 578.07, found: 577.96; R_f = 0.5 (1:1 EtOAc/hexane).

Synthesis of Compound 7



[0023] **Compound 6:** An ice cold solution of (*Z*)-but-2-ene-1,4-diol (0.93 ml, 11.3 mmol) and triethylamine (1.6 mL, 11.5 mmol) in DCM (50 ml) was treated dropwise via syringe with pivaloyl chloride (1.4 ml, 11.4 mmol) over 2 min. After 1 h, TLC showed good reaction results. The resulting mixture was partitioned by addition of water. Upon extraction, the organic layer was separated, dried ($MgSO_4$), and concentrated *in vacuo*. This crude compound was found: by TLC (Rf = 0.6, 1:1 EtOAc/hexane) to contain no starting diol and was used crude to prepare the mesylate. The crude material was taken up in DCM (50 ml) containing triethylamine (1.7 mL, 12 mmol) and cooled on an ice bath. Methanesulfonyl chloride (0.98 ml, 12.66 mmol) was added dropwise via syringe over 2 min. TLC immediately after addition indicated complete consumption of starting material. The resulting mixture was partitioned by addition of water. Upon extraction, the organic layer was separated, dried ($MgSO_4$), filtered and concentrated *in vacuo*. Column chromatography gave pure compound **6**, 1.48 g, 52%, as a colorless oil. ^1H NMR (399 MHz, $CDCl_3$) δ 5.89 – 5.75 (m, 2H), 4.89 – 4.84 (d, *J* = 5.7 Hz, 2H), 4.68 – 4.63 (d, *J* = 5.9 Hz, 2H), 3.03 (s, 3H), 1.19 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.28, 130.61, 126.11, 65.08, 59.65, 38.84, 38.21, 27.25; MS (ESI +ve): calc $(M+NH_4)^+$: 268.12, found: 268.20; R_f = 0.3 (20% EtOAc/hexane).

[0024] **Compound 7:** A MeOH (10 ml) solution of sodium methanesulfonothioate (0.63 g, 4.70 mmol) and (*Z*)-4-(methylsulfonyloxy)but-2-enyl pivalate (1.00 g, 4.00 mmol) was stirred at r.t. for 18 h with formation of a white precipitate (after 10 min). The resulting mixture was partitioned by addition of water and DCM. Upon extraction into DCM, the organic layer was separated, dried (MgSO4), filtered and concentrated *in vacuo*. Column chromatography gave compound **7**, 0.83 g, 78% as a colorless oil. ^1H NMR (399 MHz, $CDCl_3$) δ 5.82 – 5.73 (m, 2H), 4.73 – 4.66 (m, 2H), 3.95 – 3.87 (m, 2H), 3.32 (s, 3H), 1.19 (s, 9H); ^{13}C NMR (100 MHz,

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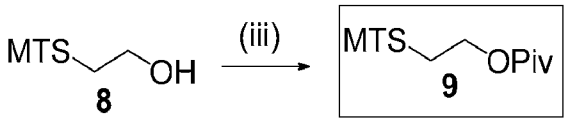
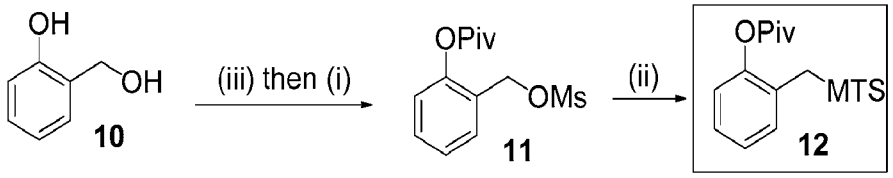
$CDCl_3$) δ 178.35, 129.37, 127.32, 59.50, 51.44, 38.84, 33.61, 27.28; MS (ESI +ve): calc $(M+NH_4)^+$: 284.10, found: 284.19; R_f = 0.4 (20% EtOAc/hexane).

Synthesis of Compound 9



[0025] **Compound 9:** Pivaloyl chloride (0.60 g, 5.0 mmol) was added in a dropwise fashion to a stirring solution of S-2-hydroxyethyl methanesulfonothioate (0.65 g, 4.16 mmol) in DCM (20 ml). After 2 h at r.t. the resulting mixture with white precipitate was partitioned with water. The organic layer was separated, dried (Na_2SO_4), filtered concentrated to an oil. Column chromatography gave compound **9** as a colorless oil (0.45 g, 45%). ^1H NMR (399 MHz, $CDCl_3$) δ 4.39 – 4.34 (t, *J* = 6.3 Hz, 2H), 3.44 – 3.39 (t, *J* = 6.3 Hz, 2H), 3.36 (s, 3H), 1.20 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 62.10, 51.11, 38.96, 35.19, 27.24; MS (ESI +ve): calc $(M+NH_4)^+$: 158.08, found: 158.04; R_f = 0.3 (20% EtOAc/hexane).

Synthesis of Compound 12



[0026] **Compound 11:** Pivaloyl chloride (4.96 ml, 40.3 mmol) was added dropwise via syringe to an ice cold DCM solution (50 mL) of 2-(hydroxymethyl)phenol (5 g, 40.3 mmol) and triethylamine (5.61 ml, 40.3 mmol). An ice-cold solution of the crude pivalate ester was treated with triethylamine (6.74 ml, 48.4 mmol) and 50 mL DCM. Methanesulfonyl chloride (3.43 ml, 44.3 mmol) was then added slowly (5 min) via syringe and the resulting mixture was warmed to r.t. The mixture was poured onto ice and the organic layer was separated then washed with sat $NaHCO_3$ (aq), dried ($MgSO_4$), filtered and concentrated to afford 10.5 g crude pale yellow oil. Column chromatography (ISCO) gave pure **11** 5.45 g, 47%. ^1H NMR (399 MHz, $CDCl_3$) δ 7.53 – 7.46 (dd, 7.7, 1.8 Hz, 1H), 7.46 – 7.40 (dt, 7.7, 1.8 Hz, 1H), 7.32 – 7.24 (t, 7.7 Hz, 1H), 7.13 – 7.06 (d, 7.7 Hz, 1H), 5.21 (s, 2H), 2.79 (s, 3H), 1.40 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.05, 150.06, 131.18, 131.07, 126.35, 125.94, 123.21, 66.88, 39.48, 38.82, 27.30, 27.26. MS (ESI +ve): calc $(M+NH_4)^+$: 304.12, found: 303.99; R_f = 0.4 (20% EtOAc/hexane).

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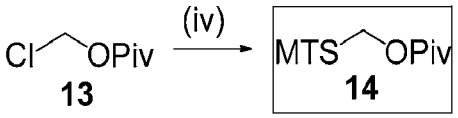
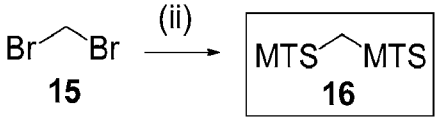
[0027] **Compound 12:** A MeOH (20 mL) solution of sodium methanesulfonothioate (0.825 g, 6.15 mmol) was treated with 2-((methylsulfonyloxy)methyl)phenyl pivalate (1.76 g, 6.15 mmol) at r.t. and left to stir for 18 h. The mixture was partitioned between water and DCM. The organic layer was separated, dried ($MgSO_4$), filtered and concentrated to afford a colorless oil. Column chromatography gave pure compound **12** as a pale colorless oil, 0.754 g, 41%. ^1H NMR (399 MHz, $CDCl_3$) δ 7.48 – 7.44 (dd, J = 7.7, 1.7 Hz, 1H), 7.39 – 7.34 (td, J = 7.8, 1.7 Hz, 1H), 7.25 – 7.20 (td, J = 7.6, 1.2 Hz, 1H), 7.10 – 7.06 (dd, J = 8.2, 1.2 Hz, 1H), 4.29 (s, 2H), 2.90 (s, 3H), 1.39 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.69, 149.59, 131.17, 129.85, 127.41, 126.18, 123.40, 51.43, 39.47, 36.01, 27.30; MS (ESI +ve): calc $(M+NH_4)^+$: 320.10, found: 320.09; R_f = 0.4 (20% EtOAc/hexane).

Synthesis of Compound 14



[0028] **Compound 14:** Chloromethyl pivalate (0.478 ml, 3.32 mmol) was added to a stirring mixture of sodium iodide (0.050 g, 0.33 mmol) and sodium methanesulfonothioate (0.445 g, 3.32 mmol) in acetone (7 ml) at r.t. After 24 h, TLC showed good conversion to product. The solvent was removed, and the residue was partitioned between water and DCM. The organic layer was separated and dried ($MgSO_4$), filtered and concentrated to afford a colorless oil. Column chromatography gave pure **14** as a slightly pink solid, 0.41 g, 55%. ^1H NMR (399 MHz, $CDCl_3$) δ 5.67 (s, 2H), 3.39 (s, 3H), 1.24 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.35, 67.84, 52.20, 38.93, 27.05; R_f = 0.5 (20% EtOAc/hexane).

Synthesis of Compound 16



[0029] **Compound 16:** Prepared from **15** and NaMTS as described previously in US 3,484,473. ^1H NMR (399 MHz, $CDCl_3$) δ 4.86 (s, 2H), 3.45 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 52.15, 41.50.

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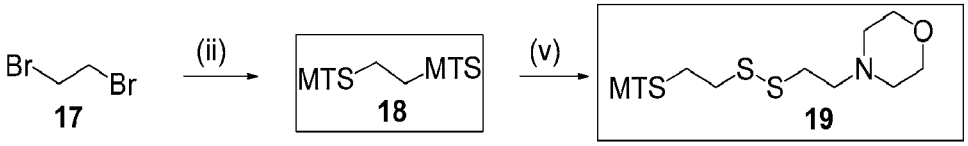
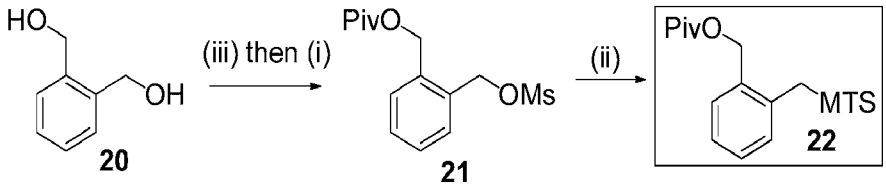
Synthesis of Compounds 18 and 19



[0030] **Compound 18:** Prepared from **17** and NaMTS as described previously: Chem. Pharm. Bull. Vol. 12(11) p. 1271, 1964. ^{1}H NMR (399 MHz, $CDCl_3$) δ 3.55 (s, 4H), 3.40 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 50.67, 35.96.

[0031] **Compound 19:** A DCM solution (1 mL) of 2-morpholinoethanethiol (0.17 g, 1.2 mmol) was added dropwise via syringe to a stirring solution of compound **18** (300 mg, 1.2 mmol) in DCM (10 mL) at r.t. Immediately after addition, TLC showed rapid formation of product and some dimer. After 0.5 h, the mixture was partitioned by addition of $NaHCO_3$ solution. Upon extraction, the organic layer was separated, dried ($MgSO_4$), filtered and concentrated *in vacuo*. Column chromatography gave pure **19** (0.20 g, 53%) as a colorless oil. ^{1}H NMR (399 MHz, $CDCl_3$) δ 3.73 – 3.67 (t, J = 4.7 Hz, 4H), 3.51 – 3.46 (m, 2H), 3.35(s, 3H), 3.07 – 3.01 (m, 2H), 2.88 – 2.83 (m, 2H), 2.69 – 2.63 (m, 2H), 2.52 – 2.43 (t, J = 4.6 Hz, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 66.96, 57.91, 53.58, 50.79, 37.66, 36.10, 35.52; MS (ESI +ve): calc $(M+H)^+$: 318.03, found: 318.04; R_f = 0.3 (EtOAc).

Synthesis of Compound 22



[0032] **Compound 21:** Compound **20** was converted to compound **21** by a procedure analogous to that described for compound **11**. ^{1}H NMR (399 MHz, $CDCl_3$) δ 7.45 – 7.36 (m, 4H), 5.37 (s, 2H), 5.21 (s, 2H), 2.93 (s, 3H), 1.21 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.20, 135.65, 131.92, 130.48, 129.98, 129.78, 128.88, 69.05, 63.39, 38.94, 38.36, 27.27; MS (ESI +ve): calc $(M+NH_4)^+$: 318.24, found: 318.14; R_f = 0.4 (20% EtOAc/hexane).

[0033] **Compound 22:** Compound **21** was converted to compound **22** by a procedure analogous to that described for compound **12**. ^{1}H NMR (399 MHz, $CDCl_3$) δ 7.46 – 7.32, (m, 4H), 5.21 (s, 2H), 4.50 (s, 2H), 3.03 (s, 3H), 1.21 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.24,

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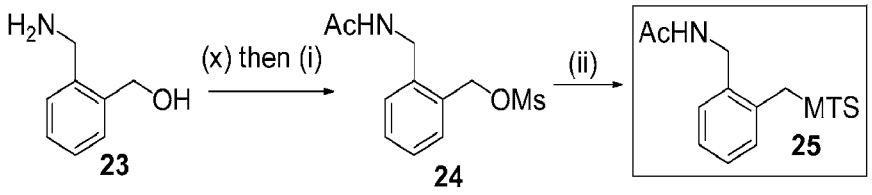
135.10, 133.15, 130.93, 130.32, 129.05, 129.00, 63.61, 51.07, 38.97, 38.03, 27.30; MS (ESI +ve): calc $(M+NH_4)^+$: 334.11, found: 334.13; R_f = 0.4 (20% EtOAc/hexane).

Synthesis of Compound 25

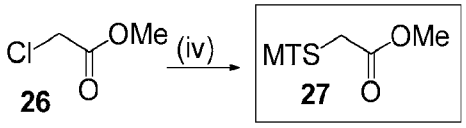


[0034] **Compound 23:** Compound **23** is prepared according to a literature method (Journal of Medicinal Chemistry, 50(23), 5568-5570, 2007).

[0035] **Compound 24:** An ice-cold pyridine solution (10 mL) of compound **23** (1 mmol) is treated successively, in a dropwise fashion with acetyl chloride (1 mmol), then after 5 min with MsCl (1.1 mmol). The solution is warmed to room temperature then the solvent is removed. The residue is dissolved in EtOAc, washed with water, dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification by column chromatography affords pure compound **24**.

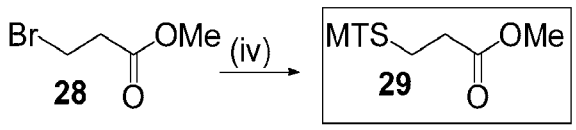
[0036] **Compound 25:** Compound **24** is converted to compound **25** by a procedure analogous to that described for compound **12**.

Synthesis of Compound 27



[0037] **Compound 27:** Compound **26** was converted to compound **27** by a procedure analogous to that described for compound **14**. ^1H NMR (399 MHz, $CDCl_3$) δ 3.97 (s, 2H), 3.79 (s, 3H), 3.48 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.84, 53.35, 51.53, 37.83; MS (ESI +ve): calc $(M+NH_4)^+$: 202.02, found: 201.96; R_f = 0.2 (20% EtOAc/hexane).

Synthesis of Compound 29



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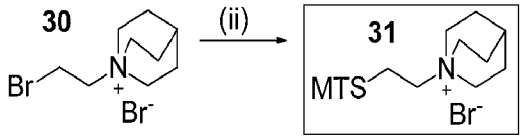
[0038] **Compound 29:** Compound **28** was converted to compound **29** by a procedure analogous to that described for compound **14**. ^{1}H NMR (399 MHz, $CDCl_3$) δ 3.72 (s, 3H), 3.39 (t, J = 6.8 Hz, 2H), 3.34 (s, 3H), 2.85 (t, J = 6.8 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.53, 52.29, 50.66, 34.51, 31.20; MS (ESI +ve): calc $(M+NH_4)^+$: 216.10, found: 216.04; R_f = 0.2 (20% EtOAc/hexane).

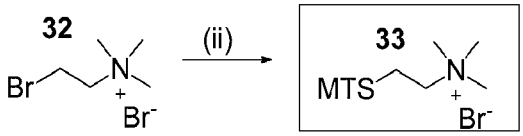
Synthesis of Compound 31



[0039] **Compound 30:** Compound **30** is prepared according to a literature method (Tetrahedron, 42(2), 601-607; 1986).

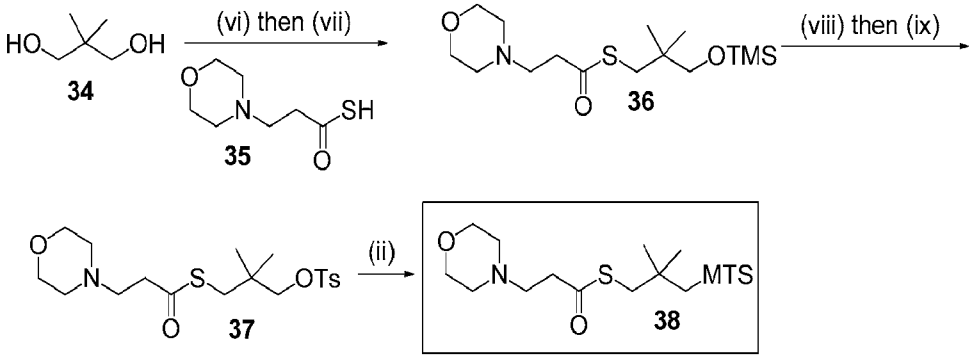
[0040] **Compound 31:** Compound **31** is prepared from compound **30** according to a patent procedure (US 20090181444).

Synthesis of Compound 33



[0041] **Compound 33:** Compound **33** is prepared from compound **32** according to a patent procedure (US 20090181444).

Synthesis of Compound 38



[0042] **Compound 36:** An ice-cold DCM (20 mL) solution of compound **34** (1 mmol) is treated with NEt_3 (1 mmol) followed by the dropwise addition of TMS-Cl (1.1 mmol). After 1 h,

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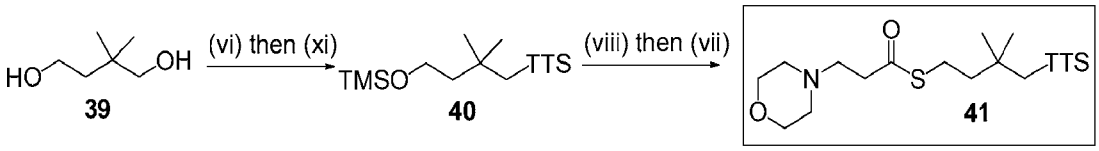
the solution is washed with water, dried ($MgSO_4$), filtered and concentrated *in vacuo*. The crude TMS protected material is re-dissolved in THF (10 mL), whereon PPh_3 (1.2 mmol), compound **35** (1.2 mmol), then DEAD (1.2 mmol, dropwise) are added in succession. After stirring at room temperature for 18 h, the solvent is removed under vacuum, the residue is re-dissolved in DCM, the solution of which is washed with water, dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification by column chromatography affords pure compound **36**.

[0043] **Compound 37:** A THF (10 mL) solution of compound **36** (0.5 mmol) is treated with TBAF (1 mmol of a 1M solution in THF), with monitoring by TLC. On completion of TMS cleavage, the solvent is removed under vacuum, and the residue is re-dissolved in DCM, the solution of which is washed with water, dried ($MgSO_4$), filtered and reduced *in vacuo*. The crude alcohol is re-dissolved in pyridine (5 mL), and TsCl (0.55 mmol) is added. After 18 h at room temperature, the solvent is removed, and the residue is re-dissolved in DCM, the solution of which is washed with water, dried ($MgSO_4$), filtered and reduced *in vacuo*. Purification by column chromatography affords pure compound **37**.

[0044] **Compound 38:** Compound **37** is converted to compound **38** by a procedure analogous to that described for compound **12**.

Synthesis of Compound 41



[0045] **Compound 40:** An ice-cold DCM (20 mL) solution of compound **39** (1 mmol) is treated with NEt_3 (1 mmol) followed by the dropwise addition of TMS-Cl (1.1 mmol). After 1 h, the solution is washed with water, dried ($MgSO_4$), filtered and concentrated *in vacuo*. The crude TMS protected material is re-dissolved in THF (10 mL), whereon PPh_3 (1.2 mmol), potassium *p*-toluenethiosulfonate (KTTS, 1.2 mmol), anhydrous $ZnCl_2$ (1 mmol) then DEAD (1.2 mmol, dropwise) are added in succession. After stirring at r.t. for 18 h, the solvent is removed under vacuum, and the residue is re-dissolved in DCM, the solution of which is washed with water, dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification by column chromatography affords pure compound **40**.

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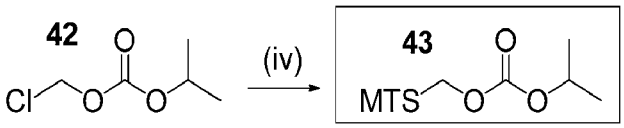
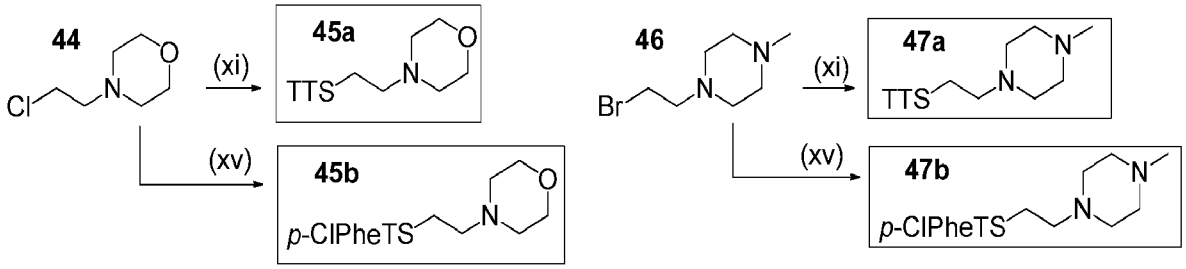
[0046] **Compound 41:** A THF (10 mL) solution of compound **40** (0.5 mmol) is treated with TBAF (1 mmol of a 1M solution in THF), with monitoring by TLC. On completion of TMS cleavage, the solvent is removed under vacuum, and the residue is re-dissolved in DCM, the solution of which is washed with water, dried ($MgSO_4$), filtered and concentrated *in vacuo*. The crude alcohol is re-dissolved in THF (10 mL), whereon PPh_3 (1.2 mmol), compound **35** (1.2 mmol), then DEAD (1.2 mmol, dropwise) are added in succession. After stirring at r.t. for 18 h, the solvent is removed under vacuum, and the residue is re-dissolved in DCM, the solution of which is washed with water, dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification by column chromatography affords pure compound **41**.

Synthesis of Compound 43



[0047] **Compound 43:** Compound **42** is converted to compound **43** by a procedure analogous to that described for compound **14**.

Synthesis of Compound 45a, 45b, 47a and 47b



[0048] **Compound 45a:** A mixture of 4-(2-chloroethyl)morpholine hydrochloride (compound **44**) (50 g, 269 mmol), sodium iodide (4.03 g, 26.9 mmol) and potassium 4-methylbenzenesulfonothioate (73.0 g, 322 mmol) was stirred in MeOH (200 ml) and heated at 60°C over 72 h. The pale yellow mixture was cooled and diluted with 200 mL water, stirred for 0.5 h then the white solid was collected by filtration, washed with water (100 mL), IPA (200 mL), EtOAc (200 mL) and ether (200 mL). Dried mass = 68 g. This microcrystalline powder was recrystallized from 90°C water (200 mL) and the crystalline mass was collected by filtration after standing at r.t. overnight (64 g, 71%). ^1H NMR (500 MHz, $CDCl_3$) δ 7.83 (d, J = 8.4 Hz, 2H), 7.36 (d, J = 8.4 Hz, 2H), 3.67 (t, J = 4.7 Hz, 4H), 3.16 (t, J = 6.9 Hz, 2H), 2.64 (t, J = 6.9 Hz,

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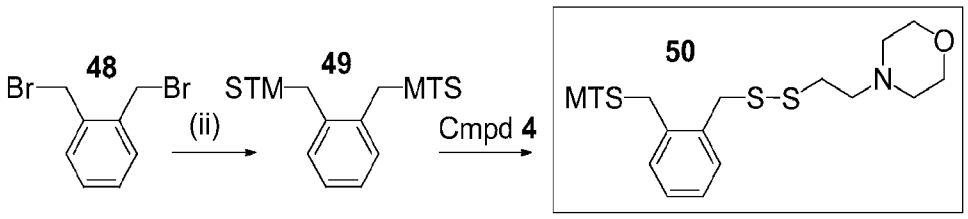
2H), 2.47 (s, 3H), 2.41 (t, J = 4.4 Hz, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 144.82, 141.99, 129.95, 127.14, 66.85, 56.54, 53.18, 33.44, 21.78; MS (ESI +ve): calc $(M+H)^+$: 302.08, found: 302.22.

[0049] **Compound 45b**: Replacing potassium 4-methylbenzenesulfonothioate with potassium 4-chlorobenzenesulfonothioate compound **45b** was synthesized by a method analogous to that described for compound **45a**. (ESI +ve): calc $(M+H)^+$: 324.03, 322.04, found: 324.22, 322.20 (^{37}Cl, ^{35}Cl isotope pattern).

[0050] **Compound 47a**: Replacing 4-(2-chloroethyl)morpholine hydrochloride with 4-(2-bromooethyl)–*N*–methylpiperazine hydrobromide (compound **46)**, compound **47a** was synthesized by a method analogous to that described for compound **45a**. MS (ESI +ve): calc $(M+H)^+$: 315.12, found: 315.07.

[0051] **Compound 47b**: Replacing 4-(2-chloroethyl)morpholine hydrochloride with 4-(2-bromooethyl)–*N*–methylpiperazine hydrobromide and potassium 4-methylbenzenesulfonothioate with potassium 4-chlorobenzenesulfonothioate compound **47b** is synthesized by a method analogous to that described for compound **45a**.

Synthesis of Compound 50



[0052] **Compound 49:** A mixture of compound **48**, (500 mg, 1.894 mmol) and sodium methanesulfonothioate (534 mg, 3.98 mmol) was dissolved in acetone (10 ml) and stirred at r.t. for 4 h. TLC indicated a complete reaction. The solvent was removed then the mixture was partitioned by addition of water and DCM. Upon extraction, the organic layer was separated then dried ($MgSO_4$), filtered and concentrated *in vacuo*. Column chromatography afforded the pure product as a colorless solid (0.60 g, 97%). ^{1}H NMR (399 MHz, $CDCl_3$) δ 7.47 (dd, J = 5.5, 3.5 Hz, 2H), 7.38 (dd, J = 5.5, 3.5 Hz, 2H), 4.55 (s, 4H), 3.13 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 133.41, 131.75, 129.51, 51.02, 37.09; MS (ESI +ve): calc $(M+NH_4)^+$: 344.01, found: 344.01; R_f = 0.5 (1:1 EtOAc/hexane).

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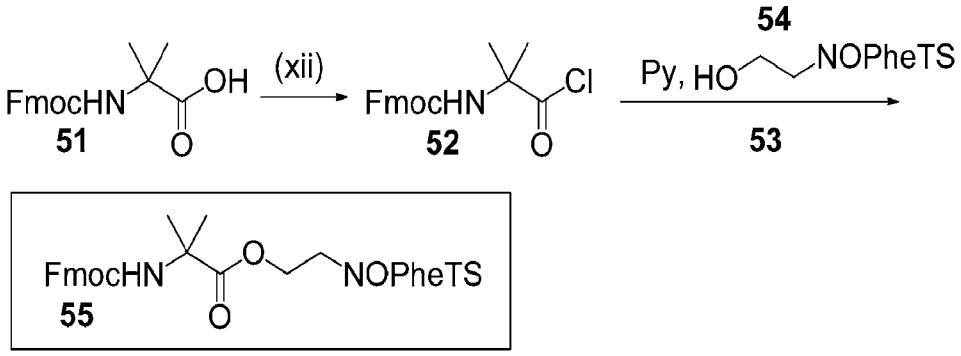
[0053] **Compound 50:** A DCM solution (2 mL) compound **4**, (180 mg, 1.225 mmol) was added dropwise via syringe to a stirring solution of compound **49** (400 mg, 1.225 mmol) in DCM (20 mL) at r.t. After 0.5 h, the mixture was partitioned by addition of $NaHCO_3$. Upon extraction, the organic layer was separated then dried ($MgSO_4$), filtered and concentrated *in vacuo*. Column chromatography gave the product (170 mg, 35%) as a colorless oil. ^{1}H NMR (399 MHz, $CDCl_3$) δ 7.43 – 7.39 (m, 1H), 7.35 – 7.27 (m, 3H), 4.54 (s, 2H), 4.03 (s, 2H), 3.67 (t, J = 4.6 Hz, 4H), 3.05 (s, 3H), 2.58 – 2.50 (m, 4H), 2.38 (t, *J* = 4.6 Hz, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 136.09, 133.20, 131.87, 131.21, 128.86, 128.54, 66.95, 57.95, 53.52, 51.04, 40.81, 38.18, 35.82; MS (ESI +ve): calc (M+H): 394.06, found: 394.23; R_f = 0.5 (EtOAc).

Synthesis of Compound 55



[0054] **Compound 54:** Bromine (0.246 ml, 4.78 mmol) in DCM (50 mL) was added in a dropwise fashion over 0.5 h to a stirring mixture of sodium 4-nitrobenzenesulfinate (2 g, 9.56 mmol) and 2-hydroxyethyl disulfide (0.585 ml, 4.78 mmol) in DCM (50 mL) at r.t. After 2 h at r.t. the mixture was filtered to remove salt. The solvent was removed then column purification by column chromatography gave the product (2.1 g, 83%) as a colorless oil. Rf = 0.4 (1:1 EA/hexane.^{1}H NMR (399 MHz, $CDCl_3$) δ 8.41 (d, *J* = 8.8 Hz, 2H), 8.13 (d, *J* = 8.8 Hz, 2H), 3.89 (t, *J* = 5.8 Hz, 2H), 3.23 (t, *J* = 5.8 Hz, 2H), 2.02 – 1.94 (s, br, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 149.73, 128.40, 124.82, 60.94, 39.28.

[0055] **Compound 55:** Compound **51** (1 g, 3.07 mmol) in DCM (50 mL) was treated dropwise with oxalyl dichloride (0.527 ml, 6.15 mmol) and stirred at r.t. During the reaction a colorless homogeneous solution was formed. After 1h at r.t. the solvent was removed under reduced pressure. The white residue (crude acid chloride, compound **52**) was re-dissolved in

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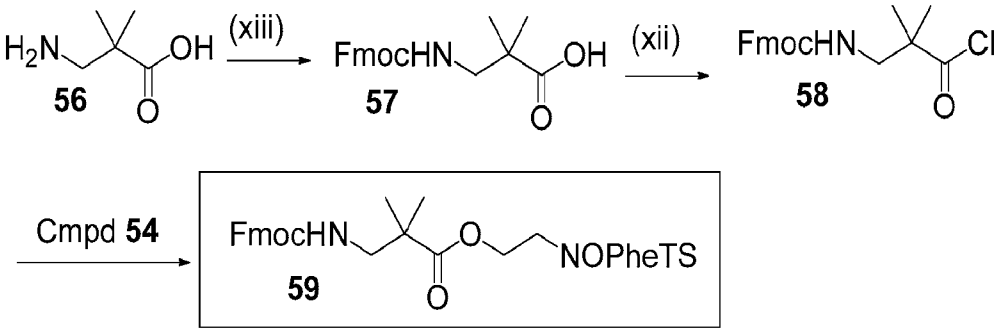
DCM (20 mL) and added to a stirring solution of compound **54** (0.48 g, 3.1 mmol) in pyridine (20 mL). After 18 h, it was judged that the reaction was complete. The solvents were removed. The residue was re-dissolved in toluene and partitioned with water. The toluene extracts were collected, washed with brine, dried ($MgSO_4$), filtered and concentrated. Column chromatography afforded pure compound **55** (0.86 g, 60%) as a colorless solid. ^{1}H NMR (399 MHz, $CDCl_3$) δ 8.31 (d, J = 8.9 Hz, 2H), 8.03 (d, J = 8.9 Hz, 2H), 7.7 (d, J = 7.5 Hz, 2H), 7.58 (d, J = 7.5 Hz, 2H), 7.42 (t, J = 7.5 Hz, 2H), 7.32 (dt, J = 7.5, 1.2 Hz, 2H), 5.22 (s, br, 1H), 4.34 (s, br, 4H), 4.19 (t, J = 6.8 Hz, 1H), 3.23 (t, J = 6.0 Hz, 2H), 4.17 (s, br, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.91, 155.10, 150.60, 149.53, 143.85, 141.42, 128.27, 128.20, 127.91, 127.22, 127.18, 125.12, 124.84, 120.17, 66.81, 62.60, 56.40, 47.25, 34.76, 25.37; MS (ESI +ve): calc $(M+H)^+$: 571.11, found: 571.00; R_f = 0.5 (EtOAc).

Synthesis of Compound 59



[0056] **Compound 57:** Compound **57** is prepared from compound **56** (1 mmol) by reaction with 1.5 eq Fmoc-OSu in pyridine at r.t. for 18 h. Aqueous workup then column chromatography affords pure compound **57.**

[0057] **Compound 59:** Compound **59** is prepared from compound **57** by a procedure analogous to that described for compound **55**.

Synthesis of Compound 63

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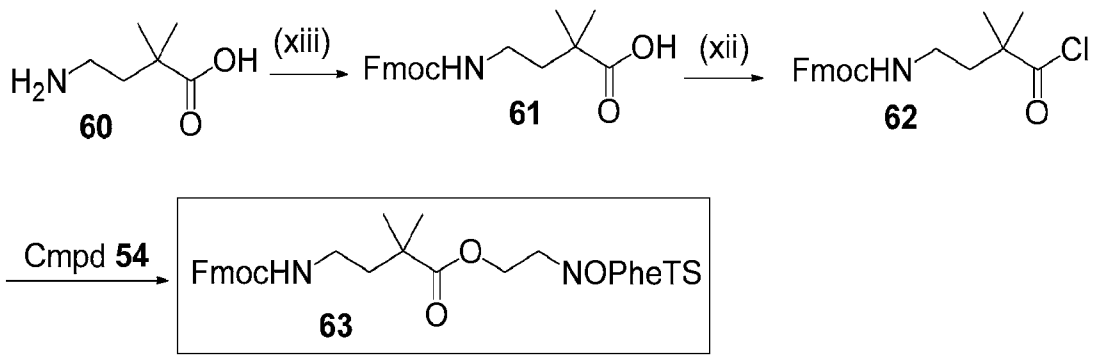
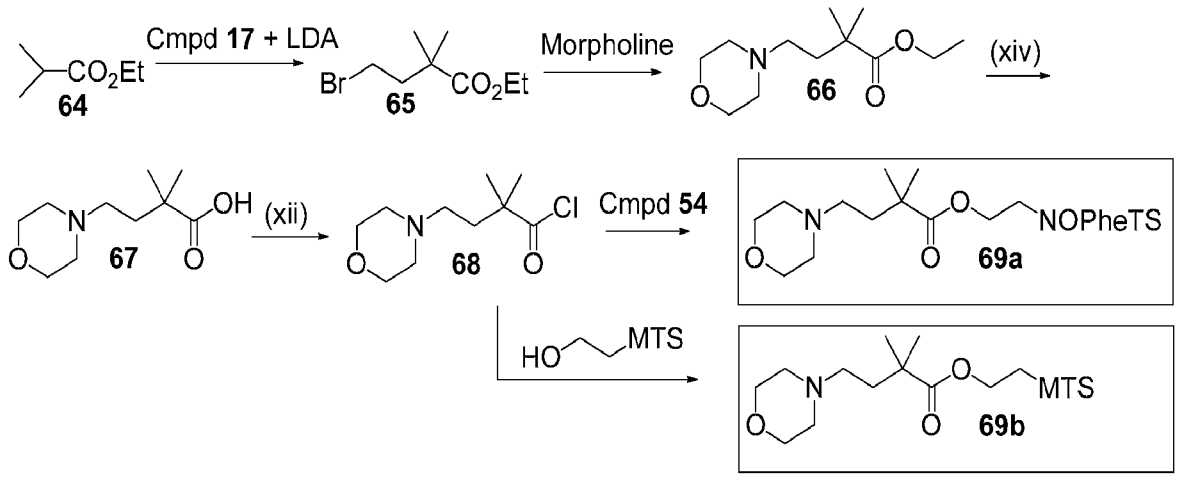


[0058] **Compound 61:** Compound **61** is prepared from compound **60** by a procedure analogous to that described for compound **57**.

[0059] **Compound 63:** Compound **63** is prepared from compound **61** by a procedure analogous to that described for compound **55**.

Synthesis of Compound 69



[0060] **Compound 65:** Ethyl isobutyrate, (11.6 g, 100 mmol) was added over 0.5 h to a cooled (-78°C) solution of LDA in THF (53 mL, 2M). The orange mixture was then warmed to 0°C briefly before re-cooling to -78°C. 1,2-dibromoethane (20 g, 106 mmol) was then added over 0.5 h to the solution which was warmed gradually to r.t. and stirred overnight. The resulting mixture was partitioned by addition of water and EA. Upon extraction into EA and washing with brine, the organic layer was separated then dried ($MgSO_4$), filtered and concentrated *in vacuo*. Column chromatography gave the pure product as a colorless oil (6.0 g, 25%). ^{1}H NMR (399 MHz, $CDCl_3$) δ 4.15 (q, J = 7.1 Hz, 2H), 3.35 (t, J = 8.4 Hz, 2H), 2.16 (t, J = 8.4 Hz, 2H), 1.27 (t, J = 7.1 Hz, 3H), 1.22 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.78, 60.83, 43.83, 42.89, 28.45, 25.20, 14.32; R_f = 0.5 (5% EtOAc/hexane).

[0061] **Compound 66:** A mixture of compound **65** (3.4 g, 15.24 mmol) and morpholine (13.28 g, 152 mmol) in THF (20 ml) was heated at 50°C in a glass pressure bottle over 72 h. A white precipitate was observed to form. The mixture was separated between water and EA. The EA extracts were dried ($MgSO_4$), filtered and concentrated. Column chromatography gave the product as a colorless liquid (3.5 g, 100%). ^{1}H NMR (399 MHz, $CDCl_3$) δ 4.11 (q, J = 7.1 Hz, 2H), 3.67 (t, J = 4.7 Hz, 4H), 2.41 (t, br, J = 4.0 Hz, 4H), 2.30 – 2.26 (m, 2H), 1.74 – 1.70 (m, 2H), 1.23 (t, J = 7.1 Hz, 3H), 1.17 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.69, 67.11, 55.15, 54.02, 41.10, 37.04, 25.49, 14.35; MS (ESI +ve): calc $(M+H)^+$: 230.18, found: 230.33; R_f = 0.5 (5% EtOAc/hexane).

[0062] **Compound 67:** Compound **66** (120 mg, 0.523 mmol) and sodium hydroxide (120 mg, 3.00 mmol) were stirred together in 1:1 $EtOH/H_2O$ (10 mL). After 36 h, the pH of the solution was adjusted to ca. 2 by addition of c. HCl, then NEt_3 was added until the pH reached ca. 10. SiO_2 (2 g) was added and the solvents/water were evaporated. Column chromatography with dry loading (MeOH/DCM, with 2% NEt_3 in the DCM), gave the product as a partial (approx 1/3 mol eq) $HNEt_3$ salt. The adjusted yield was 103 mg, 84%. ^{1}H NMR (399 MHz, $CDCl_3$ plus 3 drops of DMSO-d6) δ 3.62 (t, J = 4.7 Hz, 4H), 2.51 – 2.46 (br, 4H), 2.40 (t, J = 7.1 Hz, 2H), 1.62 (J = 7.1 Hz, 2H), 1.08 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 179.91, 65.91, 54.29, 52.87, 45.44, 41.37, 35.09, 25.93, 8.56; MS (ESI -ve): calc $(M-H)^-$: 200.13, found: 200.16; R_f = 0.5 (2% NEt_3/10% MeOH/DCM).

[0063] **Compound 69a**: A suspension of compound **67** (11 g, 54.9 mmol) in DCM (200 mL) was treated with oxalyl chloride (9.42 ml, 110 mmol) and stirred at r.t. During the reaction a colorless homogeneous solution was formed. After 1h at r.t. the solvent was removed under reduced pressure. The yellow residue was suspended in a mixture of DCM (200 mL) and Py (100 mL) then S-2-hydroxyethyl compound **54** (15.91 g, 60.4 mmol) (DB-7-5) was added all at once and the reaction was monitored by HPLC/MS. After 18 h, it was judged by MS and TLC that the reaction was virtually complete. The solvents were removed then the residue was redissolved in DCM/bicarbonate. The combined organics were dried ($MgSO_4$), filtered and reduced. Purification by column chromatography gave 12.5 g, 51% of compound **69a** as a yellow oil which crystallized on standing. ^{1}H NMR (399 MHz, $CDCl_3$) δ 8.41 (d, J = 8.5 Hz, 2H), 8.13 (d, J = 8.5 Hz, 2H), 4.27 (t, J = 6.3 Hz, 2H), 3.65 (t, J = 4.6 Hz, 4H), 3.28 (t, J = 6.3 Hz, 2H), 2.40 (s, br, 4H), 2.27 (t, J = 7.7 Hz, 2H), 1.71 (t, J = 7.7 Hz, 2H), 1.14 (s, 6H); ^{13}C NMR (100 MHz,

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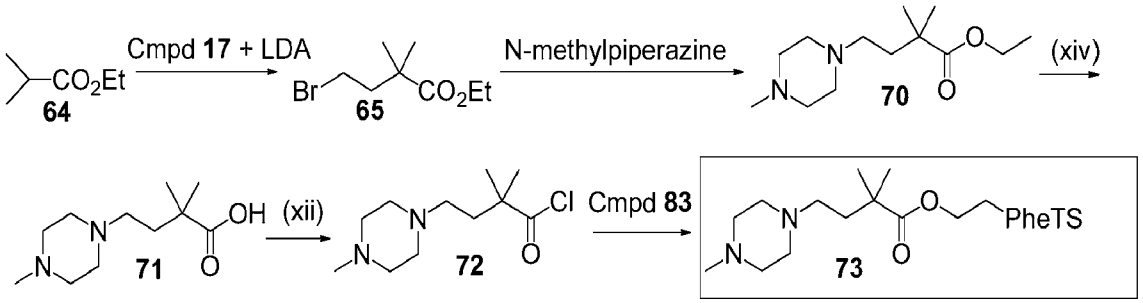
$CDCl_3$) δ 177.11, 150.71, 149.69, 128.34, 124.92, 66.96, 61.66, 54.95, 53.92, 41.25, 46.81, 35.11, 25.36; MS (ESI +ve): calc $(M+H)^+$: 447.12, found: 446.98.

[0064] **Compound 69b:** A suspension of compound **67** (128 mg, 0.636 mmol) in DCM (12 mL) was treated dropwise with oxalyl chloride (545 µl, 6.36 mmol) and stirred at r.t. During the reaction a colorless homogeneous solution was formed which soon developed a colorless precipitate. HPLC/MS indicated good conversion to acid chloride (compound **68**) as shown by conversion to the *N*-propyl amide. The solvent was removed under reduced pressure. The white residue was cooled on an ice bath and treated with a DCM (12 mL) solution of S-2-hydroxyethyl methanesulfonothioate (compound **54**) (99 mg, 0.636 mmol), followed by dropwise addition of Hunig's base (0.34 g, 2.6 mmol, 4 eq) with stirring. After 1.5 h, the solution was washed with diluted $NaHCO_3$ (aq). The combined extracts were dried ($MgSO_4$), filtered and concentrated. Column chromatography gave the product as a colorless oil (84 mg, 39%). ^1H NMR (399 MHz, $CDCl_3$) δ 4.40 (t, J = 6.2 Hz, 2H), 3.70 (t, J = 4.6 Hz, 4H), 3.44 (t, J = 6.2 Hz, 2H), 3.39 (s, 3H), 2.47 – 2.43 (br, 4H), 2.32 (t, J = 7.7 Hz, 2H), 1.77 (t, J = 7.7 Hz, 2H), 1.64 – 1.58 (br, 4H), 1.22 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.30, 67.08, 62.27, 55.03, 54.01, 51.07, 41.32, 36.99, 35.14, 25.44; MS (ESI +ve): calc $(M+H)^+$: 340.13, found: 340.27; R_f = 0.2 (EtOAc).

Synthesis of Compound 73



[0065] **Compound 70:** Compound **70** was prepared by reaction of N-methyl piperazine with compound **65** by a procedure analogous to that described for compound **66**. ^1H NMR (399 MHz, $CDCl_3$) δ 4.10 (q, J = 7.1 Hz, 2H), 2.3 – 2.6 (br, 8H), 2.28 (t, J = 8.0 Hz, 2H), 2.26 (s, 3H), 1.72 (t, J = 8.0 Hz, 2H), 1.23 (t, J = 7.1 Hz, 3H), 1.15 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.71, 60.44, 55.27, 54.64, 53.50, 46.18, 41.15, 37.36, 25.47, 14.34; MS (ESI +ve): calc $(M+H)^+$: 243.20, found: 243.31.

[0066] **Compound 71:** Compound **71** was prepared by hydrolysis of compound **70** using a procedure analogous to that described for compound **67**. ^1H NMR (399 MHz, $CDCl_3$) δ 12 – 10

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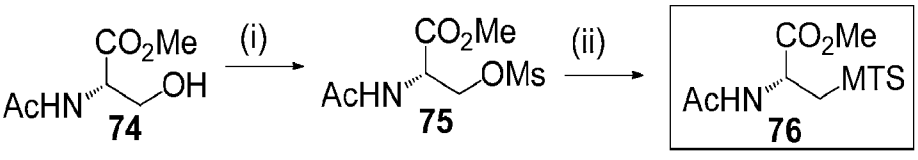
(vbr, 1H), 3.2 – 2.2 (vbr, 8H), 2.44 (t, J = 8.0 Hz, 2H), 2.36 (s, 3H), 1.71 (t, J = 8.0 Hz, 2H), 1.17 (s, 6H), ^{13}C NMR (100 MHz, $CDCl_3$) δ 181.36, 55.18, 53.51, 52.18, 44.31, 41.57, 37.19, 26.28; MS (ESI +ve): calc $(M+H)^+$: 215.17, found: 215.19.

[0067] **Compound 83**: Bromine (2.58 ml, 50.0 mmol) in DCM (50 mL) was added in a dropwise fashion over 0.5 h to a stirring mixture of sodium benzenesulfinate (16.4 g, 100 mmol) and 2-hydroxyethyl disulfide (6.11 ml, 49.9 mmol) in DCM (50 mL) at r.t. After 2 h at r.t. TLC showed good reaction (Rf = 0.4 (1:1 EA/hexane), so the mixture was filtered to remove salt. The solvent was removed then column chromatography gave the product (17.4 g, 80%) as a colorless oil.

[0068] **Compound 73:** Compound **71** (1.342 g, 6.29 mmol), in DCM (20 mL) was treated with oxalyl chloride (1.079 ml, 12.58 mmol) and stirred at r.t. During the reaction a pale yellow precipitate was observed to form. After 0.5h at r.t. the solvent was removed under reduced pressure. The pale yellow residue was suspended in a mixture of DCM (20 mL) and Py (20 mL) then compound **83** (2.060 g, 9.44 mmol) was added all at once.. After 18 h, it was judged by TLC that the reaction was complete. The solvents were removed then the residue was redissolved in water and washed with ether. The aqueous layer was reduced to a brown solid. Recrystallization from boiling EtOH (10 mL) gave a slightly impure HCl salt which was free based (NEt_3 in DCM) then loaded directly onto silica gel. Column chromatography gave 0.95 g, 36% of pure compound **73**.. ^{1}H NMR (399 MHz, $CDCl_3$) δ 7.96 (d, J = 8.0 Hz, 2H), 7.68 (t, J = 7.2 Hz, 1H), 7.59 (t, J = 7.7 Hz, 2H), 4.25 (t, J = 6.3 Hz, 2H), 3.25 (t, J = 6.3 Hz, 2H), 2.8 – 2.3 (vbr, 8H), 2.36 (s, 3H), 2.33 (t, J = 7.7 Hz, 2H), 1.74 (t, J = 7.7 Hz, 2H), 1.16 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.13, 144.68, 134.09, 129.59, 127.09, 61.99, 54.86, 54.35, 52.84, 45.95, 45.71, 41.26, 37.05, 34.66, 25.39; MS (ESI +ve): calc $(M+H)^+$: 415.17, found: 415.09.

Synthesis of Compound 76



[0069] **Compound 75:** Compound **74** was converted to compound **75** by a procedure analogous to that described for compound **2**. ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.49 – 6.41 (br, d, J = 6.3 Hz, 1H), 4.91 (dt, J = 6.3, 2.7 Hz, 1H), 4.59 (ddd, J = 31.4, 10.6, 3.0 Hz, 2H), 3.84 (s, 3H),

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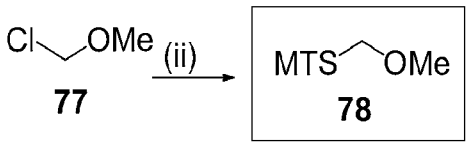
3.04 (s, 3H), 2.09 (s, 3H); 170.27, 169.05, 68.90, 53.33, 52.03, 37.63, 23.16; MS (ESI +ve): calc $(M+H)^+$: 240.06, found: 240.24.

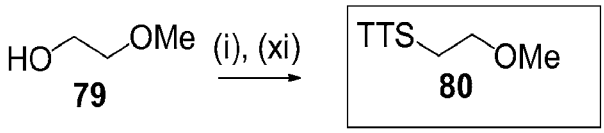
[0070] **Compound 76:** Compound **75** was converted to compound **76** by a procedure analogous to that described for compound **3**. ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.56 – 6.40 (br, d, J = 6.2 Hz, 1H), 4.93 (q, J = 5.9 Hz, 1H), 3.81 (s, 3H), 4 3.74 (dd, J = 14.6, 4.8 Hz, 1H), 3.57 (dd, J = 14.6, 5.6 Hz, 1H), 3.38 (s, 3H), 2.07 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.44, 170.19, 53.23, 52.02, 50.73, 37.77, 23.12; MS (ESI +ve): calc $(M+H)^+$: 256.03, found: 256.21.

Synthesis of Compound 78



[0071] A mixture of compound **77**, (2.49 ml, 32.8 mmol), and sodium methanesulfonothioate (4.4 g, 32.8 mmol) was stirred in acetone (80 ml) over 6h. The acetone was removed by rotary evaporation and the mixture was triturated with DCM. After filtration, DCM was removed by evaporation (5 g crude yield) and the mixture was subjected to purification by column chromatography. Yield of pure compound **78** was 2.8 g, 55%, colorless oil, Rf = 0.3 (20% EA/hexane). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.30 (s, 2H), 3.48 (s, 3H), 3.38 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 80.07, 57.40, 22.71.

Synthesis of Compound 80



[0072] An ice-cold solution of compound **79** (5 g, 65.7 mmol) and triethylamine (11 ml, 79 mmol) in DCM (150 mL) was treated with methanesulfonyl chloride (5.59 ml, 72.3 mmol) over 2 min. After 1 h the reaction was judged to be complete by TLC. Water was added and the organic extracts were washed sequentially with dilute HCl, saturated sodium bicarbonate, brine, then dried ($MgSO_4$), filtered and reduced. The product mesylate (9.4 g, 61 mmol, 96%) was taken up in acetone (200 ml), then the potassium salt of toluene thiosulfinic acid (61 mmol) was added and the solution was stirred at 50°C over 18 h. A thick white precipitate was observed to form. The mixture was filtered, reduced to an oil then extracted into DCM/water. The organic

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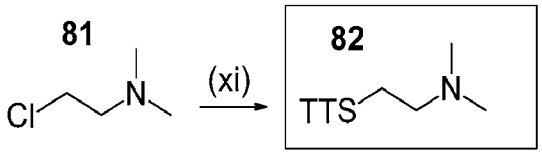
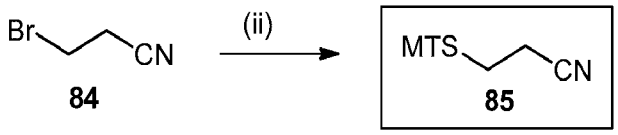
extracts were dried ($MgSO_4$), filtered and reduced. Column chromatography gave pure compound **80** as a colorless oil which crystallized on standing Rf = 0.3 (20% EA/hexane)

Synthesis of Compound 82



[0073] A mixture of 2-chloro-*N,N*-dimethylethanamine hydrochloride (10 g, 69.4 mmol), sodium iodide (1.041 g, 6.94 mmol) and potassium 4-methylbenzenesulfonothioate (18.86 g, 83 mmol) was stirred in MeOH (50 ml) and heated at 60°C over 72 h. Aqueous workup then column chromatography gave pure compound **82** (8.5 g, 47%) as a colorless oil. ^{1}H NMR (399 MHz, $CDCl_3$) δ 7.81 (d, J = 8.4 Hz, 2H), 7.33 (dd, J = 8.4, 0.6 Hz, 2H), 3.09 (t, J = 6.8 Hz, 2H), 2.53 (t, J = 6.8 Hz, 2H), 2.44 (s, 3H), 2.17 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 144.76, 142.03, 129.92, 127.18, 57.51, 45.14, 34.29, 21.77; MS (ESI +ve): calc $(M+H)^+$: 260.07, found: 260.16.

Synthesis of Compound 85



[0074] Sodium methanesulfonate (11.5 g, 86 mmol) was placed in a dry 50 mL one neck flask. 30 mL of anhydrous DMF (Aldrich) was added using a dry glass syringe. 5 mL of 3-bromopropionitrile (compound **84**) (8.2 g, 61.2 mmol) was added using a dry glass syringe. The reaction flask was closed under argon, sealed and stirred for 24 h at 50°C. The reaction was monitored using TLC (system: hexanes/ethyl acetate – 5:5 – v/v). The reaction mixture was diluted with 100 mL of ethyl acetate and washed 5 times with water (5 × 100 mL). The organic layers were dried over sodium sulfate, filtered and evaporated to dryness. The residue was dissolved in 5 mL of dichloromethane and purified by silica gel chromatography (CombiFlash) using a linear gradient of ethyl acetate in hexanes. Pure compound **85** (7.2 g, 52%) was obtained as a colorless oil. ^{1}H-NMR ($CDCl_3$, 399 MHz). δ 3.43 (s, 3H), 3.41 (t, 2H, J = 2.5 Hz), 2.93 (t, 2H, J = 2.5 Hz); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 117.7, 51.2, 31.7, 19.6.

Synthesis of Compound 87

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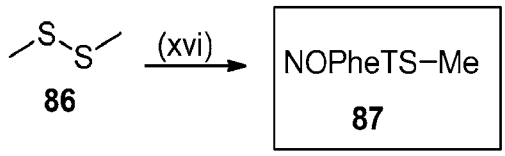
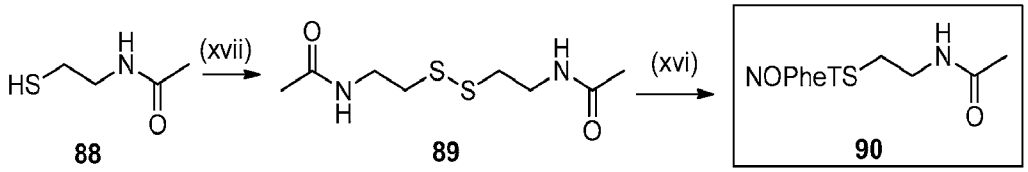


[0075] **Compound 87:** Compound **86** (1.11 mL, 12.5 mmol) and sodium 4-nitrobenzenesulfinate (5.23 g, 25.0 mmol) were added in DCM (12.5 mL) to give a white suspension. Dibromine (0.64 ml, 12.5 mmol) was added to the stirred solution. The solution was stirred for 30 min at r.t. and filtrated to remove the salt, then the filtrate was evaporated under reduced pressure. The crude product was added to a silica gel column and was eluted with hexane- EtOAc to give pure compound **87** (4.80 g, 20.6 mmol, 82% yield). ^{1}H NMR (399 MHz, $CDCl_3$) δ 8.44 - 8.40 (m, 2H), 8.15 - 8.10 (m, 2H), 2.58 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 149.00, 128.54, 124.87, 94.61, 18.55; R_f = 0.60 (1:1 EtOAc/hexane).

Synthesis of Compound 90



[0076] **Compound 89:** In a 1 L round-bottomed flask *N*-(2-mercaptoethyl)acetamide, **88** (11.92 g, 100 mmol) was dissolved in EtOAc (300 mL) to give a colorless solution. Sodium iodide (0.150 g, 1.000 mmol) and 30% hydrogen peroxide (3.40 g, 100 mmol) in H_2O (11.3 mL) were added to the solution which was stirred for 45 min at r.t. Saturated $Na_2S_2O_3$ was added to the solution. The aqueous layer was extracted with EtOAc (3 × 300 mL). The combined organic layers were dried with Na_2SO_4, filtered and concentrated by rotary evaporation. The crude product was subjected to column chromatography and was eluted with a MeOH/DCM gradient to give pure compound **89** (4.94 g, 20.90 mmol, 41.8 % yield), which was judged by TLC to be sufficiently pure for direct use in the next step of the reaction. R_f = 0.35 (9:1 DCM/Methanol)

[0077] **Compound 90:** In a 100 mL round-bottomed flask, compound **89** (2.364 g, 10.00 mmol) and sodium 4-nitrobenzenesulfinate (4.18 g, 20 mmol) were dissolved in CH_2Cl_2 (20 mL) to give a white suspension. Dibromine (0.516 ml, 10.00 mmol) was added to the stirred solution. The solution was stirred for 30 min at r.t. and filtrated to remove the salt, then the filtrate was evaporated under reduced pressure. The crude product was added to a silica gel column and was eluted with hexane- EtOAc to give pure compound **90** (2.37 g, 7.79 mmol, 39 % yield). ^{1}H

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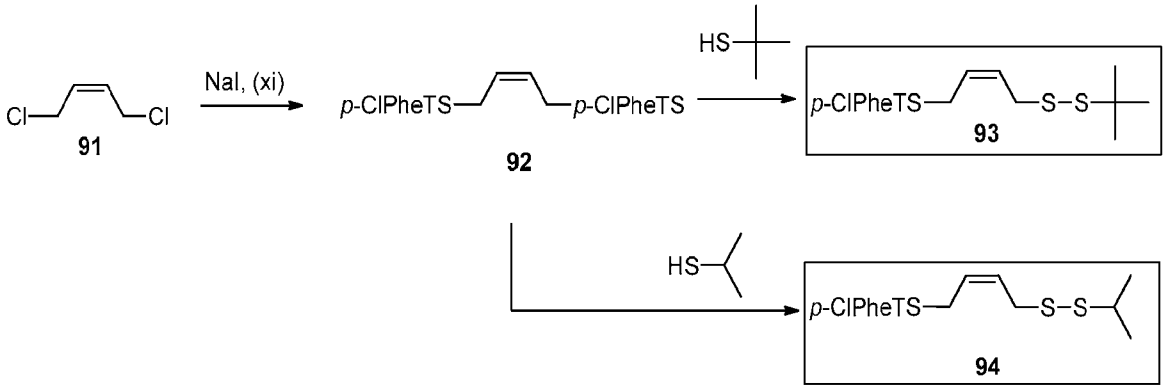
NMR (399 MHz, $CDCl_3$) δ 8.45 - 8.40 (m, 2H), 8.17 - 8.12 (m, 2H), 3.60 - 3.54 (dt, 2H), 3.21 - 3.16 (t, 2H), 2.00 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 130.35, 126.27, 66.97, 58.20, 53.67, 51.52, 36.22, 35.16, 33.67; R_f = 0.40 (EtOAc).

Synthesis of Compounds 93 and 94



[0078] **Compound 92:** In a 250 mL round-bottomed flask, sodium 4-chlorobenzenesulfonothioate (14.5 g, 63.0 mmol) was dissolved in MeOH. *Cis*-1,4-dichloro-2-butene, **91** (3.16 g, 30.0 mmol) and sodium iodide (450 mg, 3.0 mmol) was added to the stirred solution. The solution was stirred for 3 h at r.t., then heated up to 50 °C. After stirring for 18 h at 50 °C, solvents were removed under reduced pressure. The resulting crude material was diluted with DCM (300 mL) and washed with water (1 × 100 mL). The aqueous layer was extracted with DCM (1 × 100 mL). Combined organic layers were dried with Na_2SO_4, filtered and concentrated. The crude product was subjected to column chromatography and was eluted with an EtOAc/hexane gradient to give compound **92** (5.99 g, 12.8 mmol, 43 % yield). ^1H NMR (399 MHz, $CDCl_3$) δ 7.87-7.84 (m, 4H), 7.56-7.51 (m, 4H), 5.56-5.50 (m, 2H), 3.72-3.68 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$)130.35, 126.27, 66.97, 58.20, 53.67, 51.52, 36.22, 35.16, 33.67; R_f = 0.35 (1:3 EtOAc/hexane).

[0079] **Compound 93:** In a 100 mL round-bottomed flask compound **92** (3.09 g, 6.58 mmol) was dissolved in THF at 0 °C. Triethylamine (459 μL, 3.29 mmol) and 2-methyl-2-propanethiol (742 μL, 6.58 mmol) were added to the stirred solution at 0 °C. The solution was stirred for 20 min at 0 °C, then triethylamine (230 μL, 1.65 mmol) was added to the stirred solution at 0 °C. After stirring for 40 min at 0 °C, the solvents were removed under reduced pressure. The resultant was subjected to column chromatography and was eluted with an EtOAc/hexane gradient to give compound **93** (1.77 g, 4.62 mmol, 70 % yield). ^1H NMR (399

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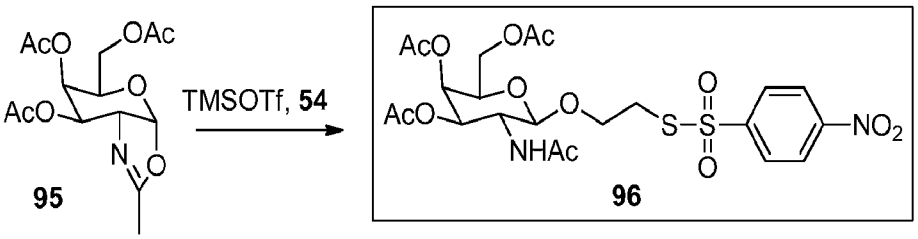
MHz, $CDCl_3$) δ 7.90 - 7.85 (m, 2H), 7.56 - 7.52 (m, 2H), 5.73 - 5.65 (m, 1H), 5.55 - 5.48 (m, 1H), 3.78 - 3.75 (d, 2H), 3.36 - 3.32 (d, 2H), 1.32 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 143.54, 140.60, 130.90, 129.83, 128.66, 124.56, 48.29, 37.05, 33.35, 30.16; R_f= 0.60 (1:3 EtOAc/hexane).

[0080] **Compound 94:** Using a procedure analogous to that described for compound **93**, and substituting 2-propanethiol for 2-methyl-2-propanethiol, pure compound **94** was obtained (1.13 g, 3.06 mmol, 72 % yield). ^1H NMR (399 MHz, $CDCl_3$) δ 7.90 - 7.82 (m, 2H), 7.56 - 7.48 (m, 2H), 5.72 - 5.64 (m, 1H), 5.57 - 5.47 (m, 1H), 3.79 - 3.72 (d, 2H), 3.33 - 3.26 (d, 2H), 3.61 - 2.92 (m, 1H), 1.27 (d, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 143.28, 140.36, 131.37, 125.63, 124.55, 124.56, 41.32, 38.02, 35.81, 33.08, 22.54; R_f= 0.55 (1:3 EtOAc/hexane).

Synthesis of Compound 96



[0081] **Compound 96:** Compounds **9** (1.81 g, 5.50 mmol) and **54** (2.17 g, 8.25 mmol) were combined and dried by co-evaporation with anhydrous toluene (3 × 2 mL) . The mixture was dissolved in dry 1,2-dichloroethane (16.5 mL). 4 Å molecular sieves were added to the solution and the mixture was stirred 30 min at r.t. Trimethylsilyltriflate (497 μL, 2.75 mmol) was added to the stirred solution. The solution was stirred for 9 h at r.t., then additional **54** (0.723 g, 2.75 mmol) was added to the stirred solution. After stirring 16 h at r.t., the solution was diluted with DCM (100 mL) and washed with sat. $NaHCO_3$ (1 × 100 mL). The aqueous layer was extracted with DCM (1 × 50 mL). The combined organic layers were dried with Na_2SO_4, filtered and concentrated by rotary evaporation. The resulting crude was subjected to column chromatography and was eluted with an EtOAc/hexane gradient to give compound **96** (2.21 g, 3.73 mmol, 68 % yield). ^1H NMR (399 MHz, $CDCl_3$) δ 8.42 - 8.36 (d, 2H), 8.12 8.06 (d, 2H), 6.07 - 6.00 (d, 1H), 5.33 - 5.30 (d, 1H), 5.23 - 5.15 (dd, 1H), 4.69 - 4.63 (d, 1H), 4.15 - 4.04 (m, 3H), 4.04-3.87 (m, 2H), 3.84-3.73 (m, 1H), 3.22-3.12 (t, 2H), 2.12-1.92 (m, 12H); ^{13}C NMR (100 MHz, $CDCl_3$)179.95, 170.74, 170.48, 150.77, 149.59, 128.47, 125.03, 101.40, 71.08, 70.02, 67.39, 66.92, 61.76, 51.12, 36.51, 23.72, 20.92; R_f = 0.25 (1:9 MeOH/DCM).

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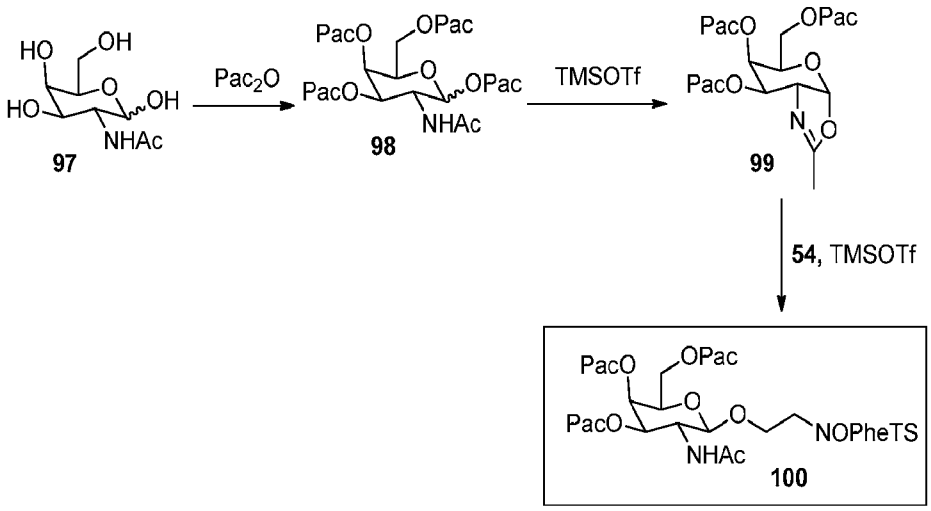
Synthesis of Compound 100



[0082] **Compound 98:** An ice-cold solution of compound **97** (4.87 g, 22.0 mmol) in dry pyridine (70 mL) was treated with phenoxyacetic anhydride (37.8 g, 132.0 mmol) and 4-dimethylaminopyridine (26.9 mg, 220 μmol). After stirring at r.t. for 12 h, the mixture was concentrated and co-evaporated with toluene *in vacuo*. The resultant was diluted by DCM (400 mL) and washed with sat. $NaHCO_3$ (1 × 400 mL). Upon extraction, the organic layer was separated, dried with Na_2SO_4, filtered and concentrated *in vacuo*. The resultant was subjected to column chromatography and was eluted with an EtOAc/hexane gradient to give pure compound **98** (16.7 g, 22.0 mmol, 100 % yield). ^{1}H NMR (399 MHz, $CDCl_3$) δ 7.36 - 6.75 (m, 20H), 6.27 - 6.20 (d, 1H), 5.43 - 5.36 (m, 1H), 5.22 - 5.06 (m, 2H), 4.81 - 4.65 (m, 3H), 4.60 - 4.52 (m, 2H), 4.45 - 4.29 (m, 2H), 4.17 - 4.02 (m, 2H), 3.97 - 3.87 (m, 1H), 1.85 and 1.74 (d, 3H, rotamers); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.20, 168.78, 168.70, 168.36, 167.52, 157.56, 157.48, 157.40, 130.09, 129.70, 129.66, 129.60, 122.32, 122.01, 121.94, 114.61, 114.58, 114.58, 114.39, 91.93, 68.37, 68.25, 67.30, 64.54, 61.25, 46.43, 22.95; R_f = 0.35 (1:1 EtOAc/hexane).

[0083] **Compound 99:** Compound **98** (15.15 g, 20.0 mmol) was dissolved in $ClCH_2CH_2Cl$ (40 mL) and trimethylsilyl trifluoromethanesulfonate (5.43 mL, 30.0 mmol) was added to the stirred solution at r.t. The solution was stirred for 24 h at 50 °C, triethylamine (12.6 mL, 90.0 mmol) was added and the mixture was concentrated *in vacuo*. The resultant was purified by silica gel column chromatography (EtOAc (2.5% Et_3N)-hexane) to give slightly

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impure compound **99** containing tiny amounts of phenoxyacetic acid. This material was used in the next step of the reaction scheme without further purification.

[0084] **Compound 100:** Compound **99** (3.63 g, 6 mmol) and *S*-2-hydroxyethyl-4-nitrobenzenesulfonothioate, **54** (2.76 g, 10.5 mmol) were dissolved in $ClCH_2CH_2Cl$ (18 mL) to give a colorless solution. 4Å Molecular sieves were added to the stirred solution at r.t. The solution was stirred at r.t. for 30 min then trimethylsilyl trifluoromethanesulfonate (0.543 ml, 3.00 mmol) was added. The mixture was stirred at r.t. for 24 h and TLC showed about 90% reaction completed. Additional **54** (237.0 mg, 0.90 mmol) was added to the mixture and it was stirred for further 36 h to complete the reaction. The mixture was diluted with DCM (100 mL) and washed with sat $NaHCO_3$ (1 × 100 mL). The aqueous layer was back-extracted with CH_2Cl_2 (1 × 50 mL). The combined organic layers were dried with Na_2SO_4, filtered, concentrated and dissolved in 120 mL of Ac_2O-pyridine (1:9, v/v). The mixture was stirred for 12 h then concentrated under reduced pressure. The resultant was diluted with CH_2Cl_2 (100 mL) and washed with sat $NaHCO_3$ (1 × 100 mL). The aqueous layer was back extracted with CH_2Cl_2 (1 × 50 mL). The combined organic layers were dried with Na_2SO_4, filtered and concentrated. The resultant was purified by silica gel column chromatography (EtOAc-hexane gradient) to give pure compound **100** (2.56 g, 2.94 mmol, 49.0 % yield). ^1H NMR (399 MHz, $CDCl_3$) δ 8.70 - 8.62 (d, 2H), 8.41 - 8.33 (d, 2H), 7.65 - 7.03 (m, 15H), 6.35 - 6.27 (d, 1H), 5.81 - 5.72 (m, 2H), 5.06 - 4.97 (m, 3H), 4.92 - 4.85 (m, 2H), 4.83 - 4.62 (m, 2H), 4.58 - 4.44 (m, 2H), 4.34 - 4.26 (m, 2H), 4.26 - 4.15 (m, 1H), 4.06 - 3.95 (m, 1H), 3.51 - 3.40 (m, 2H), 2.22 and 2.18 (d, 3H, rotamers); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.17, 169.13, 169.05, 168.92, 157.86, 157.80, 157.76, 150.82, 149.63, 130.01, 129.96, 128.47, 125.06, 122.27, 122.23, 114.93, 114.74, 100.86, 70.64, 70.54, 65.36, 64.90, 62.21, 51.30, 36.54, 23.74; R_f = 0.60 (1:1 EtOAc/hexane).

Synthesis of Compound 104

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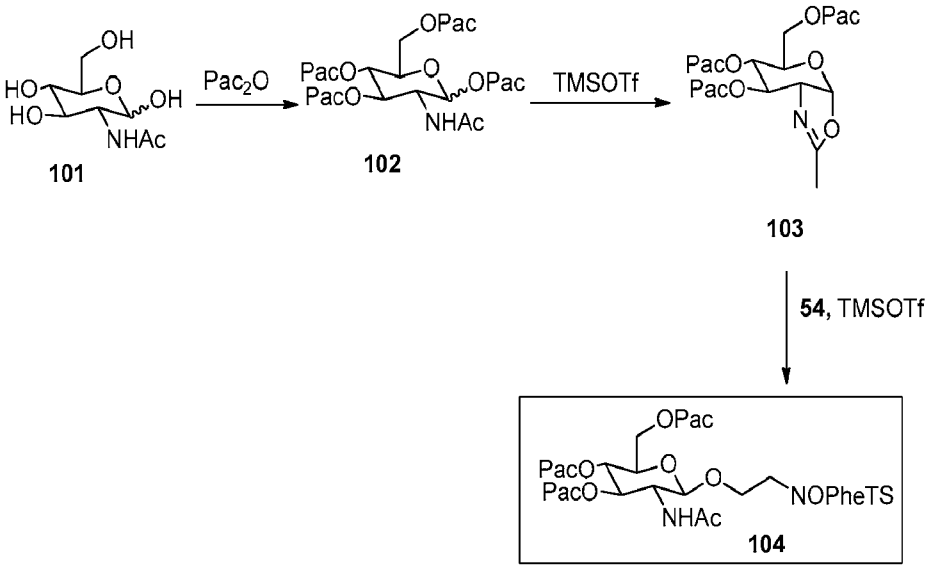
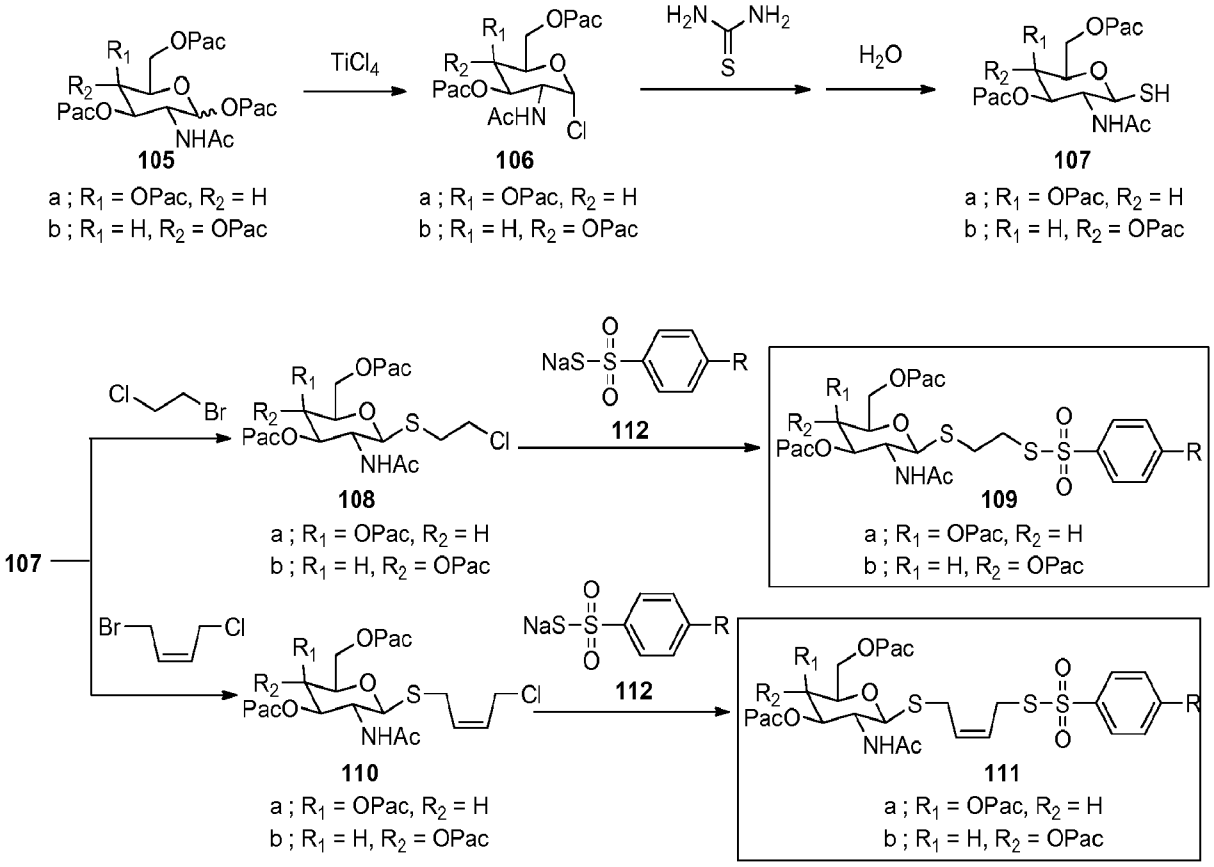


[0085] **Compound 102:** Using a procedure analogous to that described for compound **98**, and substituting compound **101** for compound **97**, pure compound **102** is obtained.

[0086] **Compound 103** Using a procedure analogous to that described for compound **99**, and substituting compound **102** for compound **98**, pure compound **103** is obtained.

[0087] **Compound 104** Using a procedure analogous to that described for compound **100**, and substituting compound **103** for compound **99**, pure compound **103** is obtained.

Synthesis of Compounds 109 and 111



[0088] **Compound 106:** A solution of compound **105** (100 mmol) in dry 1,2-dichloroethane (300 mL) is treated dropwise via syringe with $TiCl_4$ (110 mmol) over 2 min. After refluxing for 16 h , TLC shows complete reaction. The crude is diluted with DCM and washed with sat. $NaHCO_3$. The combined organic layers are dried with Na_2SO_4, filtered, concentrated and used for next reaction without further purification.

[0089] **Compound 107:** Thiourea (150 mmol) and compound **106** (100 mmol) are dissolved in acetone (200 mL) under Ar. The reaction mixture is heated to 60 °C and stirred. After 2 h, a white solid precipitation is removed by filtration. The precipitate is recrystallized. The filtered crystals and $Na_2S_2O_5$ (140 mmol) are added to a stirred mixture of DCM (500 mL) and H_2O (250 mL). The reaction mixture is heated to reflux under Ar. After 3 h, the reaction mixture is cooled to r.t. and the phases are separated. The aqueous layer is back extracted with DCM. The combined organic layers are dried over Na_2SO_4, filtered and concentrated to give compound **107**.

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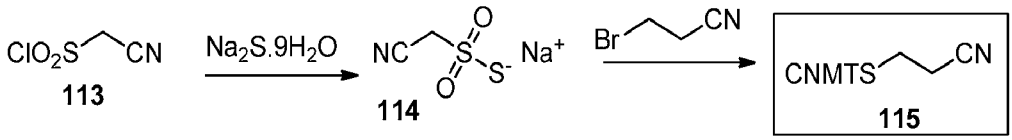
[0090] **Compound 108:** 1-Bromo-2-chloroethane (100 mmol), triethylamine (200 mmol) and compound **107** (100 mmol) are dissolved in acetonitrile (200 mL) under Ar. The reaction mixture is heated to 60 °C and stirred. TLC shows completed reaction. The mixture is diluted with DCM (500 mL) and washed with $NaHCO_3$ (250 mL). The organic layer is dried over Na_2SO_4, filtered, concentrated and purified to give compound **108**.

[0091] **Compound 109:** compound **112** (120 mmol), sodium iodide (10.0 mmol) and compound **108** (100 mmol) are dissolved in MeOH (200 mL) under Ar. The reaction mixture is heated to 60 °C and stirred. TLC shows completed reaction. The mixture is diluted with DCM (500 mL) and washed with $NaHCO_3$ (250 mL). The organic layer is dried over Na_2SO_4, filtered, concentrated and purified to give compound **109**.

[0092] **Compound 110:** 1-Bromo-4-chloro-2,3-*cis*-butene (100 mmol), triethylamine (200 mmol) and compound **107** (100 mmol) are dissolved in acetonitrile (200 mL) under Ar. The reaction mixture is heated to 60 °C. TLC shows reaction is completed. The mixture is diluted with DCM (500 mL) and washed with $NaHCO_3$ (250 mL). The organic layer is dried over Na_2SO_4, filtered, concentrated and purified to give compound **110**.

[0093] **Compound 111:** compound **112** (120 mmol), sodium iodide (10.0 mmol) and compound **110** (100 mmol) are dissolved in MeOH (200 mL) under Ar. The reaction mixture is heated to 60 °C. TLC shows completed reaction. The mixture is diluted with DCM (500 mL) and washed with $NaHCO_3$ (250 mL). The organic layer is dried over Na_2SO_4, filtered, concentrated and purified to give compound **111**.

Synthesis of compound 115



[0094] **Compound 113:** 2-Chlorosulfonylacetonitrile (**113**) is prepared following the procedure of Sammes (as described in patent GB1252903).

[0095] **Compound 114:** Sodium sulfide nonahydrate (65 mmol) is taken up in 100 mL water. The flask is kept in a water bath. Compound **113** (50 mmol) is added dropwise to the solution while stirring and gently warming the water bath. Sulfur is observed to appear and then

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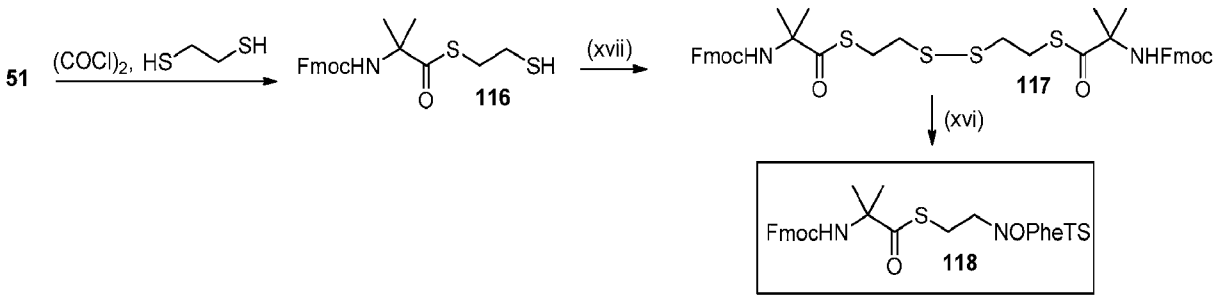
disappear in the flask. Solvent is evaporated under vacuum and the residue is recrystallized from ethanol. Sodium cyanomethanesulfonothioate (compound **114**) is thus obtained as a colorless crystalline solid.

[0096] **Comopund 115:** Compound **114** (13.69 g, 86 mmol) is taken up, with stirring, in anhydrous DMF (30 mL) in a 100 mL round bottom flask. Then, 3-bromopropionitrile (5 mL, 8.2 g, 61.2 mmol) is added and the resulting mixture is stirred for 18 h at 50 °C with monitoring by TLC. On completion of reaction (complete consumption of 3-bromopropionitrile), the reaction mixture is diluted with EtOAc (100 mL) and the organic layer is washed with 5 × 20 mL H_2O. The organic separated organic layer is then dried ($MgSO_4$), filtered and reduced to an oil by rotary evaporation. The crude oil is purified by column chromatography using an EtOAc/hexane gradient and the fractions containing pure material are collected and the solvent is removed by rotary evaporation then further drying *in vacuo* to furnish the pure compound **115** as a colorless oil.

Synthesis of compound 118



[0097] **Compound 116:** Compound **51** (2.9 g, 8.91 mmol) in DCM (50 mL) was treated dropwise with oxalyl dichloride (0.53 ml, 6.15 mmol) then DMF (10 μL) and stirred at r.t. During the reaction a colorless homogeneous solution was formed. After 2 h at r.t. the solvent was removed under reduced pressure. The white residue was redissolved in DCM (20 mL) then added dropwise to a stirring pyridine (10 mL) solution of ethane-1,2-dithiol (8.40 g, 89 mmol), with monitoring by UPLC/MS. After 18 h, it was judged that the reaction was complete. The solvents were removed and the remaining ethanethiol was removed by trap-to-trap distillation then the residue was subjected to purification by column chromatography with DCM to provide the pure compound **116** as a colorless solid. Yield was 1.82 g, 51 %. MS (ESI +): calc $(M+Na)^+$:

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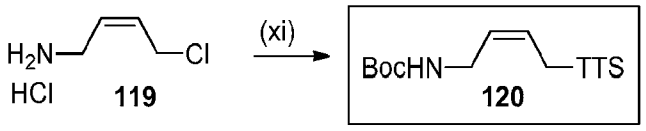
424.10, found: 424.06. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.79 (d, J = 7.5 Hz, 2H), 7.63 (d, J = 5.7 Hz, 2H), 7.43 (t, J = 7.5 Hz, 2H), 7.34 (t, J = 7.4 Hz, 2H), 5.40 – 5.15 (br, 1H), 4.60 – 4.35 (br, 2H), 4.31 – 4.19 (m, 1H), 3.15 – 3.04 (br, 2H), 2.95 – 2.80 (m, 1H), 2.75 – 2.60 (br, 2H), 1.72 – 1.43 (m, 6H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 203.14, 154.75, 143.92, 141.49, 127.84, 127.19, 125.17, 120.13, 66.81, 62.69, 47.41, 33.01, 25.78, 24.60.

[0098] **Compound 117**: To a stirred solution of compound **116** (1.7 g, 4.23 mmol) in EtOAc (12 mL) was added sodium iodide (6.35 mg, 0.042 mmol) and hydrogen peroxide (0.144 g, 4.23 mmol) (0.45 mL of a 30% aqueous solution) and the mixture was stirred at r.t. for 15 min. TLC showed complete consumption of the starting material. Aqueous sodium thiosulfate was added until the solution became colorless. The solution was washed with water, dried ($MgSO_4$), then column chromatography (EtOAc/hexane), gave pure compound **117** as a colorless solid foam (1.45 g, 86%). MS (ESI +): calc $(M+H)^+$: 802.07, found: 802.08. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.78 (d, J = 7.6 Hz, 4H), 7.63 (d, J = 5.8 Hz, 4H), 7.42 (t, J = 7.5 Hz, 4H), 7.33 (t, J = 7.5 Hz, 4H), 5.42 – 5.25 (br, 2H), 4.55 – 4.35 (br, 4H), 4.30 – 4.18 (br, 2H), 3.25 – 3.10 (br, 4H), 2.95 – 2.70 (br, 4H), 1.65 – 1.45 (br, 12H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 203.19, 154.71, 143.90, 141.45, 127.80, 127.17, 125.16, 120.10, 66.76, 62.65, 47.37, 37.90, 28.56, 25.73.

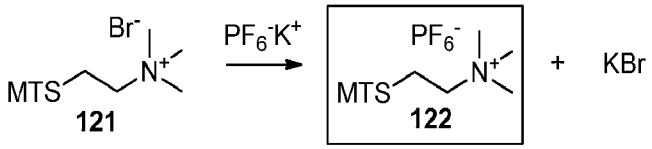
[0099] **Compound 118**: Dibromine (0.091 ml, 1.77 mmol) was added in a dropwise fashion over 2 min to a stirring mixture of sodium 4-nitrobenzenesulfinate (0.742 g, 3.55 mmol) and compound **117** (1.42 g, 1.773 mmol) in DCM (10 mL) at r.t. After 30 min stirring at r.t., the mixture was filtered and the filtrate was reduced to an orange solid foam in vacuo. Column chromatography (DCM/hexane) gave pure compound **118** (1.26 g, 60 %) as a pale yellow solid foam. MS (ESI +): calc $(M+H)^+$: 587.71, found: 802.08. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.36 (d, J = 8.2 Hz, 2H), 8.14 (d, J = 7.3 Hz, 2H), 7.79 (d, J = 7.0 Hz, 2H), 7.63 (s, 2H), 7.43 (t, J = 6.7 Hz, 2H), 7.35 (d, J = 6.8 Hz, 2H), 5.35 – 5.15 (br, 1H), 4.55 – 4.35 (br, 2H), 4.30 – 4.20 (br, 1H), 3.30 – 3.00 (br, 4H), 1.70 – 1.25 (br, 6H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 202.71, 154.66, 150.60, 149.63, 143.78, 141.46, 128.40, 127.91, 127.21, 125.12, 124.81, 120.18, 66.86, 62.59, 47.35, 35.84, 28.42, 25.61.

Synthesis of compound 120



[00100] **Compound 120:** Commercially available compound **119** (1 g, 7.04 mmol) and potassium 4-methylbenzenesulfonothioate (1.753 g, 7.75 mmol) were stirred in MeOH (10 mL) over 5 days at r.t. then at 40 °C over 24 h. TLC showed good conversion to product. The MeOH was evaporated and the residue was taken up in DCM (30 mL) and Boc_2O (1.54 g, 7.04 mmol) was added. The mixture was treated with triethylamine (1.030 ml, 7.39 mmol) in a dropwise fashion over 10 min at r.t. until the solution became practically homogeneous. Washing with water (50 mL), drying ($MgSO_4$), filtration and evaporation gave the crude product which was further purified by column chromatography to give pure compound **120** (1.64 g, 65 %) as a colorless solid. MS (ESI +): calc $(M+Na)^+$: 380.10, found: 380.11. ^{1}H NMR (399 MHz, $CDCl_3$) δ 7.80 (d, J = 8.2 Hz, 2H), 7.34 (dd, J = 8.5, 0.8 Hz, 2H), 5.54 (ddt, J = 24.7, 16.6, 7.9 Hz, 2H), 4.65 (s, 1H), 3.77 – 3.66 (m, 4H), 2.45 (s, 3H), 1.43 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.83, 145.05, 142.06, 131.80, 130.01, 127.15, 124.55, 79.72, 37.29, 32.75, 28.51, 21.79.

Synthesis of compound 122



[00101] **Compound 122**: A solution of commercially available compound **121** (1 g, 3.59 mmol) in water (1 mL) was treated all at once with a solution of potassium hexafluorophosphate (0.662 g, 3.59 mmol) in water (10 mL) and agitated by shaking at r.t. for 5 min. The resulting solid was collected by filtration, washed with 2 × 3mL water and dried in vacuo over KOH to give pure compound **122** as a colorless solid (1.01 g, 82 %). ^{1}H NMR (300 MHz, CD_3CN) δ 3.67 – 3.60 (m, 2H), 3.49 (s, 3H), 3.53 – 3.45 (m, 2H), 3.10 (s, 9H); ^{13}C NMR (126 MHz, CD_3CN) δ 65.98, 54.05 (q, J = 4.1 Hz), 51.11, 28.99; ^{19}F NMR (376 MHz, CD_3CN) δ -73.15 (d, J = 706.5 Hz); ^{31}P NMR (162 MHz, CD_3CN) δ -143.50 (hept, J = 706.6 Hz).

Synthesis of compound 125

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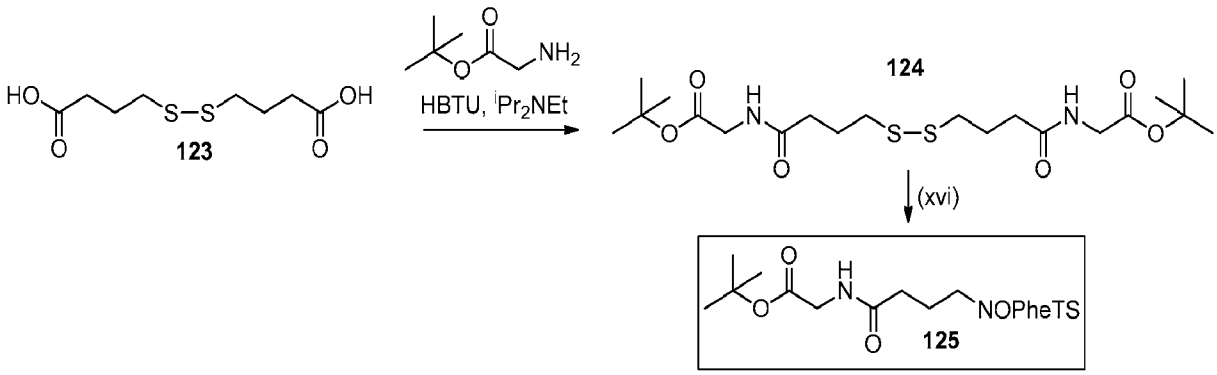
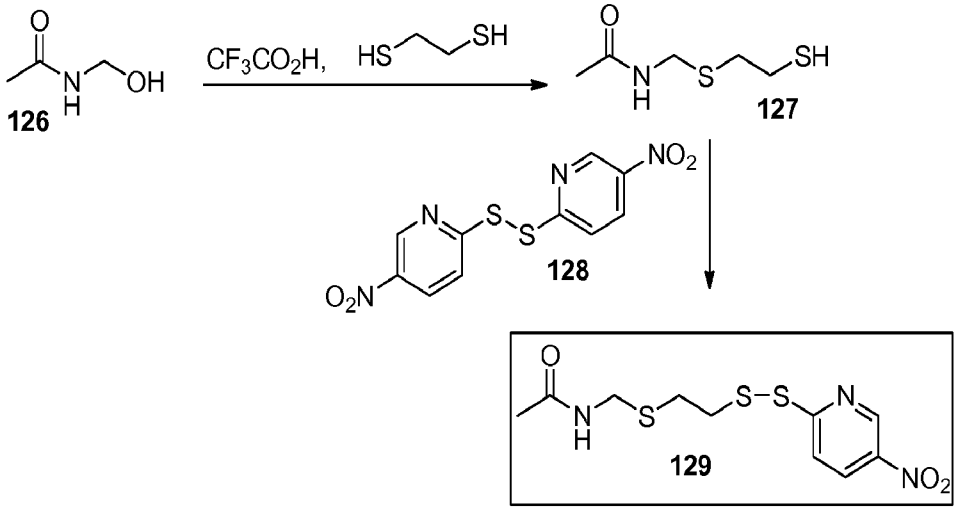


[00102] **Compound 124:** A DMF (50 mL) solution of commercially available compound **123** (10 mmol) is treated successively with *tert*-butyl-2-aminoacetate (22 mmol), di-isopropylethylamine (50 mmol) and HBTU (24 mmol). After 1 h stirring at r.t., water (100 mL) is added and the resulting solution is extracted with EtOAc (2 × 50 mL) and washed with 5 % $NaHCO_3$ (2 × 25 mL) then dilute brine (2 × 25 mL), dried ($MgSO_4$), filtered and reduced by rotary evaporation. Purification by column chromatography provides the pure compound **124**.

[00103] **Compound 125:** Dibromine (0.091 ml, 1.77 mmol) is added in a dropwise fashion over 2 min to a stirring mixture of sodium 4-nitrobenzenesulfinate (0.742 g, 3.55 mmol) and di-*tert*-butyl-2,2'-((4,4'-disulfanediyl-bis(butanoyl))bis(azanediyl))diacetate (824 mg, 1.77 mmol) in DCM (10 mL) at r.t. After 30 min stirring at r.t., the mixture is filtered and the filtrate is reduced to a solid foam *in vacuo*. Column chromatography gives the pure product as a solid foam.

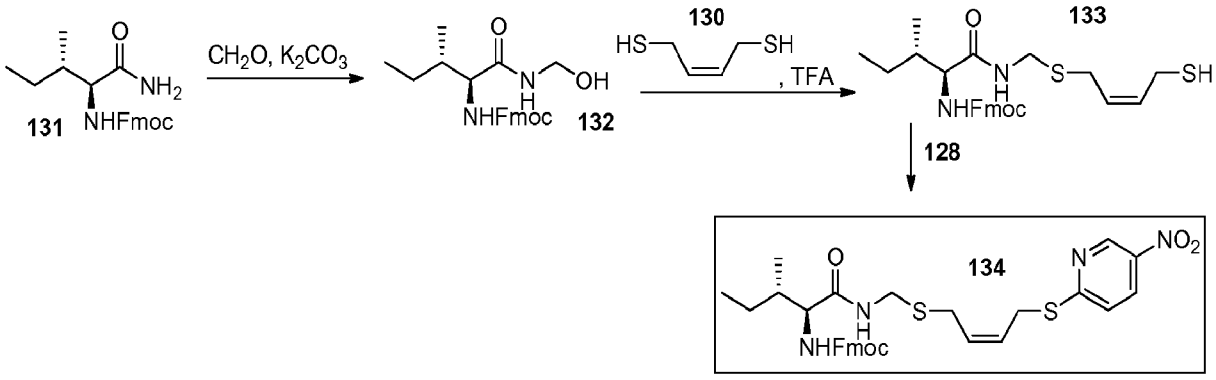
Synthesis of compound 129



[00104] **Compound 127:** A solution of ethane-1,2-dithiol (1.130 g, 12 mmol) in TFA (10 mL) was treated all at once with commercially available compound **126** (0.89 g, 9.99 mmol). The solution became warm. After 1 h stirring at r.t. the volatiles were removed and the residue was subjected to column chromatography to give pure compound **127** as a colorless oil (0.95 g, 48 %). R_f = 0.6 (5%MeOH/DCM); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.09 – 5.93 (br, 1H), 4.41 (d, *J* = 6.4 Hz, 2H), 2.87 – 2.83 (m, 2H), 2.79 (ddd, *J* = 9.7, 7.3, 3.5 Hz, 2H), 2.04 (s, 3H), 1.71 (t, *J* = 8.0 Hz, 1H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.25, 40.89, 35.22, 24.91, 23.44.

[00105] **Compound 129**: A warm (35 °C) DMF (10 mL) solution of commercially available compound **128** (939 mg, 3.0 mmol) was treated in a dropwise fashion over 5 min with a DCM (5 mL) solution of compound **127** (250 mg, 1.513 mmol). After checking TLC, the solvents were removed by rotary evaporation under high vacuum then the residue was triturated with DCM (50 mL), filtered, and the filtrate was washed with 5 × 5% $NaHCO_3$, then was dried ($MgSO_4$), filtered and reduced to a pale yellow solid. Column chromatography gave pure compound **129** as a pale yellow solid. MS (ESI +): calc $(M+H)^+$: 320.02, found: 320.34; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.28 (dd, *J* = 2.7, 0.7 Hz, 1H), 8.43 (dd, *J* = 8.9, 2.6 Hz, 1H), 7.93 (dd, *J* = 8.9, 0.7 Hz, 1H), 6.10 – 5.90 (br, 1H), 4.41 (d, *J* = 6.5 Hz, 2H), 3.13 (dd, *J* = 8.6, 6.3 Hz, 2H), 2.93 (dd, *J* = 8.6, 6.4 Hz, 2H), 2.00 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.18, 168.57, 145.22, 142.22, 131.81, 119.59, 40.65, 38.59, 30.07, 23.39.

Synthesis of compound 134



[00106] **Compound 130:** Compound **130** is prepared by a previously described method (Heterocycles, Vol 54, p 139, 2001).

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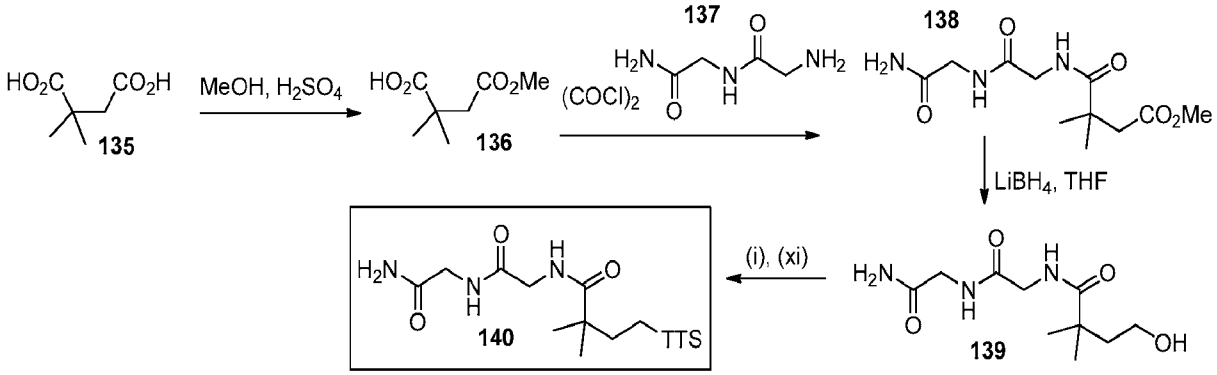
[00107] **Compound 132:** Using an analogous procedure described previously for the synthesis of compound **126** (Organic Syntheses, Coll. Vol. 6, p.5 (1988)), compound **131** (Tetrahedron Letters, 48(39), 7038-7041; 2007) is converted to compound **132** by gently heating in the presence of an aqueous formaldehyde solution containing K_2CO_3.

[00108] **Compound 133:** Using a procedure analogous to that described for the synthesis of compound **127** and substituting compound **132** for compound **126** and compound **130** for ethane-1,2-dithiol, pure compound **133** is thus obtained.

[00109] **Compound 134**: Using a procedure analogous to that described for the synthesis of compound **129** and substituting compound **133** for compound **127**, pure compound **134** is thus obtained.

Synthesis of compound 140



[00110] **Compound 136:** An ice-cold solution of commercially available compound **135** (50 g, 342 mmol), and methanol (277 mL) was treated dropwise with sulfuric acid (3.36 g, 34.2 mmol) over 10 min then warmed to r.t. gradually overnight. Most of the solvent was removed by rotary evaporation, then saturated aqueous $NaHCO_3$ was carefully added. The pH of the solution was adjusted to 1.9 by addition of 1 M HCl then extracted into EtOAc (200 mL), washed with diluted brine (50 mL), dried ($MgSO_4$), filtered and reduced to 29 g of colorless oil. Column chromatography gave pure compound **136** as a colorless solid (12.5 g, 23 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ 12.5 – 10.5 (vbr, 1H), 3.69 (s, 3H), 2.63 (s, 2H), 1.32 (s, 6H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 183.32, 171.76, 51.72, 43.88, 40.60, 25.34.

[00111] **Synthesis of compound 138:** Compound **136** (10 mmol) in DCM (50 mL) is treated dropwise with oxalyl dichloride (10 mmol) then DMF (10 μL) and stirred at r.t. During

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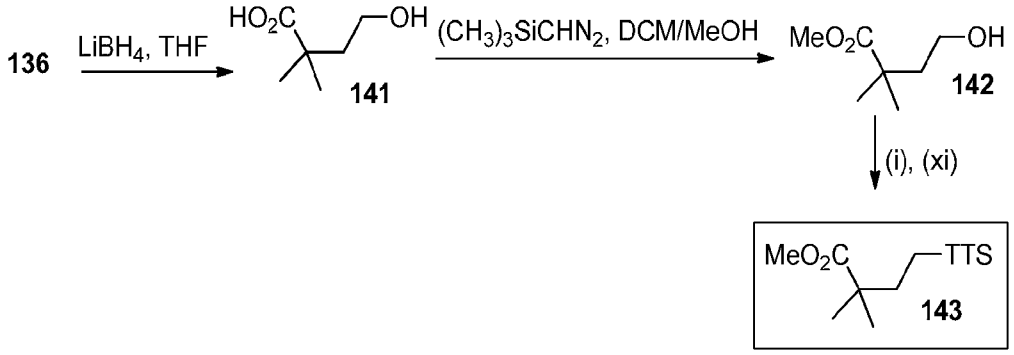
the reaction a colorless homogeneous solution is formed. After 2 h at r.t. the solvent is removed under reduced pressure. The white residue is redissolved in DCM (20 mL) then added dropwise to a stirring pyridine (10 mL) solution of compound **137** (10 mmol), with monitoring by TLC. After 18 h, the solvents are removed and the residue is subjected to purification by column chromatography with DCM to provide the pure compound **137.**

[00112] **Compound 139:** Compound **138** (5 mmol) as a solution in THF (20 mL) is added to an ice-cold solution of $LiBH_4$ (5 mmol) in THF (20 mL). Progress of the reaction at r.t. is judged by TLC. After completion of the reaction (complete consumption of starting material), water (100 mL) is added carefully to the ice-cold solution which is subsequently extracted with EtOAc (2 × 100 mL). The combined organic extracts are dried ($MgSO_4$), filtered and the solvents are removed by rotary evaporation. The residue is subjected to column chromatography the pure compound **139** is thus obtained.

[00113] **Compound 140:** Using a procedure analogous to that described for the synthesis of compound **80**, and substituting compound **139** for compound **79**, pure compound **140** is thus obtained.

Synthesis of compound 143



[00114] **Compound 139**: Using a procedure analogous to that described for the synthesis of compound **139** and substituting compound **136** for compound **138**, pure compound **141** is thus obtained.

[00115] **Compound 142:** A solution of compound **141** (8.39 mmol) in DCM/MeOH (12/3 mL) is treated dropwise at 0 °C with a 2 M solution of (diazomethyl)trimethylsilane (1.05 g, 9.23 mmol) in ether over 30 min. After quenching excess reagent with AcOH, washing with water (2

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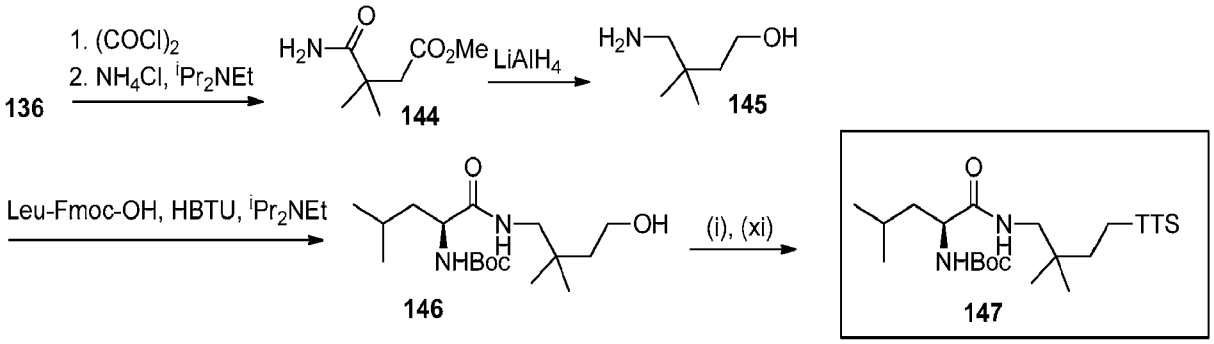
× 5 mL), drying ($MgSO_4$), filtration, removal of solvent by rotary evaporation, then column chromatography the pure compound **142** is obtained.

[00116] **Compound 143:** Using a procedure analogous to that described for the synthesis of compound **80**, and substituting compound **142** for compound **79**, pure compound **143** is thus obtained.

Synthesis of compound 147



[00117] **Compound 144:** Using a procedure analogous to that described for the synthesis of compound **69a**, and substituting compound **136** for compound **67**, and compound **54** for NH_4Cl plus iPr_2NEt, pure compound **144** is thus obtained.

[00118] **Compound 145:** $LiAlH_4$ (100 mL of a 2 M solution in THF) is added in a dropwise fashion to an ice-cold THF (500 mL) solution of compound **144** (86 mmol). The mixture is warmed to r.t. then refluxed for 0.5 h. The residual $LiAlH_4$ is destroyed by careful addition of saturated Na_2SO_4 to the ice-cold solution until a granular precipitate is formed. The mixture is filtered and reduced then redissolved in EtOAc, dried (MgSO4), filtered and reduced *in vacuo* to furnish compound **145** which is sufficiently pure for direct use in the next step of the reaction.

[00119] **Compound 146:** Using a procedure analogous to that described for the synthesis of compound **124**, and substituting compound **145** for compound **123**, and Leu-Fmoc-OH for *tert*-butyl 2-aminoacetate, pure compound **146** is thus obtained.

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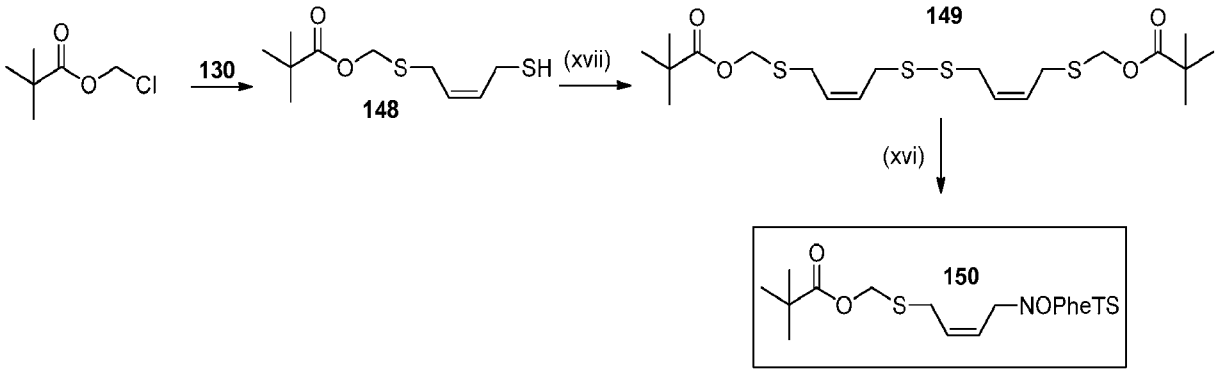
[00120] **Compound 147:** Using a procedure analogous to that described for the synthesis of compound **80**, and substituting compound **146** for compound **79**, and compound **114** for potassium 4-methylbenzenesulfonothioate, pure compound **147** is thus obtained.

Synthesis of compound 150



[00121] **Compound 148:** Commercially available chloromethyl pivalate (10 mmol) is added in a dropwise fashion to a stirring solution of compound **130** (20 mmol) in 1,2-dichloroethane (100 mL) under Ar. The resulting solution is heated in order to force to completion (noted by disappearance of chloromethylpivalate material by TLC). Solvent is removed by rotary evaporation, then the residue is subjected to column chromatography to provide the pure compound **148**.

[00122] **Compound 149:** Using a procedure analogous to that described for the synthesis of compound **89**, and substituting compound **148** for compound **88**, pure compound **149** is thus obtained.

[00123] **Compound 150:** Using a procedure analogous to that described for the synthesis of compound **87**, and substituting compound **149** for compound **86**, pure compound **150** is thus obtained.

[00124] **Additional sulfurization reagents:**

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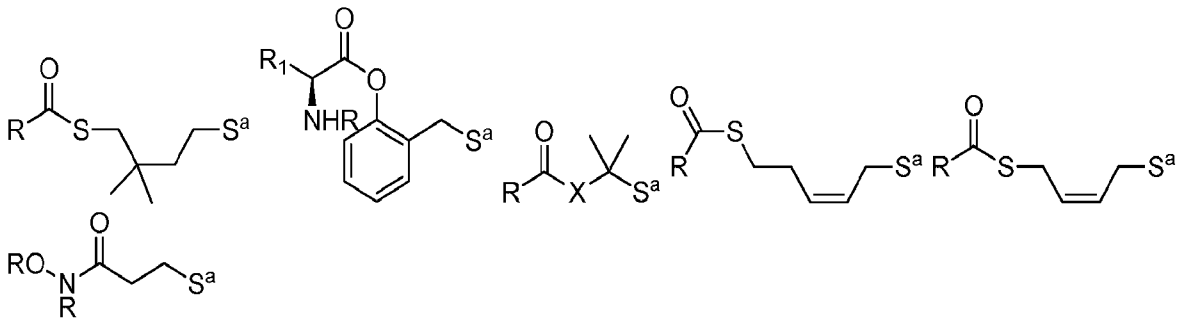
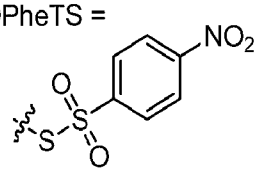
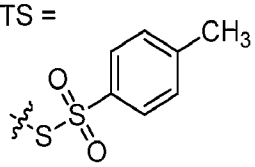


wherein S^a denotes any of the following structures:

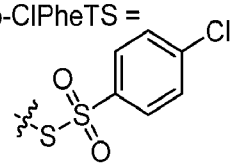
NOPheTS =



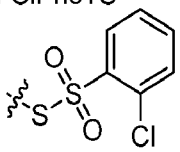
TTS =



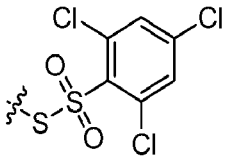
p-ClPheTS =



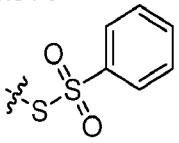
m-ClPheTS =



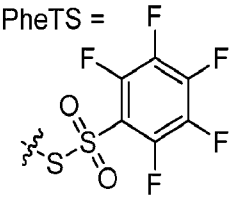
2,4,6-TriClPheTS =



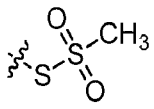
PheTS =



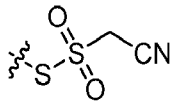
PFPheTS =



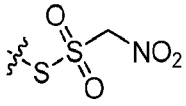
MTS =



α–CNMTS =



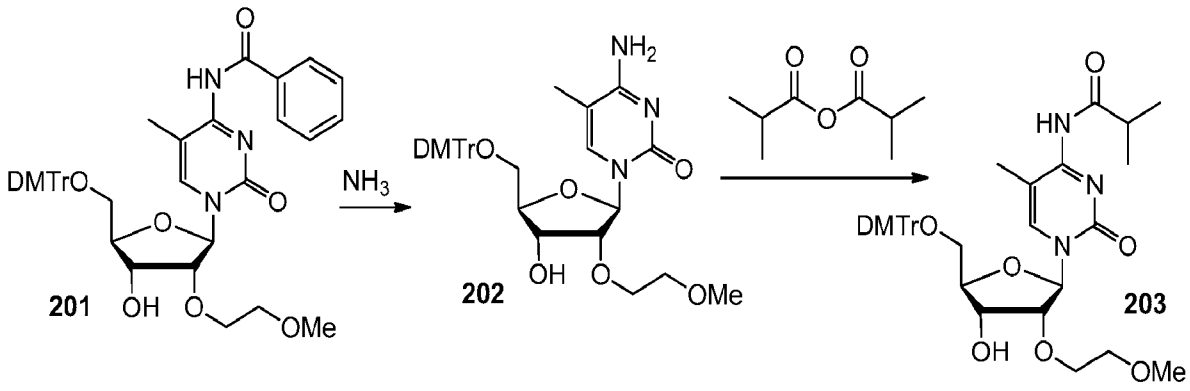
α–NO2MTS =



Synthesis of Phosphoramidites

[00125] In some embodiments, the present invention provides phosphoramidites, and methods of making the same. In some embodiments, the provided phosphoramidites were used in the synthesis of oligonucleotides described in the present application. Exemplary phosphoramidites and their synthesis are described below.

Synthesis of compound 203



[00126] **Compound 202:** N^4-Bz-5′-*O*-DMTr-2′-*O*-MOE-5-methylcytidine (**201**) (2.00 g, 2.77 mmol) was treated with 2 M ammonia in 2-propanol (Aldrich, anhydrous, 45 mL) and Pyridine (22.5 mL) at 60 °C for 5 h then at r.t. overnight, with monitoring by TLC and UPLC/MS. Solvent was removed (3× azeotrope with toluene) then column chromatography (0 – 10% MeOH/DCM) gave the pure compound **202** (1.62 g, 95 %) as a colorless solid which was homogeneous by TLC and HPLC. (ESI +): calc $(M+H)^+$: 618.28, found: 618.53. ^{1}H NMR (399 MHz, $CDCl_3$) δ 7.82 (d, *J* = 1.3 Hz, 1H), 7.47 – 7.40 (m, 2H), 7.37 – 7.18 (m, 8H), 6.83 (dd, *J* = 8.9, 1.7 Hz, 4H), 5.93 (d, *J* = 1.1 Hz, 1H), 4.43 (td, *J* = 8.3, 4.9 Hz, 1H), 4.24 (ddd, *J* = 11.7, 5.1, 3.0 Hz, 1H), 4.07 (dt, *J* = 8.6, 2.4 Hz, 1H), 4.03 – 3.98 (m, 1H), 3.90 (ddd, *J* = 11.7, 6.3, 3.3 Hz, 1H), 3.79 (d, *J* = 1.0 Hz, 6H), 3.63 – 3.52 (m, 3H), 3.44 (dd, *J* = 11.0, 3.0 Hz, 1H), 3.37 (s, 3H), 3.32 (d, *J* = 9.4 Hz, 1H), 1.80 – 1.64 (s, br, 1H), 1.13 (s, 3H).

[00127] **Compound 203:** Isobutyric anhydride (0.48 ml, 2.9 mmol) was added dropwise to a solution of compound **202** (1.61 g, 2.61 mmol) in DMF (13 ml) at r.t. The solution was allowed to stir at r.t. over 24 h then the solvent was removed in vacuo at 35 °C. Extraction into ether/bicarbonate then drying ($MgSO_4$), filtration and removal of the solvent gave the crude product as a colorless solid foam. Column chromatography (0 – 4% MeOH/DCM) gave the pure product (1.75 g, 98 %) as a colorless solid foam which was homogenous by TLC and HPLC. (ESI +): calc $(M+H)^+$: 688.32, found: 688.56. ^{1}H NMR (399 MHz, $CDCl_3$) δ 8.00 – 7.75 (br, 1H), 7.45 – 7.39 (m, 2H), 7.34 – 7.20 (m, 8H), 6.84 (dd, *J* = 9.0, 1.2 Hz, 4H), 5.97 (s, 1H), 4.43 (d, *J* = 6.1 Hz, 1H), 4.20 – 4.04 (m, 3H), 3.79 (d, *J* = 0.9 Hz, 6H), 3.64 – 3.58 (m, 1H), 3.64 – 3.52 (m, 2H), 3.47 – 3.40 (m, 2H), 3.38 (s, 3H), 1.70 – 1.55 (br, 1H), 1.39 (d, *J* = 1.0 Hz, 3H), 1.18 (d, *J* = 6.9 Hz, 6H).

Synthesis of compound 205

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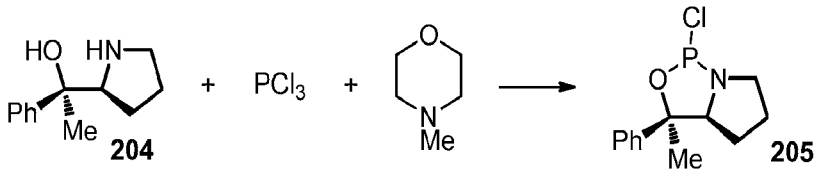
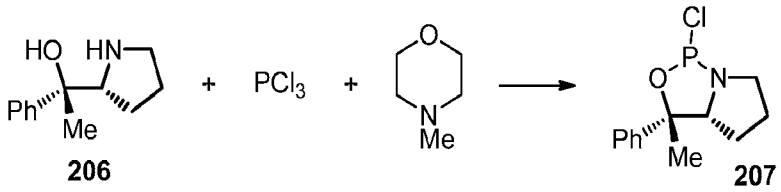


[00128] **Compound 205:** (*R*)-1-phenyl-1-((*S*)-pyrrolidin-2-yl)ethanol (**4**) was dried by azeotropic distillation with toluene (3×3 mL). A solution dried compound **4** (0.725 g, 3.79 mmol) and 4-methylmorpholine (0.833 ml, 7.58 mmol) in toluene (5 mL) was added to an ice-cold solution of trichlorophosphine (0.331 ml, 3.79 mmol) in toluene (5 mL) which was then warmed to r.t. for 1 h then filtered under Ar and reduced to an oil which was used in the next step of the reaction without further purification.

Synthesis of compound 207



[00129] **Compound 207:** Using a procedure analogous to that described for the synthesis of compound **205**, and substituting compound **206** for compound **204**, compound **207** was obtained as a crude brown oil and was used in the next step of the reaction without further purification.

Synthesis of compound 208

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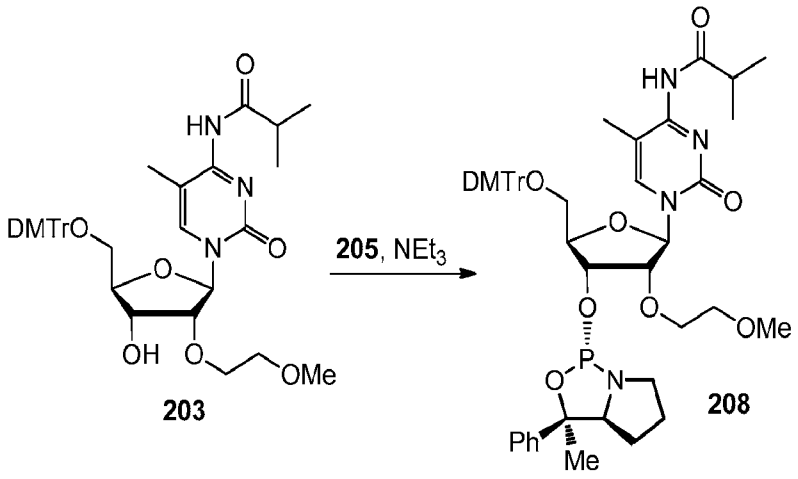

[00130] **Compound 208:** Compound **203** (1.74 g, 2.53 mmol) was dried by co-evaporation with pyridine (3×5 mL) then toluene (5×5 mL). The resulting dried **203** was dissolved in THF (15 mL), then triethylamine (2.47 ml, 17.7 mmol) was added and the solution was cooled to -78 °C by means of CO_2(s)/acetone cooling bath. A THF solution (15 mL) of the crude compound **205** (3.79 mmol) was added dropwise over 0.5 h then gradually warmed to r.t. After 1 h at r.t., TLC indicated complete conversion of compound **203** to product. The mixture was washed into a separation funnel with chloroform (100 mL) then was extracted with $NaHCO_3$ (saturated, aqueous, 50 mL then 2×25 mL). At each extraction, the aqueous extracts were washed with an additional 1×10 mL of chloroform. The combined chloroform extracts were dried ($MgSO_4$), filtered, and concentrated by rotary evaporation at 32 °C. The crude solid thus obtained was redissolved in DCM containing a few drops of triethylamine then subjected to column chromatography with a hexane/EtOAc gradient containing a steady concentration of 2% triethylamine to give pure compound **208** as a white solid foam. ^{1}H NMR (399 MHz, CD_3CN) δ 7.92 – 7.80 (s, 1H), 7.52 – 7.47 (m, 2H), 7.40 – 7.24 (m, 12H), 6.88 (dd, J = 9.0, 1.1 Hz, 4H), 5.94 (d, J = 3.9 Hz, 1H), 4.79 (dt, J = 9.6, 5.6 Hz, 1H), 4.31 – 4.27 (m, 1H), 4.27 – 4.22 (m, 1H), 3.87 (t, J = 4.7 Hz, 2H), 3.75 (d, J = 2.0 Hz, 6H), 3.66 (td, J = 5.9, 5.5, 2.2 Hz, 1H), 3.58 – 3.53 (m, 2H), 3.50 (dd, J = 11.1, 2.4 Hz, 1H), 3.38 (dd, J = 11.0, 3.3 Hz, 1H), 3.31 (s, 3H), 2.85 (dq, J = 10.5, 7.4, 6.9 Hz, 2H), 1.73 (s, 3H), 1.52 (d, J = 1.0 Hz, 3H), 1.47 – 1.32 (m, 2H), 1.17 (dd, J = 6.9, 0.7 Hz, 6H), 1.05 – 0.90 (m, 2H); ^{31}P NMR (162 MHz, CD_3CN) δ 155.35.

Synthesis of compound 210

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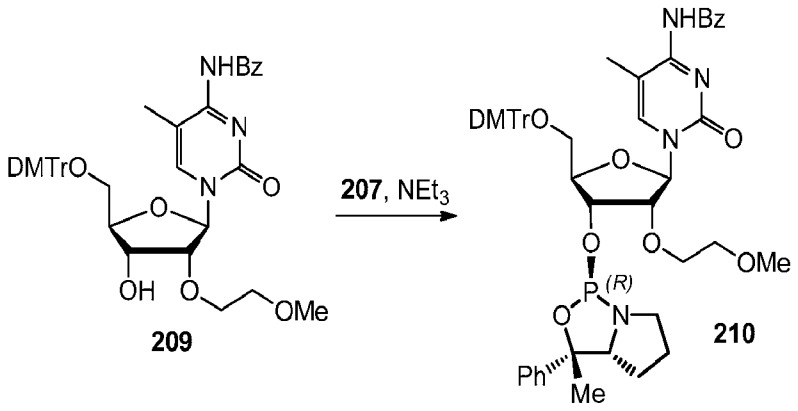
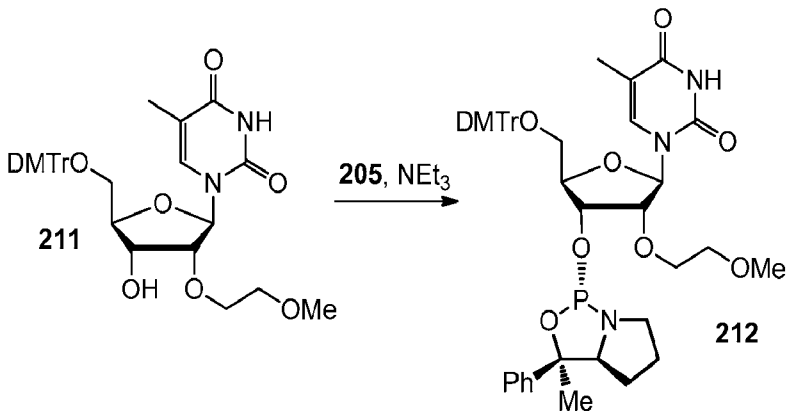


[00131] **Compound 210:** Using a procedure analogous to that described for compound **208** and substituting compound **209** for compound **203** and compound **207** for compound **205**, pure compound **210** was obtained as a colorless solid foam. ^{1}H NMR (399 MHz, CD_3CN) δ 8.31 – 8.27 (m, 2H), 7.86 (d, *J* = 1.2 Hz, 1H), 7.62 – 7.55 (m, 1H), 7.55 – 7.45 (m, 5H), 7.44 – 7.24 (m, 12H), 6.94 – 6.90 (m, 4H), 5.96 (d, *J* = 3.7 Hz, 1H), 4.86 – 4.76 (m, 1H), 4.29 (dd, *J* = 5.0, 3.8 Hz, 1H), 4.23 (dt, *J* = 5.8, 2.8 Hz, 1H), 3.92 – 3.80 (m, 2H), 3.78 (s, 6H), 3.74 (ddd, *J* = 7.1, 5.3, 2.1 Hz, 1H), 3.55 (t, *J* = 4.7 Hz, 2H), 3.51 (d, *J* = 2.3 Hz, 1H), 3.41 (dd, *J* = 11.1, 3.4 Hz, 1H), 3.30 (s, 3H), 3.26 (ddd, *J* = 10.3, 6.1, 2.2 Hz, 1H), 2.86 (ddt, *J* = 10.1, 8.2, 7.2 Hz, 1H), 1.72 (d, *J* = 0.7 Hz, 3H), 1.62 (d, *J* = 1.1 Hz, 3H), 1.56 – 1.41 (m, 2H), 1.09 – 0.87 (m, 2H); ^{31}P NMR (162 MHz, CD_3CN) δ 155.22.

Synthesis of compound 212



[00132] **Compound 212:** Using a procedure analogous to that described for compound **208** and substituting compound **211** for compound **203**, pure compound **212** was obtained as a colorless solid foam. ^{1}H NMR (399 MHz, $CDCl_3$) δ 9.25 – 8.70 (br, 1H), 7.69 (d, *J* = 1.3 Hz, 1H), 7.46 – 7.40 (m, 2H), 7.40 – 7.17 (m, 12H), 6.81 (dd, *J* = 9.0, 2.5 Hz, 4H), 6.09 (d, *J* = 4.3

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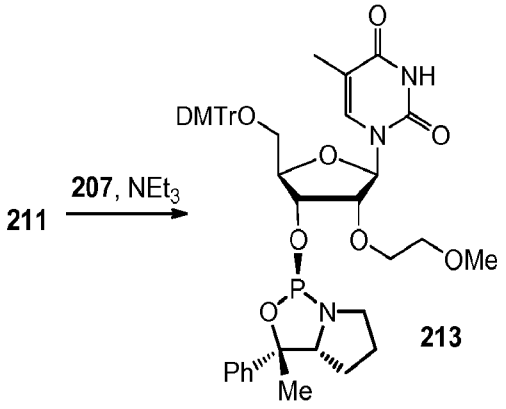
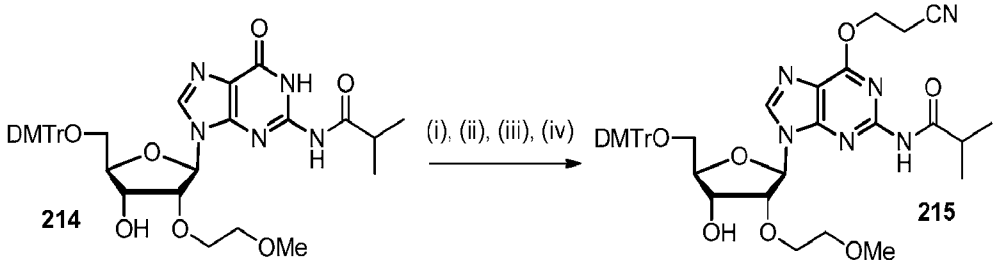
Hz, 1H), 4.78 (dt, J = 9.6, 5.2 Hz, 1H), 4.36 – 4.26 (m, 2H), 3.94 – 3.88 (m, 2H), 3.74 (dd, J = 4.0, 0.9 Hz, 6H), 3.69 (td, J = 6.4, 2.9 Hz, 1H), 3.64 – 3.56 (m, 3H), 3.44 – 3.37 (m, 2H), 3.36 (d, J = 0.9 Hz, 3H), 3.00 (dtd, J = 10.3, 7.9, 6.4 Hz, 1H), 1.72 (s, 3H), 1.54 – 1.44 (m, 1H), 1.38 (dd, J = 6.8, 5.4 Hz, 1H), 1.34 (d, J = 1.2 Hz, 3H), 1.23 – 1.13 (m, 1H), 0.97 – 0.87 (m, 1H); ^{31}P NMR (162 MHz, $CDCl_3$) δ 158.18.

Synthesis of compound 213



[00133] **Compound 213:** Using a procedure analogous to that described for compound **210** and substituting compound **211** for compound **209**, pure compound **213** was obtained as a colorless solid foam. ^{1}H NMR (399 MHz, $CDCl_3$) δ 9.55 – 9.10 (br, 1H), 8.09 (d, J = 1.5 Hz, 1H), 7.80 – 7.71 (m, 2H), 7.69 – 7.47 (m, 12H), 7.20 – 7.08 (m, 4H), 6.32 (d, J = 2.9 Hz, 1H), 5.20 – 5.11 (m, 1H), 4.66 – 4.59 (m, 1H), 4.49 – 4.39 (m, 1H), 4.33 – 4.24 (m, 1H), 4.16 – 4.02 (m, 8H), 4.00 – 3.93 (m, 1H), 3.91 – 3.84 (m, 2H), 3.73 (dd, J = 11.0, 2.5 Hz, 1H), 3.65 – 3.52 (m, 4H), 3.27 – 3.16 (m, 1H), 2.15 (s, 3H), 1.88 – 1.76 (m, 1H), 1.76 – 1.65 (m, 1H), 1.63 – 1.49 (m, 4H), 1.29 – 1.18 (m, 1H); ^{31}P NMR (162 MHz, $CDCl_3$) δ 160.08.

Synthesis of compound 215



(i) TMSCl, NEt3, 0°C; (ii) 2,4,6-trimethylbenzene-1-sulfonyl chloride, DMAP; (iii) 1-methylpyrrolidine, 3-hydroxypropanenitrile, DBU, 0°C; (iv) MeOH/H2O, 4 d, room temperature

[00134] Compound 215: (i) Transient TMS Protection: A solution of N^2-isobutyryl-5′-*O*-DMTr-2′-*O*-MOE-guanosine (compound **214**) (15.04 g, 21.1 mmol) and triethylamine (11.8 ml, 85 mmol) was taken up in ACN (200 mL) then chlorotrimethylsilane (10.4 ml, 82 mmol) was added dropwise to the ice-cold solution which was stirred at r.t. for 1 h with monitoring by TLC. Filtration and removal of solvent, then extraction (DCM/$NaHCO_3$ 500 mL/200 mL), drying ($MgSO_4$), filtration, and removal of the solvent gave the crude compound which was used in the next step of the reaction without further purification. The compound was homogeneous by TLC (R_f= 0.6, (5% MeOH/DCM)). (ii) Activation of O^6 position for Cyanoethyl protection: The residue was redissolved in DCM (500 mL) then triethylamine (13.1 ml, 94 mmol) was added, followed by 2,4,6-trimethylbenzene-1-sulfonyl chloride (6.15 g, 28.1 mmol) and DMAP (0.14 g, 1.146 mmol). The mixture was stirred at r.t. for 3 h with monitoring by TLC until full disappearance of the starting material and emergence of a new spot by TLC (R_f= 0.9, (5% MeOH/DCM)). (iii) Cyanoethyl protection: 1-methylpyrrolidine (22.43 ml, 211 mmol) was added slowly in a dropwise fashion to the ice-cold solution with further reaction at 0 °C over 1 h, monitoring by TLC (new spot, R_f = 0.2, streak, (5% MeOH/DCM)). 3-hydroxypropanenitrile (14.40 ml, 211.0 mmol) then DBU (6.32 mL, 42.1 mmol) were added in a dropwise fashion successively to the still ice-cold solution and stirring was continued for additional 90 min, monitoring by TLC (new spot, R_f = 0.6, (5% MeOH/DCM)). In order to force the reaction almost to completion, a further 1.6 mL of DBU was added dropwise with careful monitoring by TLC. The mixture was poured onto NaH_2PO_4 (50 g in 400 mL water), the organics were separated, and washed with dilute NaH_2PO_4 (10 g in 200 mL water), dried ($MgSO_4$), filtered and reduced by rotary evaporation. (iv) TMS Deprotection: The residue thus obtained in the previous step was redissolved in MeOH (250 mL) then water was added in a dropwise fashion being careful not to induce precipitation. The dropwise addition was continued over 4 days with monitoring by TLC. Most of the solvent was removed and the residue was extracted (DCM/$NaHCO_3$ (500/200 mL), filtered and reduced. The residue was subjected to column purification (0-5% MeOH/DCM) to give pure compound **215** as a white foam which was homogeneous by TLC and HPLC. (ESI +): calc $(M+H)^+$: 767.34, found: 767.03. ^{1}H NMR (300 MHz, CD_3CN) δ 8.63 (s, 1H), 8.07 (s, 1H), 7.44 – 7.36 (m, 2H), 7.33 – 7.18 (m, 7H), 6.84 – 6.72 (m, 4H), 6.02 (d, J = 3.2 Hz, 1H), 4.74 (td,

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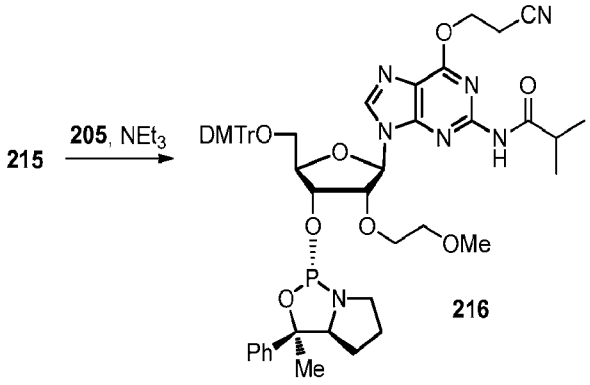
J = 6.2, 0.9 Hz, 2H), 4.71 – 4.64 (m, 2H), 4.14 – 4.06 (m, 1H), 3.88 – 3.73 (m, 8H), 3.57 – 3.41 (m, 4H), 3.32 – 3.23 (m, 3H), 3.08 (t, *J* = 6.2 Hz, 2H), 2.77 (dt, *J* = 13.7, 6.9 Hz, 1H), 1.14 (dd, *J* = 6.9, 1.2 Hz, 6H).

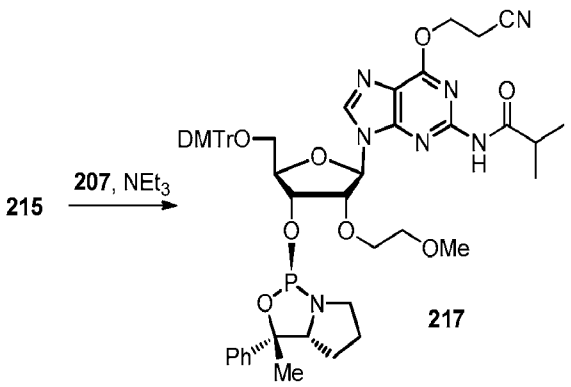
Synthesis of compound 216



216

[00135] **Compound 216:** Using a procedure analogous to that described for compound **208** and substituting compound **215** for compound **203**, pure compound **216** was obtained as a colorless solid foam. ^{1}H NMR (399 MHz, CD_3CN) δ 8.41 (s, 1H), 8.12 (s, 1H), 7.50 – 7.44 (m, 2H), 7.40 – 7.19 (m, 12H), 6.80 (dd, *J* = 8.9, 5.2 Hz, 4H), 6.04 (d, *J* = 5.7 Hz, 1H), 4.99 (t, *J* = 5.5 Hz, 1H), 4.94 (ddd, *J* = 9.9, 5.2, 3.7 Hz, 1H), 4.75 (t, *J* = 6.2 Hz, 2H), 4.29 (q, *J* = 3.8 Hz, 1H), 3.89 – 3.82 (m, 1H), 3.76 – 3.68 (m, 7H), 3.64 (ddd, *J* = 6.8, 5.5, 2.3 Hz, 1H), 3.50 – 3.46 (m, 2H), 3.42 (s, 2H), 3.38 – 3.28 (m, 1H), 3.21 (s, 3H), 3.09 (t, *J* = 6.2 Hz, 2H), 2.84 (dq, *J* = 10.2, 7.3 Hz, 1H), 2.70 – 2.58 (m, 1H), 1.76 (s, 3H), 1.46 – 1.27 (m, 2H), 1.12 (d, *J* = 6.8 Hz, 3H), 1.07 (d, *J* = 6.8 Hz, 3H), 1.04 – 0.89 (m, 2H); ^{31}P NMR (162 MHz, CD_3CN) δ 154.40.

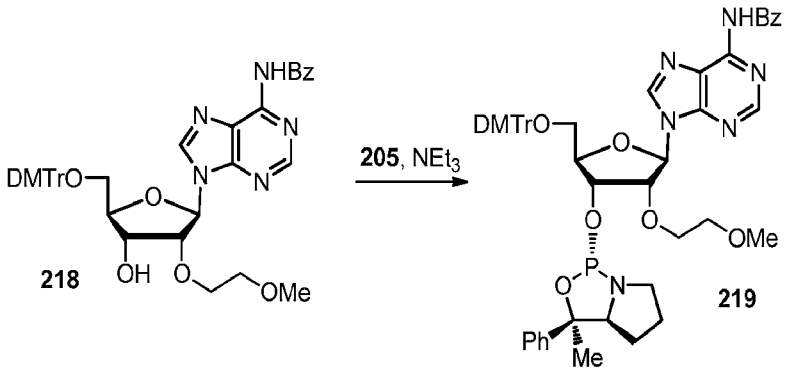
Synthesis of compound 217



217

[00136] **Compound 217:** Using a procedure analogous to that described for compound **210** and substituting compound **215** for compound **209**, pure compound **217** was obtained as a colorless solid foam.^{1}H NMR (399 MHz, CD_3CN) δ 8.56 (s, 1H), 8.13 (s, 1H), 7.44 (dd, *J* = 8.2, 1.5 Hz, 2H), 7.39 – 7.19 (m, 12H), 6.81 (dd, *J* = 10.4, 8.9 Hz, 4H), 6.06 (d, *J* = 4.5 Hz, 1H), 4.96 – 4.86 (m, 2H), 4.75 (t, *J* = 6.2 Hz, 2H), 4.30 (td, *J* = 5.1, 4.7, 2.4 Hz, 1H), 3.83 (dt, *J* = 11.2, 4.3 Hz, 1H), 3.79- 3.70 (m, 7H), 3.67 (ddd, *J* = 7.1, 5.2, 2.0 Hz, 1H), 3.52 – 3.45 (m, 3H), 3.41 (dd, *J* = 10.8, 2.7 Hz, 1H), 3.34 – 3.24 (m, 1H), 3.21 (s, 3H), 3.08 (t, *J* = 6.1 Hz, 2H), 2.89 – 2.72 (m, 2H), 1.68 (s, 3H), 1.54 – 1.38 (m, 2H), 1.14 (dd, *J* = 6.8, 5.4 Hz, 6H), 1.09 – 1.00 (m, 1H), 0.95 – 0.83 (m, 1H); ^{31}P NMR (162 MHz, CD_3CN) δ 154.93.

Synthesis of compound 219



[00137] **Compound 219:** Using a procedure analogous to that described for compound **208** and substituting compound **218** for compound **203**, pure compound **219** was obtained as a colorless solid foam. ^{1}H NMR (399 MHz, $CDCl_3$) δ 9.31 (s, 1H), 8.72 (s, 1H), 8.26 (s, 1H), 8.07 – 7.98 (m, 2H), 7.60 – 7.53 (m, 1H), 7.52 – 7.43 (m, 4H), 7.38 – 7.15 (m, 12H), 6.83 – 6.74 (m, 4H), 6.24 (d, *J* = 5.2 Hz, 1H), 5.03 – 4.92 (m, 2H), 4.45 (q, *J* = 3.8 Hz, 1H), 3.93 (dt, *J* = 11.4, 4.2 Hz, 1H), 3.77 (ddd, *J* = 7.5, 5.8, 3.2 Hz, 2H), 3.73 (s, 6H), 3.60 – 3.49 (m, 3H), 3.47 – 3.37 (m, 2H), 3.26 (s, 3H), 3.02 – 2.92 (m, 1H), 1.81 (s, 3H), 1.56 – 1.43 (m, 1H), 1.37 (dq, *J* = 13.2, 6.5 Hz, 1H), 1.22 – 1.09 (m, 1H), 0.96 (ddt, *J* = 13.1, 7.9, 6.8 Hz, 1H); ^{31}P NMR (162 MHz, $CDCl_3$) δ 157.73.

Synthesis of compound 220

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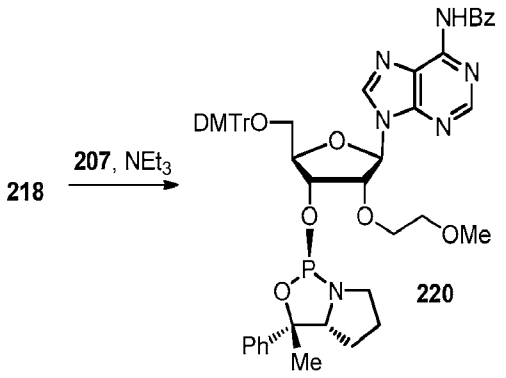
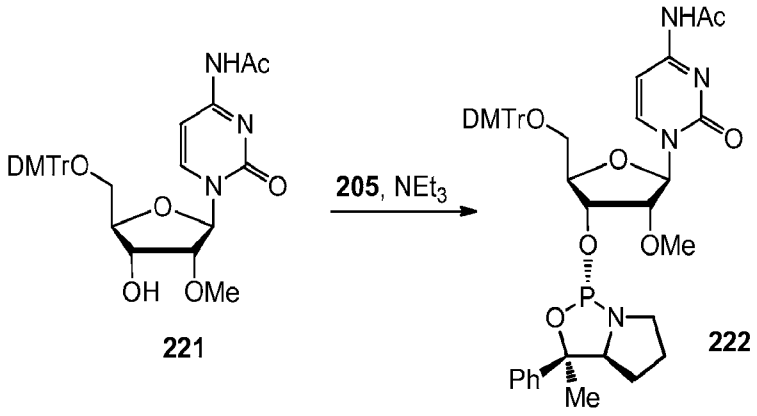


[00138] **Compound 220**: Using a procedure analogous to that described for compound **210** and substituting compound **218** for compound **209**, pure compound **220** was obtained as a colorless solid foam. ^{1}H NMR (399 MHz, $CDCl_3$) δ 9.26 (s, 1H), 8.73 (s, 1H), 8.31 (s, 1H), 8.02 (d, J = 7.6 Hz, 2H), 7.60 – 7.53 (m, 1H), 7.52 – 7.40 (m, 4H), 7.39 – 7.13 (m, 12H), 6.84 – 6.76 (m, 4H), 6.24 (d, J = 4.5 Hz, 1H), 4.99 (dt, J = 10.0, 5.0 Hz, 1H), 4.84 (t, J = 4.7 Hz, 1H), 4.46 (q, J = 4.0 Hz, 1H), 3.91 (dt, J = 11.1, 4.2 Hz, 1H), 3.85 – 3.69 (m, 8H), 3.63 – 3.50 (m, 3H), 3.42 (dd, J = 10.7, 4.0 Hz, 1H), 3.40 – 3.31 (m, 1H), 3.27 (s, 3H), 3.00 – 2.89 (m, 1H), 1.82 (s, 3H), 1.58 – 1.38 (m, 2H), 1.20 – 1.09 (m, 1H), 1.00 (ddt, J = 12.3, 8.8, 5.9 Hz, 1H); ^{31}P NMR (162 MHz, $CDCl_3$) δ 158.98.

Synthesis of compound 222



[00139] **Compound 222:** Using a procedure analogous to that described for compound **208** and substituting compound **221** for compound **203**, pure compound **222** was obtained as a colorless solid foam. ^{1}H NMR (499 MHz, $CDCl_3$) δ 10.46 (s, br, 1H), 8.62 (d, J = 7.5 Hz, 1H), 7.42 (d, J = 7.1 Hz, 2H), 7.36 – 7.21 (m, 12H), 7.00 (d, J = 7.5 Hz, 1H), 6.82 (dd, J = 9.0, 7.1 Hz, 4H), 6.03 (s, 1H), 4.77 (td, J = 8.7, 4.8 Hz, 1H), 4.34 (dt, J = 9.4, 2.3 Hz, 1H), 3.97 (d, J =

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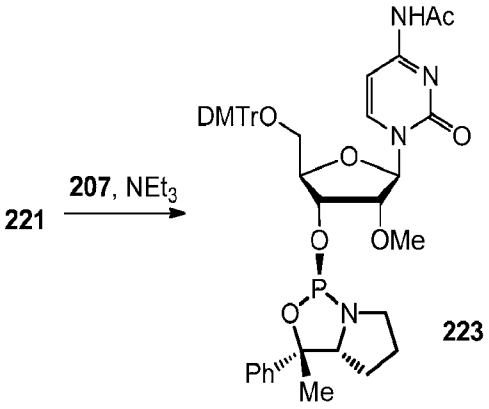
4.8 Hz, 1H), 3.78 (dd, *J* = 6.7, 3.4 Hz, 1H), 3.74 (s, 3H), 3.71 (s, 6H), 3.68 – 3.58 (m, 2H), 3.48 – 3.38 (m, 1H), 3.01 (p, *J* = 7.8 Hz, 1H), 2.27 (s, 3H), 1.72 (s, 3H), 1.49 (tt, *J* = 12.3, 7.0 Hz, 1H), 1.40 (dq, *J* = 13.2, 6.6 Hz, 1H), 1.18 (dq, *J* = 13.9, 7.0 Hz, 1H), 1.02 – 0.90 (m, 1H); ^{31}P NMR (202 MHz, $CDCl_3$) δ 158.71.

Synthesis of compound 223



[00140] **Compound 223:** Using a procedure analogous to that described for compound **210** and substituting compound **221** for compound **209**, pure compound **223** was obtained as a colorless solid foam. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.76 (s, 1H), 8.70 (d, *J* = 7.4 Hz, 1H), 7.48 – 7.41 (m, 2H), 7.41 – 7.18 (m, 12H), 7.06 (d, *J* = 7.4 Hz, 1H), 6.87 (dd, *J* = 8.9, 1.9 Hz, 4H), 6.00 (s, 1H), 4.80 (td, *J* = 9.2, 4.7 Hz, 1H), 4.33 (d, *J* = 9.5 Hz, 1H), 3.84 (d, *J* = 4.7 Hz, 1H), 3.81 (s, 3H), 3.80 (s, 3H), 3.72 (td, *J* = 6.6, 3.4 Hz, 1H), 3.70 – 3.62 (m, 4H), 3.53 (dd, *J* = 11.3, 2.2 Hz, 1H), 3.34 (tdd, *J* = 10.7, 7.8, 4.9 Hz, 1H), 2.96 (dq, *J* = 10.3, 7.6 Hz, 1H), 2.25 (s, 3H), 1.83 (s, 3H), 1.56 – 1.48 (m, 1H), 1.43 (dq, *J* = 13.1, 6.4 Hz, 1H), 1.24 – 1.16 (m, 1H), 1.00 – 0.91 (m, 1H). ^{31}P NMR (202 MHz, $CDCl_3$) δ 157.17.

Synthesis of compound 225

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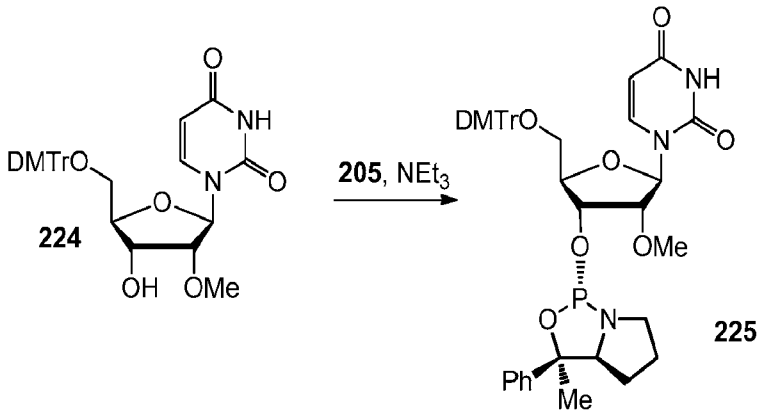
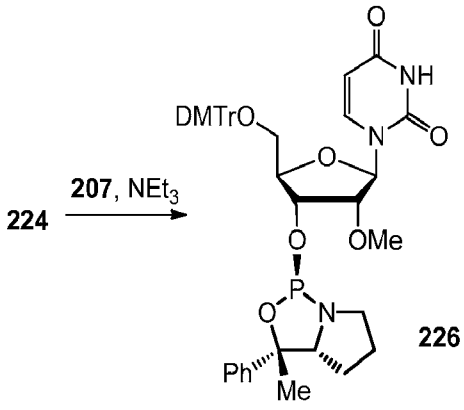


[00141] **Compound 225:** Using a procedure analogous to that described for compound **208** and substituting compound **224** for compound **203**, pure compound **225** was obtained as a colorless solid foam. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.76 (s, br, 1H), 8.70 (d, J = 7.4 Hz, 1H), 7.48 – 7.41 (m, 2H), 7.41 – 7.18 (m, 12H), 7.06 (d, J = 7.4 Hz, 1H), 6.87 (dd, J = 8.9, 1.9 Hz, 4H), 6.00 (s, 1H), 4.80 (td, J = 9.2, 4.7 Hz, 1H), 4.33 (d, J = 9.5 Hz, 1H), 3.84 (d, J = 4.7 Hz, 1H), 3.81 (s, 3H), 3.80 (s, 3H), 3.72 (td, J = 6.6, 3.4 Hz, 1H), 3.69 – 3.62 (m, 4H), 3.53 (dd, J = 11.3, 2.2 Hz, 1H), 3.34 (tdd, J = 10.7, 7.8, 4.9 Hz, 1H), 2.96 (dq, J = 10.3, 7.6 Hz, 1H), 2.25 (s, 3H), 1.83 (s, 3H), 1.56 – 1.48 (m, 1H), 1.43 (dq, J = 13.1, 6.4 Hz, 1H), 1.24 – 1.16 (m, 1H), 1.00 – 0.91 (m, 1H); ^{31}P NMR (202 MHz, $CDCl_3$) δ 158.97.

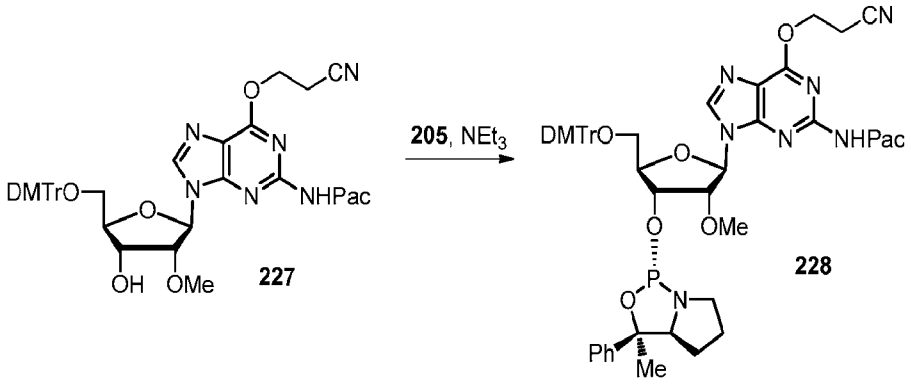
Synthesis of compound 226



[00142] **Compound 226:** Using a procedure analogous to that described for compound **210** and substituting compound **224** for compound **209**, pure compound **226** was obtained as a colorless solid foam. ^{1}H NMR (499 MHz, $CDCl_3$) δ 9.8 – 9.0 (br, 1H), 8.09 (d, J = 8.2 Hz, 1H), 7.40 (d, J = 7.4 Hz, 2H), 7.36 – 7.19 (m, 12H), 6.80 (dd, J = 11.4, 8.8 Hz, 4H), 6.02 (d, J = 2.2

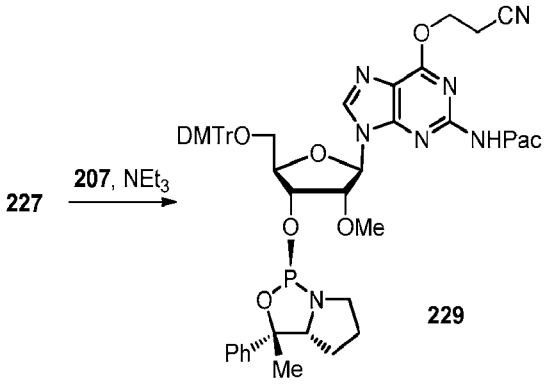
Hz, 1H), 5.22 (d, J = 8.1 Hz, 1H), 4.82 (td, J = 8.1, 4.9 Hz, 1H), 4.27 (d, J = 7.4 Hz, 1H), 3.96 (dd, J = 5.0, 2.2 Hz, 1H), 3.81 – 3.76 (m, 1H), 3.74 (s, 3H), 3.70 (s, 3H), 3.62 (s, 3H), 3.61 – 3.54 (m, 2H), 3.50 – 3.41 (m, 1H), 3.04 (dtd, J = 10.2, 8.0, 6.3 Hz, 1H), 1.75 (s, 3H), 1.56 – 1.47 (m, 1H), 1.44 – 1.35 (m, 1H), 1.26 – 1.18 (m, 1H), 0.95 (dq, J = 12.3, 7.5 Hz, 1H); ^{31}P NMR (202 MHz, $CDCl_3$) δ 158.96.

Synthesis of compound 228



[00143] **Compound 228:** Using a procedure analogous to that described for compound **208** and substituting compound **227** for compound **203**, pure compound **228** was obtained as a colorless solid foam. ^{31}P NMR (202 MHz, $CDCl_3$) δ 158.16.

Synthesis of compound 229



[00144] **Compound 229:** Using a procedure analogous to that described for compound **210** and substituting compound **227** for compound **209**, pure compound **229** was obtained as a colorless solid foam. ^{31}P NMR (202 MHz, $CDCl_3$) δ 158.84.

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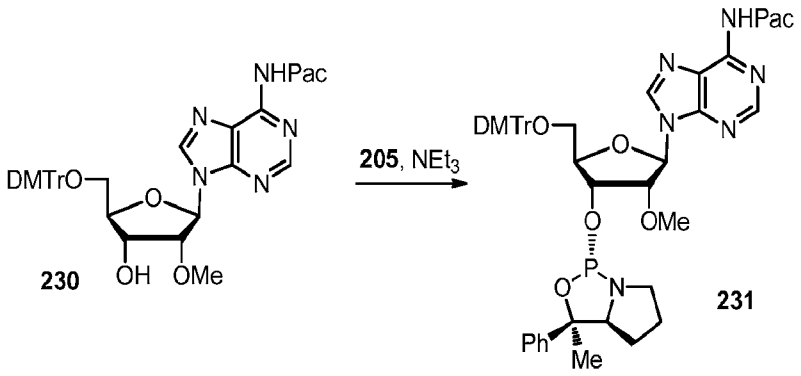
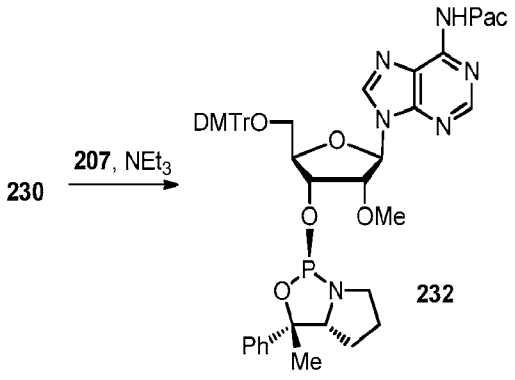
Synthesis of compound 231



[00145] **Compound 231:** Using a procedure analogous to that described for compound **208** and substituting compound **230** for compound **203**, pure compound **231** was obtained as a colorless solid foam. ^{1}H NMR (499 MHz, $CDCl_3$) δ 9.6 – 9.3 (s, 1H), 8.73 (s, 1H), 8.32 (s, 1H), 7.50 – 7.41 (m, 2H), 7.39 – 7.16 (m, 14H), 7.10 – 7.01 (m, 3H), 6.82 (d, J = 8.8 Hz, 4H), 6.21 (d, J = 4.1 Hz, 1H), 4.96 (dt, J = 10.1, 5.1 Hz, 1H), 4.86 (s, 2H), 4.54 (t, J = 4.5 Hz, 1H), 4.43 (q, J = 3.9 Hz, 1H), 3.81 – 3.75 (m, 7H), 3.62 (dd, J = 10.8, 3.3 Hz, 1H), 3.54 (s, 3H), 3.46 – 3.42 (m, 1H), 3.41 – 3.33 (m, 1H), 3.01 – 2.93 (m, 1H), 1.82 (s, 3H), 1.56 – 1.47 (m, 1H), 1.44 (dq, J = 13.2, 6.5 Hz, 1H), 1.22 – 1.11 (m, 1H), 1.03 – 0.94 (m, 1H); ^{31}P NMR (202 MHz, $CDCl_3$) δ 158.11.

Synthesis of compound 232



[00146] **Compound 232:** Using a procedure analogous to that described for compound **210** and substituting compound **230** for compound **209**, pure compound **232** was obtained as a colorless solid foam. ^{1}H NMR (499 MHz, $CDCl_3$) δ 9.8 – 9.2 (br, 1H), 8.71 (s, 1H), 8.23 (s, 1H), 7.49 – 7.43 (m, 2H), 7.40 – 7.15 (m, 14H), 7.09 – 7.02 (m, 3H), 6.79 (d, J = 8.9 Hz, 4H), 6.20 (d, J = 5.6 Hz, 1H), 4.93 (ddd, J = 9.3, 5.0, 3.8 Hz, 1H), 4.86 (s, 2H), 4.73 (t, J = 5.3 Hz, 1H), 4.43

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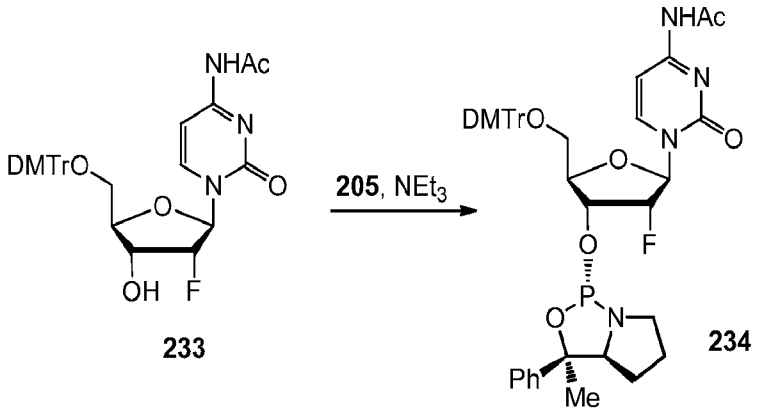
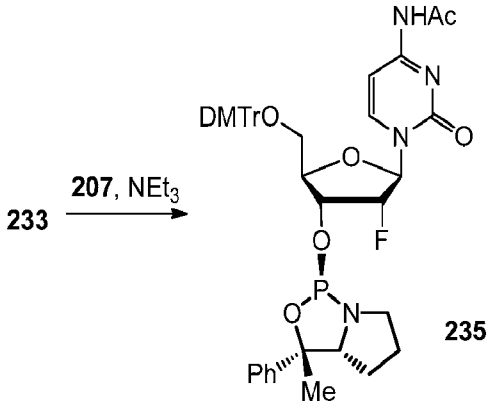
(q, J = 3.9 Hz, 1H), 3.79 – 3.75 (m, 1H), 3.74 (s, 3H), 3.74 (s, 3H), 3.57 (dt, J = 6.8, 3.8 Hz, 1H), 3.52 (s, 3H), 3.46 – 3.36 (m, 2H), 2.99 (dtd, J = 10.1, 7.9, 6.5 Hz, 1H), 1.80 (s, 3H), 1.54 – 1.45 (m, 1H), 1.39 – 1.31 (m, 1H), 1.23 – 1.13 (m, 1H), 0.99 – 0.90 (m, 1H); ^{31}P NMR (202 MHz, $CDCl_3$) δ 158.13.

Synthesis of compound 234



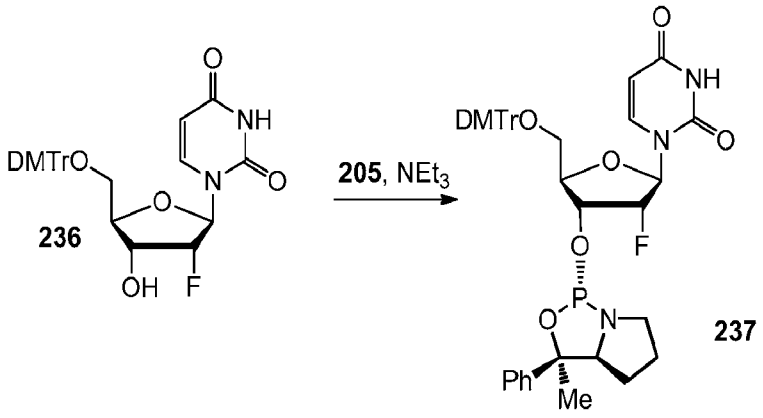
[00147] **Compound 234:** Using a procedure analogous to that described for compound **208** and substituting compound **233** for compound **203**, pure compound **234** was obtained as a colorless solid foam.

Synthesis of compound 235



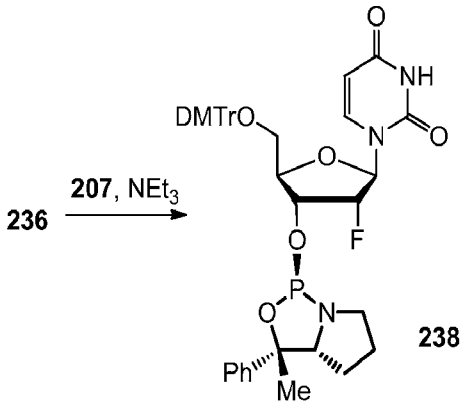
[00148] **Compound 235:** Using a procedure analogous to that described for compound **210** and substituting compound **233** for compound **209**, pure compound **235** was obtained as a colorless solid foam. ^{31}P NMR (202 MHz, $CDCl_3$) δ 158.75.

Synthesis of compound 237



[00149] **Compound 237:** Using a procedure analogous to that described for compound **208** and substituting compound **236** for compound **203**, pure compound **237** was obtained as a colorless solid foam. ^{31}P NMR (202 MHz, Chloroform-*d*) δ 159.51 (d, $J_{P\text{-}F}$ = 9.5 Hz).

Synthesis of compound 238



[00150] **Compound 238:** Using a procedure analogous to that described for compound **210** and substituting compound **236** for compound **209**, pure compound **238** was obtained as a colorless solid foam. ^{31}P NMR (202 MHz, Chloroform-*d*) δ 159.48.

Synthesis of compound 240

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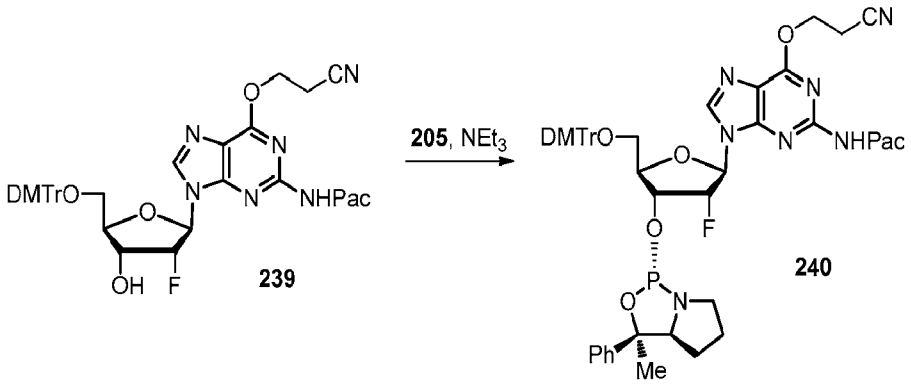
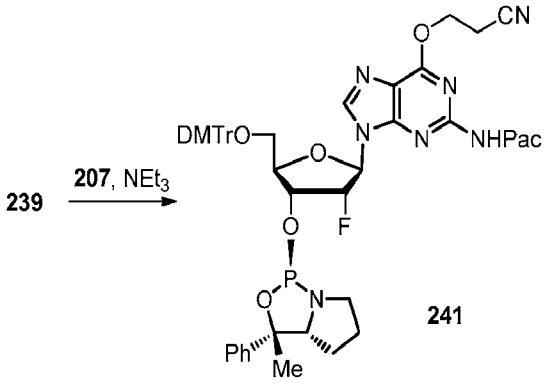


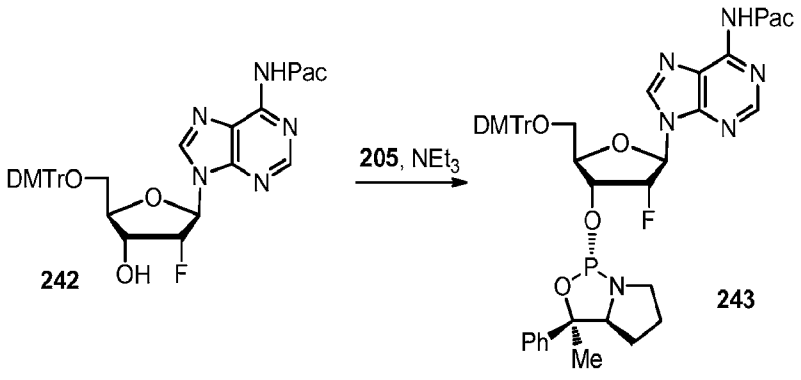
[00151] **Compound 240:** Using a procedure analogous to that described for compound **208** and substituting compound **239** for compound **203**, pure compound **240** was obtained as a colorless solid foam. ^{31}P NMR (202 MHz, $CDCl_3$) δ 160.20 (d, $J_{P\text{-}F}$ = 11.0 Hz).

Synthesis of compound 241



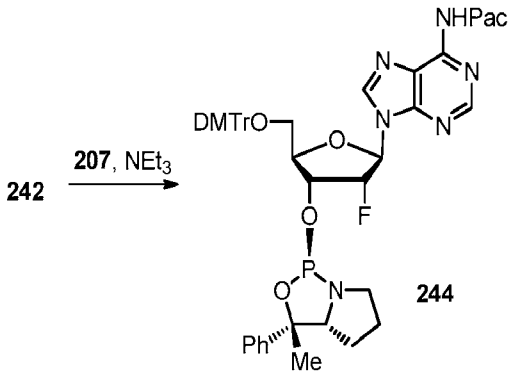
[00152] **Compound 241:** Using a procedure analogous to that described for compound **210** and substituting compound **239** for compound **209**, pure compound **241** was obtained as a colorless solid foam. ^{31}P NMR (202 MHz, $CDCl_3$) δ 159.66 (d, $J_{P\text{-}F}$ = 8.4 Hz).

Synthesis of compound 243



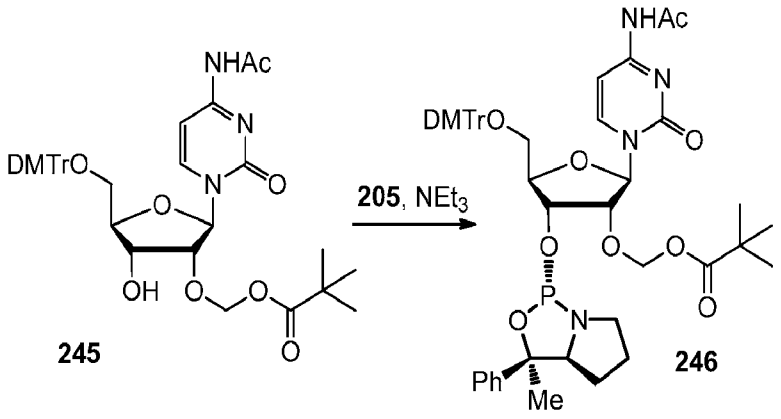
[00153] **Compound 243:** Using a procedure analogous to that described for compound **208** and substituting compound **242** for compound **203**, pure compound **243** was obtained as a colorless solid foam. ^{31}P NMR (202 MHz, $CDCl_3$) δ 160.20 (d, J = 11.0 Hz).

Synthesis of compound 244



[00154] **Compound 244:** Using a procedure analogous to that described for compound **210** and substituting compound **242** for compound **209**, pure compound **244** was obtained as a colorless solid foam. ^{31}P NMR (202 MHz, $CDCl_3$) δ 156.52 (d, $J_{P\text{-}F}$ = 8.5 Hz).

Synthesis of compound 246



[00155] **Compound 246:** Using a procedure analogous to that described for compound **208** and substituting compound **245** for compound **203**, pure compound **246** is obtained as a colorless solid foam.

Synthesis of compound 247

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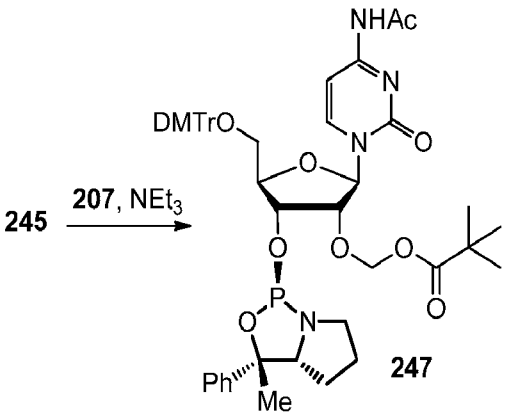
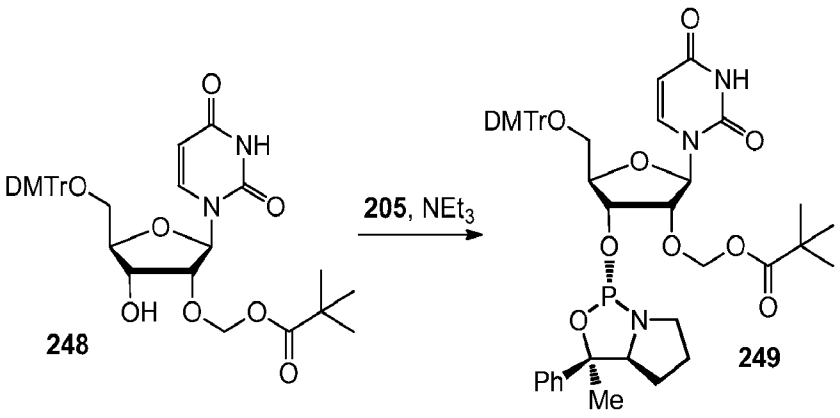


[00156] **Compound 247:** Using a procedure analogous to that described for compound **210** and substituting compound **245** for compound **209**, pure compound **247** is obtained as a colorless solid foam.

Synthesis of compound 249



[00157] **Compound 249:** Using a procedure analogous to that described for compound **208** and substituting compound **248** for compound **203**, pure compound **249** is obtained as a colorless solid foam.

Synthesis of compound 250

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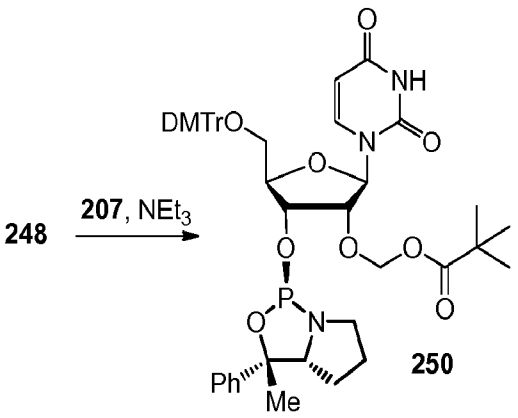
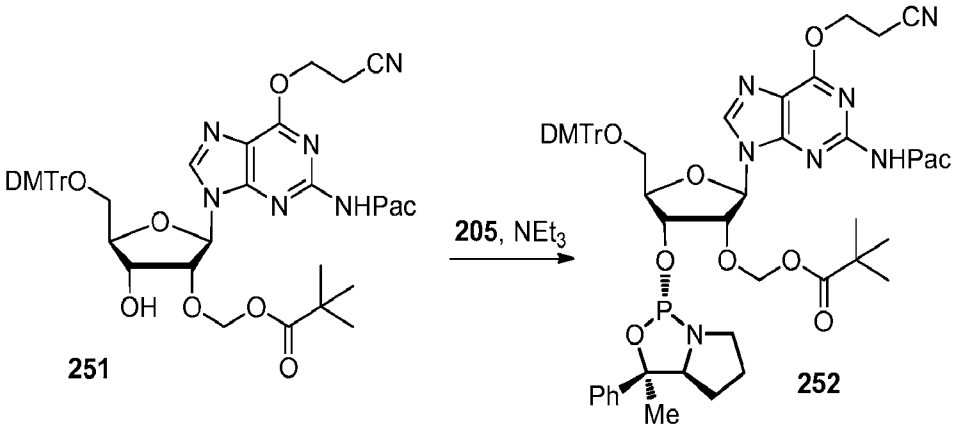


[00158] **Compound 250:** Using a procedure analogous to that described for compound **210** and substituting compound **248** for compound **209**, pure compound **250** is obtained as a colorless solid foam.

Synthesis of compound 252



[00159] **Compound 252:** Using a procedure analogous to that described for compound **208** and substituting compound **251** for compound **203**, pure compound **252** is obtained as a colorless solid foam.

Synthesis of compound 253

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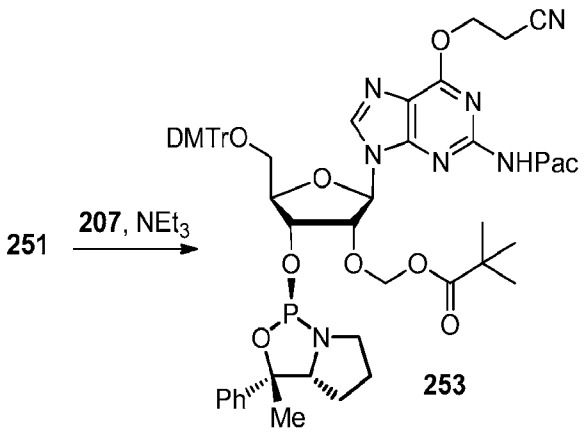
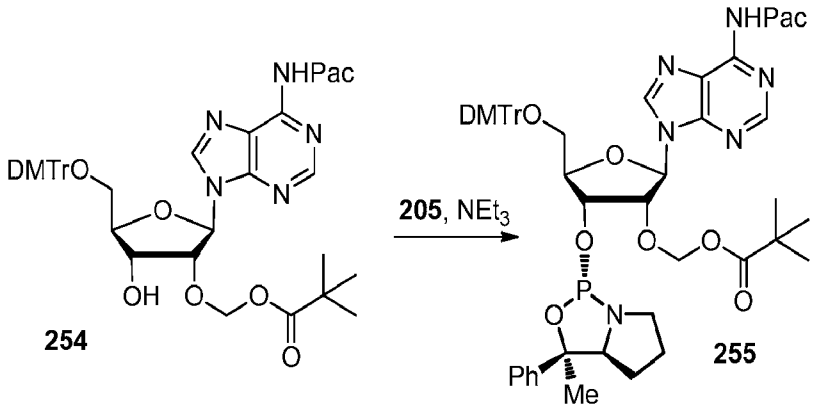


[00160] **Compound 253:** Using a procedure analogous to that described for compound **210** and substituting compound **251** for compound **209**, pure compound **253** is obtained as a colorless solid foam.

Synthesis of compound 255



[00161] **Compound 255:** Using a procedure analogous to that described for compound **208** and substituting compound **254** for compound **203**, pure compound **255** is obtained as a colorless solid foam.

Synthesis of compound 256

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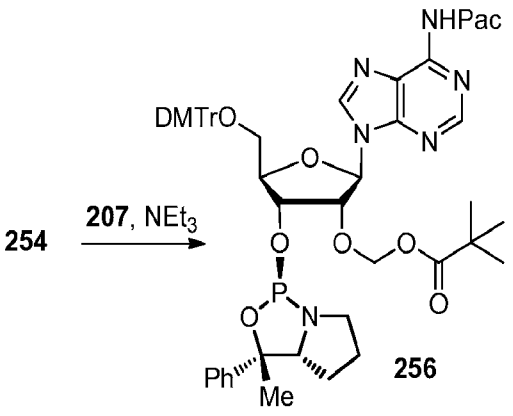
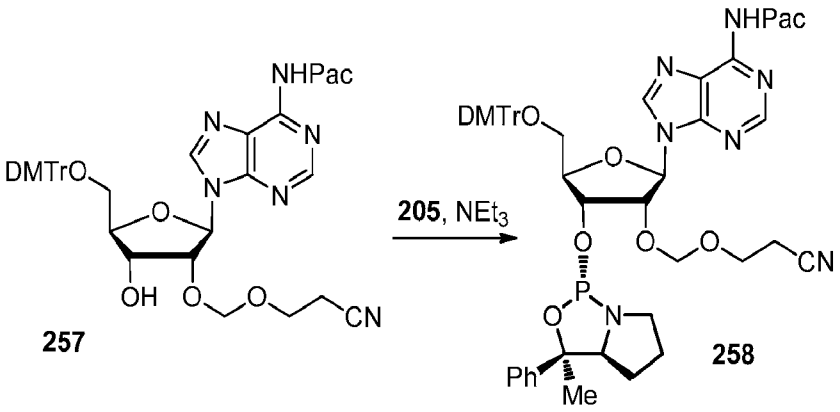


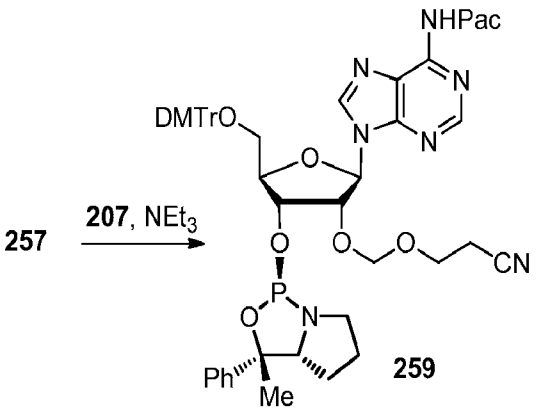
[00162] **Compound 256:** Using a procedure analogous to that described for compound **210** and substituting compound **254** for compound **209**, pure compound **256** is obtained as a colorless solid foam.

Synthesis of compound 258



[00163] **Compound 258:** Using a procedure analogous to that described for compound **208** and substituting compound **257** for compound **203**, pure compound **258** is obtained as a colorless solid foam.

Synthesis of compound 259



[00164] **Compound 259:** Using a procedure analogous to that described for compound **210** and substituting compound **257** for compound **209**, pure compound **259** is obtained as a colorless solid foam.

Examples 2-16.

[00165] Summarized below in Table E-1 is the synthetic procedure for Examples 2-16 on the DNA/RNA Synthesizer ABI-394.

Table E-1. Summary for Oligonucleotide Synthesis on a DNA/RNA Synthesizer ABI-394 Used for the Synthesis of Examples 2-16.

step	reaction	reagent	delivery time (sec)		wait time (sec)	
			1 μmol	10 μmol	1 μmol	10 μmol
1	detritylation	3% TCA in DCM	3 + 60 + 10	3 + 90 + 10	N.A.	N.A.
2	coupling	0.15 M phosphoramidite in ACN + 1.2 M CMPT in ACN	5 + 4	10 + 6	30 + 600	30 + 600
3	capping 1	5% Pac_2O in THF/2,6-lutidine	20	30	60	60
4	capping 2	5% Pac_2O in THF/2,6-lutidine + 16% NMI in	20	30	60	60

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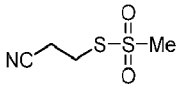
		THF				
5	sulfurization	0.3 M S-cyanoethyl-MethylThio-Sulfonate  in ACN/BSTFA	10 + 4×2	15 + 4×4	300 + 3×150 + 600	300 + 3×150 + 600

[00166] Examples 2-9: The oligonucleotides containing stereodefined phosphorothioate diester internucleotidic linkages were synthesized on an ABI-394 DNA/RNA synthesizer according to the cycle summarized in Table E-1 using a 10 μmol synthesis column and 6.5 μmol of succinyl linked dC on CPG. The synthesis cycle was performed with removal of the terminal 5′-*O*-DMTr group (DMT Off). The solid support was washed with dry ACN and dried under a flux of argon. The dry solid support was then treated with 5 mL of anhydrous 1 M solution of 1,8-diazabicycloundec-7-ene (DBU) in dry ACN-trimethylsilyl chloride – 16:1 (v/v) for 10 min at r.t., during which time the solution was slowly pushed through the column by means of a plastic luer syringe fixed to the outlet of the column. The support was then washed with dry ACN and dried under vacuum. The dry CPG was placed in a plastic vial and then treated with 3 mL of 28% aqueous ammonia for a period of 18 h at r.t. The solvents were evaporated to dryness, the residue was re-suspended in 10% aqueous DMSO, and the solid support was filtered off. The crude product was purified by anion exchange preparative HPLC (Gradient of 0.25 to 1.75 M NaCl in 20 mM NaOH). The fractions having purity above 95% were pooled, concentrated and desalted by reverse-phase HPLC (Gradient of 0 to 80% ACN). The final desalted product was lyophilized from water.

[00167] Examples 10-16: The oligonucleotides containing stereodefined phosphorothioate diester internucleotidic linkages were synthesized on an ABI-394 DNA/RNA synthesizer according to the cycle summarized in Table E-1 using 1 μmol synthesis column and 3 μmol of succinyl linked dC on CPG. The synthesis cycle was performed with removal of the terminal 5′-*O*-DMTr group (DMT Off). The solid support was washed with dry ACN and dried under a flux

of argon. The dry solid support was then treated with 5 mL of anhydrous 1 M solution of 1,5-diazabicyclo(4.3.0) non-5-ene (DBN) in dry ACN-trimethylsilyl chloride – 16:1 (v/v) for 10 min at r.t. The DBN solution was slowly pushed through the column by means of a plastic luer syringe fixed to the outlet of the column. The support was then washed with dry ACN and dried under vacuum. The dry CPG was placed in a plastic vial and was treated with 2 mL of 28% aqueous ammonia for a period of 18 h at r.t. The solvents were evaporated to dryness, the residue was re-suspended in 10% aqueous DMSO, and the solid support was filtered off.

[00168] Purification and desalting of examples 2-16:

The crude product was purified by Waters 2525 BGM HPLC system, equipped with a 2487 dual wavelength detector and with FCO and Flex inject. An AP-1 glass column from Waters, 10×200 mm was filled with Source 15Q Support (GE Healthcare, Part no. 17-0947-01) and was used with flow rate of 4 mL/min. Column was heated during all the purifications using a TL600 mobile phase heater and TL150 Temperature controller (Timberline Instruments) set at 75 °C. Buffer A: 20 mM NaOH and Buffer B: 20 mM NaOH, 2.5 M NaCl were used with step gradients starting from 30% B to 70% B. The fractions having purity above 95 % were pooled, concentrated and desalted on the same HPLC system by reverse-phase column (XBridge Semi Prep, 250×10mm, C_{18}, 5μm) with a gradient of water to 80% ACN and 4 mL/min flow rate. The final desalted product was concentrated in speedvac followed by lyophilization from water.

[00169] HPLC analysis of purified oligonucleotides: Quality of oligonucleotides was determined using DNA Pac 100 (10×250 mm) and using the following conditions:

Buffer A: 10 mM Tris HCl, 50% HCl, pH=8.0

Buffer B: A + 0.8 M $NaClO_4$, pH=8.0

Column temperature: 60 °C

Gradient method:

	Time	Flow	%A	%B	Curve
1	0.01	1.00	85.0	15.0	
2	3.00	1.00	85.0	15.0	1
3	23.00	1.00	40.0	60.0	6
4	25.00	1.00	5.0	95.0	6
5	25.50	1.00	85.0	15.0	6
6	30.00	1.00	85.0	15.0	1

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LCMS analysis method:

Eluent A: 15 mM TEA, 400 mM HFIP, Water

Eluent B: 50:50 Buffer A/Methanol

Column: UPLC@OST C_{18} 1.7 μm, 2.1×500mm

Column temperature = 50 °C

Gradient method:

Time	Flow	%A	%B	Curve
0	0.2	95	5	
10	0.2	35	65	6
12	0.2	5	95	6
12.5	0.2	95	5	6
15	0.2	95	5	1

[00170] **Example 2. Synthesis of Oligonucleotide 101 (All-(*Rp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC])**

[00171] Oligonucleotide 101 was synthesized as described above. RT in IEX-HPLC: 14.70 min. UPLC/ESI-MS: Calcd for $C_{191}H_{246}N_{67}O_{102}P_{19}S_{19}$: 6310.2; Found: 6310.4.

[00172] **Example 3. Synthesis of Oligonucleotide 102 (All-(*Sp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC])**

[00173] Oligonucleotide 102 was synthesized as described above. RT in IEX-HPLC: 15.49 min. UPLC/ESI-MS: Calcd for $C_{191}H_{246}N_{67}O_{102}P_{19}S_{19}$: 6310.2; Found: 6310.2.

[00174] **Example 4. Synthesis of Oligonucleotide 103 ((*Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC] (5R-9S-5R))**

Oligonucleotide 103 was synthesized as described above. RT in IEX-HPLC: 15.10 min. UPLC/ESI-MS: Calcd for $C_{191}H_{246}N_{67}O_{102}P_{19}S_{19}$: 6310.2; Found: 6310.3.

[00175] **Example 5. Synthesis of Oligonucleotide 104 ((*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC] (5S-9R-5S))**

[00176] Oligonucleotide 104 was synthesized as described above. RT in IEX-HPLC: 15.04 min. UPLC/ESI-MS: Calcd for $C_{191}H_{246}N_{67}O_{102}P_{19}S_{19}$: 6310.2; Found: 6307.2.

[00177] **Example 6. Synthesis of Oligonucleotide 105 ((*Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC] (1S-17R-1S))**

[00178] Oligonucleotide 105 was synthesized as described above. RT in IEX-HPLC: 14.75 min. UPLC/ESI-MS: Calcd for $C_{191}H_{246}N_{67}O_{102}P_{19}S_{19}$: 6310.2; Found: 6310.2.

[00179] **Example 7. Synthesis of Oligonucleotide 106 ((*Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC] (1R-17S-1R))**

[00180] Oligonucleotide 106 was synthesized as described above. RT in IEX-HPLC: 15.43 min. UPLC/ESI-MS: Calcd for $C_{191}H_{246}N_{67}O_{102}P_{19}S_{19}$: 6310.2; Found: 6309.6.

[00181] **Example 8. Synthesis of Oligonucleotide 107 ((*Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC] ((R/S)$_9$R))**

[00182] Oligonucleotide 107 was synthesized as described above. RT in IEX-HPLC: 15.02 min. UPLC/ESI-MS: Calcd for $C_{191}H_{246}N_{67}O_{102}P_{19}S_{19}$: 6310.2; Found: 6310.7.

[00183] **Example 9. Synthesis of Oligonucleotide 108 ((*Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC] ((S/R)$_9$S))**

[00184] Oligonucleotide 108 was synthesized as described above. RT in IEX-HPLC: 15.10 min. UPLC/ESI-MS: Calcd for $C_{191}H_{246}N_{67}O_{102}P_{19}S_{19}$: 6310.2; Found: 6307.9.

[00185] **Example 10. Synthesis of Oligonucleotide 109 ((*Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*)d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC] (3S-13R-3S))**

[00186] Oligonucleotide 109 was synthesized as described above. RT in IEX-HPLC: 14.91 min. UPLC/ESI-MS: Calcd for $C_{191}H_{246}N_{67}O_{102}P_{19}S_{19}$: 6310.2; Found: 6309.5.

[00187] **Example 11. Synthesis of Oligonucleotide 110 ((*Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC] (3R-13S-3R))**

[00188] Oligonucleotide 110 was synthesized as described above. RT in IEX-HPLC: 15.24 min. UPLC/ESI-MS: Calcd for $C_{191}H_{246}N_{67}O_{102}P_{19}S_{19}$: 6310.2; Found: 6309.3.

[00189] **Example 12. Synthesis of Oligonucleotide 111 ((*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC] ((18S/R^{19}))**

[00190] Oligonucleotide 111 was synthesized as described above. RT in IEX-HPLC: 15.69 min. UPLC/ESI-MS: Calcd for $C_{191}H_{246}N_{67}O_{102}P_{19}S_{19}$: 6310.2; Found: 6309.4.

[00191] **Example 13. Synthesis of Oligonucleotide 113 ((*Sp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC] (18S/R^{2}))**

[00192] Oligonucleotide 113 was synthesized as described above. RT in IEX-HPLC: 15.72 min. UPLC/ESI-MS: Calcd for $C_{191}H_{246}N_{67}O_{102}P_{19}S_{19}$: 6310.2; Found: 6311.0.

[00193] **Example 14. Synthesis of Oligonucleotide 114 ((*Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC] ((RRS)$_6$-R))**

[00194] Oligonucleotide 114 was synthesized as described above. RT in IEX-HPLC: 14.14 min. UPLC/ESI-MS: Calcd for $C_{191}H_{246}N_{67}O_{102}P_{19}S_{19}$: 6310.2; Found: 6313.7.

[00195] **Example 15. Synthesis of Oligonucleotide 115 ((*Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*)-d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC] (S-(RRS)$_6$))**

[00196] Oligonucleotide 115 was synthesized as described above. RT in IEX-HPLC: 14.30 min. UPLC/ESI-MS: Calcd for $C_{191}H_{246}N_{67}O_{102}P_{19}S_{19}$: 6310.2; Found: 6313.7.

[00197] **Example 16. Synthesis of Oligonucleotide 116 ((*Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*)d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC] (RS-(RRS)$_5$-RR))**

[00198] Oligonucleotide 116 was synthesized as described above. RT in IEX-HPLC: 14.17 min. UPLC/ESI-MS: Calcd for $C_{191}H_{246}N_{67}O_{102}P_{19}S_{19}$: 6310.2; Found: 6312.4.

[00199] Results of Examples 2-16 are summarized in Table E-2, below:

Table E-2. Summary of Examples 2-16.

Oligonu-cleotide	5′-sequence-3′	Description of stereo-chemistry	RT – IEX (min)
101	d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	All-(*R*p)	14.70
102	d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	All-(*S*p)	15.49
103	d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	5R-9S-5R	15.10
104	d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	5S-9R-5S	15.04
105	d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	1S-17R-1S	14.75
106	d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	1R-17S-1R	15.43
107	d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	$(R/S)_9R$	15.02
108	d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	$(S/R)_9S$	15.10
109	d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	3S-13R-3S	14.91
110	d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	3R-13S-3R	15.24
111	d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	$18S/R^{19}$	15.69
113	d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	$18S/R^{2}$	15.72
114	d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	$(RRS)_6$-R	14.14
115	d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	S-$(RRS)_6$	14.30
116	d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCsAsCsC]	RS-$(RRS)_5$-RR	14.17

[00200] Example 17. Synthesis of Control Oligonucleotides

[00201] The control oligonucleotides (see Table E-3) were synthesized using the standard chemistry methods for automated solid-phase oligonucleotide synthesis (Beaucage, S.L. and Iyer, R.P., *Tetrahedron*, 1992, *48*, 2223-2311). More specifically, stereorandom DNA was synthesized using standard DNA phosphoramidites (ChemGenes Co.), ethylthiotetrazole (ETT, Muang *et al.*, *Tetrahedron Lett.*, **2004**, *45*, 6497–6499) as the activator (Glen Research) and *N,N*-dimethyl-*N*′-(3-thioxo-3*H*-1,2,4-dithiazol-5-yl)methanimidamide (DDTT, AM Chemicals) as the sulfurizing reagent (Guzaev, A.; *Tetrahedron Lett.*, **2011**, *52*, 434–437). The phosphoramidite coupling time was 2 min and the sulfurization time was 10 min. The oligonucleotide was deprotected and purified using standard methods. DNA phosphodiester was synthesized using standard DNA phosphoramidites, ETT as the activator and iodine/pyridine/water as the oxidizing reagent. 2′-*O*-Methoxyethyl (MOE) DNA was synthesized using in-house prepared 2′-*O*-Methoxyethyl (MOE) phosphoramidites (Martin, P.; *Helv. Chim. Acta.* **1995**, *78*, 486–504; Ross, B.; Song, Q.; **2004**, US patent publication No. 20040082775), ETT as the activator and DDTT as the sulfurizing reagent. The times for coupling were 10 min and the times for sulfurization were 10 min. RNA was synthesized using standard RNA phosphoramidites (ChemGenes Co.), ETT as the activator and iodine/pyridine/water as the oxidizing reagent. The coupling times were 10 min.

[00202] All oligonucleotides were deprotected and purified using standard methods.

[00203] Purification of RNA (Oligonucleotide 117):

Waters 2525 BGM, 2487 Dual wavelength detector equipped with FCO and Flex injector

Buffer A: 20 mM Sodium Phosphate pH=8.5

Buffer B: 20 mM Sodium Phosphate, 1 M NaBr pH=8.5

Column: AP-1 glass column from Waters, 10×200mm, filled with Super Q-5PW (20), TSK Gel (Anion Exchange) from TOSOH

Column Temperature: 70 °C (Timberline Instruments, TL600 mobile phase heater and TL150 Temperature controller)

Gradient used:

Time	Flow (mL/min)	% A	% B	Curve
Initial		100	0	
10	4	100	0	1
25	4	80	20	6

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115	4	55	45	6
125	4	0	100	6
130	4	100	0	6
140	4	100	0	1

[00204] As shown in Figure 1, the chirally controlled phosphorothioate diester 20-mer oligonucleotide (All-(*R*p), Oligonucleotide 101, Figure 1, A) has a different retention time than that of the phosphorothioate diester 20-mer standard stereorandom oligonucleotide (Oligonucleotide 118, Figure 1, C) and has a sharper peak. One of skill in the art understands that during purification of the stereorandom oligonucleotide 108, it is likely that most of the all-(*R*p) oligonucleotide (101, present in an approximately $1/2^{19}$ fraction of the mixture stereorandom oligonucleotide 108) would be lost.

[00205] Results of Example 17 are summarized in Table E-3, below.

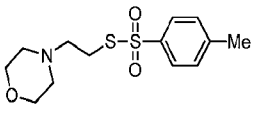
Table E-3. Summary of Example 17.

Oligonu-cleotide	5′-sequence-3′	Description	RT (min)
117	GGUGCGAAGCAGACUGAGGC	RNA	5.10
118	d[GsCsCsTsCsAsGsTsCsTsGsCsTsTsCsGsCs AsCsC]	stereorandom	15.04
119	d[GCCTCAGTC TGC TTC GCACC]	DNA	6.90
120	(Gs5mCs5mCsTs5mCs)$_{MOE}$ d[AsGsTs5mCsTsGs5mCsTsTs5mCs](Gs5mCs As 5mCs5mC)$_{MOE}$	stereorandom	15.49
121	d[GsAsTsGsCsCsTsCsTsCsCsTsAsCsGsCsGs CsCsT]	scrambled	15.09

[00206] Procedures for Examples 18-21: The oligonucleotides containing stereodefined morpholinoethyl phosphorothioate triester internucleotidic linkages were synthesized on an ABI-394 DNA/RNA synthesizer according to the cycle summarized in Table E-4 using 1 μmol synthesis column and 0.8 μmol of oxalyl linked dC on HCP. The synthesis cycle was performed with removal of the terminal 5′-*O*-DMTr group (DMT Off). The solid support was washed with

dry ACN and dried under a flux of argon. The dry HCP was placed in a plastic vial and was treated with 1 mL of dry propylamine in dry pyridine (in a 1:4 ratio) for a period of 18 h at r.t. The solvents were then evaporated and the residue was re-suspended with ~pH 1.5 aqueous solution containing 10% DMSO and the HCP support was filtered off. The crude product was purified by reverse phase preparative HPLC. The fractions having purity above 95% were pooled, concentrated and desalted by reverse-phase HPLC (Gradient of 0 to 80% ACN). The final desalted product was lyophilized from water.

Table E-4. Summary for Oligonucleotide Synthesis on a DNA/RNA Synthesizer ABI-394 Used for the Synthesis of Examples 18-21.

step	reaction	reagent	delivery time (sec)	wait time (sec)
1	detritylation	3% TCA in DCM	3 + 60 + 10	N.A.
2	coupling	0.15 M phosphoramidite in ACN + 1.2 M CMPT in ACN	5 + 4	30 + 600
3	capping 1	5% Pac_2O in THF/2,6-lutidine	20	60
4	capping 2	5% Pac_2O in THF/2,6-lutidine + 16% NMI in THF	20	60
5	sulfurization	0.3 M S-Morpholinoethyl-ToluylThioSulfonate  in ACN/BSTFA	10 + 4×2	300 + 3×150 + 600

[00207] General Purification Method for Examples 18-21:

Buffer A: 20 mM Phosphate pH=6.0 (adjusted with phosphoric acid)

Buffer B: ACN

Column: XBridge Prep C_{18}, 5 μm, C_{18}, 250×10mm, Part #186003256

Buffer heater set temperature = 50 °C

Signal monitored at 254 and 280 nm

Gradient used:

Time	Flow (ml/min)	% A	% B	Curve
Initial		99	1	
5	4	99	1	1
10	4	77	23	6
60	4	70	30	6
65	4	20	80	6
70	4	20	80	6
71	4	99	1	6
80	4	99	1	1

[00208] Analytical HPLC methods for oligonucleotides.

HPLC method 1:

Buffer A: 20 mM Phosphate pH=6.0 (adjusted with phosphoric acid)

Buffer B: ACN

Column: XBridge C_{18}, 3.5 μm, C_{18}, 4.6x50mm, Part #186003034

Buffer heater set temperature = 35 °C

Signal monitored at 254 and 280 nm

Gradient used:

Time	Flow (ml/min)	% A	% B	Curve
Initial		95	5	
3	1	95	5	1
23	1	60	40	6
25	1	40	60	6
25.5	1	95	5	6
30	1	95	5	1

[00209] HPLC method 2:

Buffer A: 50 mM TEAA, pH 7.8

Buffer B: ACN

Column: XBridge C_{18}, 3.5 μm, C_{18}, 4.6x50mm, Part #186003034

Buffer heater set temperature = 60 °C

Signal monitored at 254 and 280 nm

Gradient used:

Time	Flow (ml/min)	% A	% B	Curve
Initial		99	1	
2	1	99	1	1
22	1	65	35	6
25	1	5	95	6
25.5	1	5	95	6
30	1	99	1	1

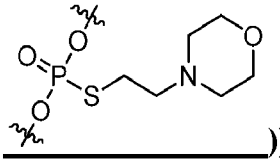
HPLC method 3:

Time	Flow (ml/min)	% A	% B	Curve
Initial		85	15	
2	1	85	15	1
20	1	60	40	6
22	1	5	95	6
25	1	5	95	6
25.5	1	85	15	6
30	1	85	15	1

HPLC method 4:

Time	Flow (ml/min)	% A	% B	Curve
Initial		85	15	
2	1	85	15	1
20	1	40	60	6
22	1	5	95	6
25	1	5	95	6
25.5	1	85	15	6
30	1	85	15	1

[00210] **Example 18. Synthesis of Oligonucleotide 122 (All-(*Rp*)-d[Gs1Cs1Cs1Ts1Cs1As1Gs1Ts1Cs1Ts1Gs1Cs1Ts1Ts1Cs1Gs1Cs1As1Cs1C] (s1 =**



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[00211] Oligonucleotide 122 was synthesized as described above. RT in RP-HPLC: (HPLC method 1): 15.2 min. UPLC/ESI-MS: Calcd for $C_{305}H_{455}N_{86}O_{121}P_{19}S_{19}$: 8460.25; Found: 8462.0.

[00212] **Example 19. Synthesis of Oligonucleotide 123 ((*Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*)-d[Gs1Cs1Cs1Ts1Cs1As1Gs1Ts1Cs1Ts1Gs1Cs1Ts1Ts1Cs1Gs1Cs1As1Cs1C] ((1S-17R-1S)**

[00213] Oligonucleotide 123 was synthesized as described above. RT in RP-HPLC (HPLC method 1): 16.2 min. UPLC/ESI-MS: Calcd for $C_{305}H_{455}N_{86}O_{121}P_{19}S_{19}$: 8460.3; Found: 8461.5.

[00214] **Example 20. Synthesis of Oligonucleotide 124 (All-(*Sp*)-d[Gs1Cs1Cs1Ts1Cs1As1Gs1Ts1Cs1Ts1Gs1Cs1Ts1Ts1Cs1Gs1Cs1As1Cs1C])**

[00215] Oligonucleotide 124 was synthesized as described above. RT in RP-HPLC (HPLC method 1): 18.3 min. UPLC/ESI-MS: Calcd for $C_{305}H_{455}N_{86}O_{121}P_{19}S_{19}$: 8460.3; Found: 8461.8.

[00216] **Example 21. Synthesis of Oligonucleotide 125 (All-(*Rp*)-d[5mCs1As1Ts1G])**

[00217] Oligonucleotide 125 was synthesized as described above. RT in RP-HPLC (HPLC method 2): 16.32 min. UPLC/ESI-MS: Calcd for $C_{58}H_{85}N_{18}O_{22}P_3S_3$: 1575.5; Found: 1575.2.

[00218] In Examples 22 and 42 the oligonucleotide containing stereodefined methoxyethyl phosphorothioate triester internucleotidic linkages was synthesized on an ABI-394 DNA/RNA synthesizer according to the cycle summarized in Table E-5 using 1 μmol synthesis column and 0.8 μmol of oxalyl linked dC on HCP. The synthesis cycle was performed with removal of the terminal 5′-*O*-DMTr group (DMT Off). The solid support was washed with dry ACN and dried under a flux of argon. The dry HCP was placed in a plastic vial and was treated with 1 mL of dry propylamine in dry pyridine (in a 1:4 ratio) for a period of 18 h at r.t. The solvents were then evaporated and the residue was re-suspended with ~pH 1.5 aqueous solution

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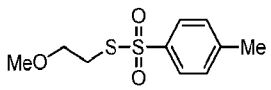
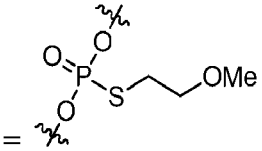
containing 10% DMSO and the HCP support was filtered off. The crude product is purified by reverse phase preparative HPLC (Gradient of 5 to 65% ACN in 20 mM sodium phosphate buffer, pH = 6.0). The fractions having purity above 95% are pooled, concentrated and desalted by reverse-phase HPLC (Gradient of 0 to 80% ACN). The final desalted product is lyophilized from water.

Table E-5. Summary for Oligonucleotide Synthesis on a DNA/RNA Synthesizer ABI-394 Used for the Synthesis of Examples 22 and 42.

step	reaction	reagent	delivery time (sec)	wait time (sec)
1	detritylation	3% TCA in DCM	3 + 60 + 10	N.A.
2	coupling	0.15 M phosphoramidite in ACN + 1.2 M CMPT in ACN	5 + 4	30 + 600
3	capping 1	5% Pac_2O in THF/2,6-lutidine	20	60
4	capping 2	5% Pac_2O in THF/2,6-lutidine + 16% NMI in THF	20	60
5	sulfurization	0.3 M S-MOE Toluylthiosulfonate  in ACN/BSTFA	10 + 4×2	300 + 3×150 + 600

[00219] **Example 22. Synthesis of Oligonucleotide 126 (All-(*Rp*)-d[Cs2As2Gs2T] (s2**



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[00220] Oligonucleotide 126 was synthesized as described above. RT in RP-HPLC (HPLC method 2): 16.23 min. UPLC/ESI-MS: Calcd for $C_{48}H_{68}N_{15}O_{22}P_3S_3$: 1396.2; Found: 1395.2.

[00221] Example 23: The oligonucleotide containing stereodefined *N*-methylpiperazino bulky ester phosphorothioate triester internucleotidic linkages was synthesized on an ABI-394 DNA/RNA synthesizer according to the cycle summarized in Table E-6 using 1 μmol synthesis column and 1.5 μmol of oxalyl linked dT on HCP. The synthesis cycle was performed with removal of the terminal 5′-*O*-DMTr group (DMT Off). The solid support was washed with dry ACN and dried under a flux of argon. The dry HCP was placed in a plastic vial and was treated with 1 mL of dry propylamine in dry pyridine (in a 1:4 ratio) for a period of 18 h at r.t. The solvents were then evaporated and the residue was re-suspended with ~pH 1.5 aqueous solution containing 10% DMSO and the HCP support was filtered off. The crude product was purified by reverse phase preparative HPLC (Gradient of 5 to 65% ACN in 20 mM sodium phosphate buffer, pH = 6.0). The fractions having purity above 95% were pooled, concentrated and desalted by reverse-phase HPLC (Gradient of 0 to 80% ACN). The final desalted product was lyophilized from water.

Table E-6. Summary for Oligonucleotide Synthesis on a DNA/RNA Synthesizer ABI-394 Used for the Synthesis of Example 23.

step	reaction	reagent	delivery time (sec)	wait time (sec)
1	detritylation	3% TCA in DCM	3 + 60 + 10	N.A.
2	coupling	0.15 M phosphoramidite in ACN + 1.2 M CMPT in ACN	5 + 4	30 + 600
3	capping 1	5% Pac_2O in THF/2,6-lutidine	20	60
4	capping 2	5% Pac_2O in THF/2,6-lutidine + 16% NMI in THF	20	60

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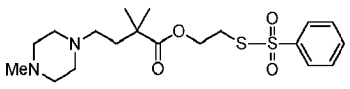
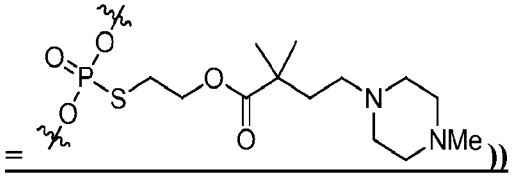
5	sulfurization	0.3 M *N*-Methyl Piperazino ester PhenylThioSulfonate  in ACN/BSTFA	10 + 4×2	300 + 3×150 + 600

[00222] **Example 23. Synthesis of Oligonucleotide 127 (All-(*Rp*)-d[Cs3As3Gs3T] (s3**

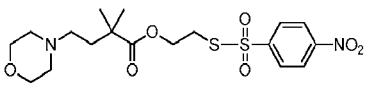


[00223] Oligonucleotide 127 was synthesized as described above. RT in RP-HPLC (HPLC method 2): 20.24 min. UPLC/ESI-MS: Calcd for $C_{78}H_{122}N_{21}O_{25}P_3S_3$: 1943.1; Found: 1941.0.

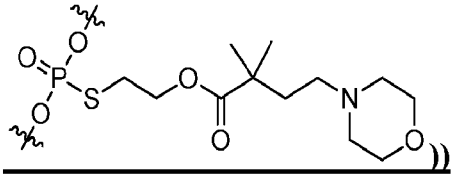
[00224] Examples 24 and 43 The oligonucleotide containing stereodefined morpholino bulky ester phosphorothioate triester internucleotidic linkages was synthesized on an ABI-394 DNA/RNA synthesizer according to the cycle summarized in Table E-7 using 1 μmol synthesis column and 1.5 μmol of oxalyl linked dT on HCP. The synthesis cycle was performed with removal of the terminal 5′-*O*-DMTr group (DMT Off). The solid support was washed with dry ACN and dried under a flux of argon. The dry HCP was placed in a plastic vial and was treated with 1 mL of dry propylamine in dry pyridine (in a 1:4 ratio) for a period of 18 h at r.t. The solvents were then evaporated and the residue was re-suspended with ~pH 1.5 aqueous solution containing 10% DMSO and the HCP support was filtered off. The crude product is purified by reverse phase preparative HPLC (Gradient of 5 to 65% ACN in 20 mM sodium phosphate buffer, pH = 6.0). The fractions having purity above 95% are pooled, concentrated and desalted by reverse-phase HPLC (Gradient of 0 to 80% ACN). The final desalted product is lyophilized from water.

Table E-7. Summary for Oligonucleotide Synthesis on a DNA/RNA Synthesizer ABI-394 Used for the Synthesis of Examples 24 and 43.

step	reaction	reagent	delivery time (sec)	wait time (sec)

1	detritylation	3% TCA in DCM	3 + 60 + 10	N.A.
2	coupling	0.15 M phosphoramidite in ACN + 1.2 M CMPT in ACN	5 + 4	30 + 600
3	capping 1	5% Pac_2O in THF/2,6-lutidine	20	60
4	capping 2	5% Pac_2O in THF/2,6-lutidine + 16% NMI in THF	20	60
5	sulfurization	0.3 M morpholino ester NitroPhenylThioSulfonate  in ACN/BSTFA	10 + 4×2	300 + 3×150 + 600

[00225] **Example 24. Synthesis of Oligonucleotide 128 (All-(*Sp*)-d[Cs4As4Gs4T] (s4 =**

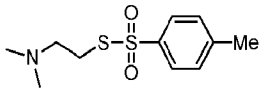


[00226] Oligonucleotide 128 was synthesized as described above. RT in RP-HPLC (HPLC method 3): 19.75 min. UPLC/ESI-MS: Calcd for $C_{75}H_{113}N_{18}O_{28}P_3S_3$: 1902.9; Found: 1904.0.

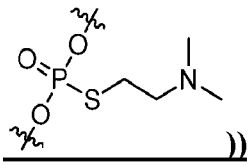
[00227] Example 25: The oligonucleotide containing stereodefined dimethylaminoethyl phosphorothioate triester internucleotidic linkages was synthesized on an ABI-394 DNA/RNA synthesizer according to the cycle summarized in Table E-8 using 1 μmol synthesis column and 1.5 μmol of oxalyl linked dT on HCP. The synthesis cycle was performed with removal of the terminal 5′-*O*-DMTr group (DMT Off). The solid support was washed with dry ACN and dried under a flux of argon. The dry HCP was placed in a plastic vial and was treated with 1 mL of dry propylamine in dry pyridine (in a 1:4 ratio) for a period of 18 h at r.t. The solvents were then evaporated and the residue was re-suspended with ~pH 1.5 aqueous solution containing 10%

DMSO and the HCP support was filtered off. The crude product was purified by reverse phase preparative HPLC (Gradient of 5 to 65% ACN in 20 mM sodium phosphate buffer, pH = 6.0). The fractions having purity above 95% were pooled, concentrated and desalted by reverse-phase HPLC (Gradient of 0 to 80% ACN). The final desalted product was lyophilized from water.

Table E-8. Summary for Oligonucleotide Synthesis on a DNA/RNA Synthesizer ABI-394 Used for the Synthesis of Example 25.

step	reaction	reagent	delivery time (sec)	wait time (sec)
1	detritylation	3% TCA in DCM	3 + 60 + 10	N.A.
2	coupling	0.15 M phosphoramidite in ACN + 1.2 M CMPT in ACN	5 + 4	30 + 600
3	capping 1	5% Pac_2O in THF/2,6-lutidine	20	60
4	capping 2	5% Pac_2O in THF/2,6-lutidine + 16% NMI in THF	20	60
5	sulfurization	0.3 M dimethylaminoethyl-ToluylThioSulfonate  in ACN/BSTFA	10 + 4×2	900 + 3×600 + 900

[00228] **Example 25. Synthesis of Oligonucleotide 129 (All-(*Sp*)-d[Cs5As5Gs5T] (s5 =**



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[00229] Oligonucleotide 129 was synthesized as described above. RT in RP-HPLC (HPLC method 2): 17.25 min. UPLC/ESI-MS: Calcd for $C_{51}H_{77}N_{18}O_{19}P_3S_3$: 1435.4; Found: 1435.0.

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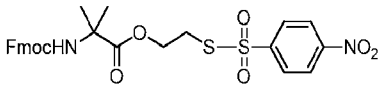
[00230] Example 26: The oligonucleotide containing stereodefined dimethylalanine ester phosphorothioate triester internucleotidic linkages was synthesized on an ABI-394 DNA/RNA synthesizer according to the cycle summarized in Table E-9using 1 μmol synthesis column and 1.5 μmol of oxalyl linked dT on HCP. The synthesis cycle was performed with removal of the terminal 5′-*O*-DMTr group (DMT Off). The solid support was washed with dry ACN and dried under a flux of argon. The dry HCP was placed in a plastic vial and was treated with 1 mL of dry propylamine in dry pyridine (in a 1:4 ratio) for a period of 18 h at r.t. The solvents were then evaporated and the residue was re-suspended with ~pH 1.5 aqueous solution containing 10% DMSO and the HCP support was filtered off. The crude product is purified by reverse phase preparative HPLC (Gradient of 5 to 65% ACN in 20 mM sodium phosphate buffer, pH = 6.0). The fractions having purity above 95% are pooled, concentrated and desalted by reverse-phase HPLC (Gradient of 0 to 80% ACN). The final desalted product is lyophilized from water.

Table E-9. Summary for Oligonucleotide Synthesis on a DNA/RNA Synthesizer ABI-394 Used for the Synthesis of Example 26.

step	reaction	reagent	delivery time (sec)	wait time (sec)
1	detritylation	3% TCA in DCM	3 + 60 + 10	N.A.
2	coupling	0.15 M phosphoramidite in ACN + 1.2 M CMPT in ACN	5 + 4	30 + 600
3	capping 1	5% Pac_2O in THF/2,6-lutidine	20	60
4	capping 2	5% Pac_2O in THF/2,6-lutidine + 16% NMI in THF	20	60
5	sulfurization	0.3 M dimethylalanine-Fmoc ester NitroPhenylThioSulfonate  in ACN/BSTFA	10 + 4×2	900 + 3×600 + 900

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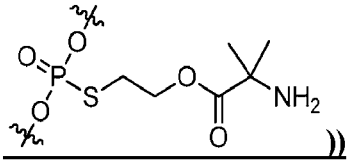
[00231] **Example 26. Synthesis of Oligonucleotide 130 (All-(*Sp*)-d[Cs6As6Gs6T] (s6 =**



[00232] Oligonucleotide 130 was synthesized as described above. RT in RP-HPLC (HPLC method 2): 16.45 min. UPLC/ESI-MS: Calcd for $C_{57}H_{83}N_{18}O_{25}P_3S_3$: 1609.5; Found: 1609.6.

[00233] Examples 27 and 28: The oligonucleotide containing stereodefined *S*-methyl phosphorothioate triester internucleotidic linkages was synthesized on an ABI-394 DNA/RNA synthesizer according to the cycle summarized in Table E-10 using 1 μmol synthesis column and 0.8 μmol of oxalyl linked dC on HCP. The synthesis cycle was performed with removal of the terminal 5′-*O*-DMTr group (DMT Off). The solid support was washed with dry ACN and dried under a flux of argon. The dry HCP was placed in a plastic vial and was treated with 1 mL of dry propylamine in dry pyridine (in a 1:4 ratio) with 50% DMSO for a period of 18 h at r.t. The solvents were then evaporated and the residue was re-suspended with ~pH 1.5 aqueous solution containing 10% DMSO and the HCP support was filtered off. The crude product is purified by reverse phase preparative HPLC (Gradient of 5 to 65% ACN in 20 mM sodium phosphate buffer, pH = 6.0). The fractions having purity above 95% are pooled, concentrated and desalted by reverse-phase HPLC (Gradient of 0 to 80% ACN). The final desalted product is lyophilized from water.

Table E-10. Summary for Oligonucleotide Synthesis on a DNA/RNA Synthesizer ABI-394 Used for the Synthesis of Examples 27 and 28.

step	reaction	reagent	delivery time (sec)	wait time (sec)
1	detritylation	3% TCA in DCM	3 + 60 + 10	N.A.
2	coupling	0.15 M phosphoramidite in ACN + 1.2 M CMPT in ACN	5 + 4	30 + 600

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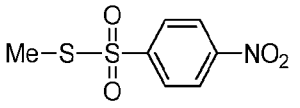
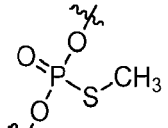
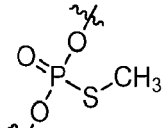
3	capping 1	5% Pac_2O in THF/2,6-lutidine	20	60
4	capping 2	5% Pac_2O in THF/2,6-lutidine + 16% NMI in THF	20	60
5	sulfurization	0.3 M *S*-methyl NitroPhenylThioSulfonate  in ACN	10 + 4×2	300 + 3×150 + 600

[00234] **Example 27. Synthesis of Oligonucleotide 131 (All-(*Rp*)-d[Gs7Cs7Cs7Ts7Cs7As7Gs7Ts7Cs7Ts7Gs7Cs7Ts7Ts7Cs7Gs7Cs7As7Cs7C] (s7 =**  **))**

[00235] Oligonucleotide 131 was synthesized as described above. RT in RP-HPLC: 27.65 min. UPLC/ESI-MS: Calcd for $C_{210}H_{284}N_{67}O_{102}P_{19}S_{19}$: 6576.71; Found: 6575. 6.

[00236] **Example 28. Synthesis of Oligonucleotide 132 (All-(*Sp*)-d[Gs7Cs7Cs7Ts7Cs7As7Gs7Ts7Cs7Ts7Gs7Cs7Ts7Ts7Cs7Gs7Cs7As7Cs7C] (s7 =**  **))**

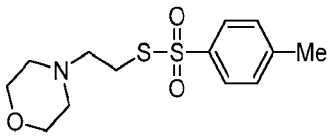
[00237] Oligonucleotide 132 was synthesized as described above. RT in RP-HPLC: 32.65 min. UPLC/ESI-MS: Calcd for $C_{210}H_{284}N_{67}O_{102}P_{19}S_{19}$: 6576.71; Found: 6574.8.

Synthesis of chirally controlled oligonucleotides comprising modified nucleobases

[00238] As generally described above and herein, in some embodiments, the present invention provides chirally controlled oligonucleotides comprising oligonucleotides other than A, T, C and G. In some embodiments, such chirally controlled oligonucleotides comprise 5-methylcytosine (5mC). Non-limiting examples are presented in Examples 21 and below.

[00239] Examples 29-41 were synthesized using the automated synthesis on ABI-394 DNA/RNA synthesizer according to the synthetic cycle summarized on Table E-11 , using 1 μmol synthesis column and 1.75 μmol of oxalyl linked dG on HCP. The synthesis cycle was performed with removal of the terminal 5′-*O*-DMTr group (DMT Off). After completion of the automated oligonucleotide synthesis, the HCP support was washed with dry ACN and dried under vacuum. The dry HCP was placed in a plastic vial and was treated with 1 mL of dry propylamine in dry pyridine (in a 1:4 ratio) for a period of 18 h at r.t. The solvents were then evaporated and the residue was re-suspended with ~pH 1.5 aqueous solution containing 10% DMSO and the HCP support was filtered off. The crude product is purified by reverse phase preparative HPLC (According to the procedure described below). The fractions having purity above 95% are pooled, concentrated and desalted by reverse-phase HPLC (Gradient of 0 to 30% ACN). The final desalted product is lyophilized from water.

[00240] **Table E-11. Summary for Oligonucleotide Synthesis on a DNA/RNA Synthesizer ABI-394 Used for the Synthesis of Examples 29-41.**

step	reaction	reagent	delivery time (sec)	wait time (sec)
1	detritylation	3% TCA in DCM	3 + 60 + 10	N.A.
2	coupling	0.15 M phosphoramidite in ACN + 1.2 M CMPT in ACN	5 + 4	30 + 600
3	capping 1	5% Pac_2O in THF/2,6-lutidine	20	60
4	capping 2	5% Pac_2O in THF/2,6-lutidine + 16% NMI in THF	20	60
5	sulfurization	0.3 M S-Morpholinoethyl ToluylThioSulfonate  in ACN/BSTFA	10 + 4×2	300 + 3×150 + 600

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[00241] General Purification Method for Examples 29-41:

Buffer A: 20 mM Phosphate pH=6.0 (adjusted with phosphoric acid)

Buffer B: ACN

Column: XBridge Prep C_{18}, 5 μm, C_{18}, 250×10mm, Part #186003256

Buffer heater set temperature = 50 °C

Signal monitored at 254 and 280 nm

Gradient used:

Time	Flow (ml/min)	% A	% B	Curve
Initial		99	1	
5	4	99	1	1
10	4	77	23	6
60	4	70	30	6
65	4	20	80	6
70	4	20	80	6
71	4	99	1	6
80	4	99	1	1

[00242] **Example 29. Synthesis of Oligonucleotide 135 (All-(*Rp*)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G])**

[00243] Oligonucleotide 135 was synthesized as described above. RT in RP-HPLC (HPLC method 1): 17.50 min. UPLC/ESI-MS: Calcd for $C_{186}H_{278}N_{51}O_{73}P_{11}S_{11}$: 5090.0; Found: 5091.9.

[00244] **Example 30. Synthesis of Oligonucleotide 136 (All-(*Sp*)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G])**

[00245] Oligonucleotide 136 was synthesized as described above. RT in RP-HPLC (HPLC method 1): 19.25 min. UPLC/ESI-MS: Calcd for $C_{186}H_{278}N_{51}O_{73}P_{11}S_{11}$: 5090.0; Found: 5090.8.

[00246] **Example 31. Synthesis of Oligonucleotide 137 ((*Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G] (1S-9R-1S))**

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[00247] Oligonucleotide 137 was synthesized as described above. RT in RP-HPLC (HPLC method 1): 17.85 min. UPLC/ESI-MS: Calcd for $C_{186}H_{278}N_{51}O_{73}P_{11}S_{11}$: 5090.0; Found: 5091.9.

[00248] **Example 32. Synthesis of Oligonucleotide 138 ((*S*p, *S*p, *R*p, *R*p, *R*p, *R*p, *R*p, *R*p, *S*p, *S*p)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G] (2S-7R-2S))**

[00249] Oligonucleotide 138 was synthesized as described above. RT in RP-HPLC (HPLC method 1): 18.10 min. UPLC/ESI-MS: Calcd for $C_{186}H_{278}N_{51}O_{73}P_{11}S_{11}$: 5090.0; Found: 5091.9.

[00250] **Example 33. Synthesis of Oligonucleotide 139 ((*R*p, *S*p, *S*p, *S*p, *S*p, *S*p, *S*p, *S*p, *S*p, *R*p)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G] (1R-9S-1R))**

[00251] Oligonucleotide 139 was synthesized as described above. RT in RP-HPLC (HPLC method 1): 18.75 min. UPLC/ESI-MS: Calcd for $C_{186}H_{278}N_{51}O_{73}P_{11}S_{11}$: 5090.0; Found: 5088.9.

[00252] **Example 34. Synthesis of Oligonucleotide 140 ((*R*p, *R*p, *S*p, *S*p, *S*p, *S*p, *S*p, *S*p, *S*p, *R*p, *R*p)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G] (2R-7S-2R))**

[00253] Oligonucleotide 140 was synthesized as described above. RT in RP-HPLC (HPLC method 1): 18.72 min. UPLC/ESI-MS: Calcd for $C_{186}H_{278}N_{51}O_{73}P_{11}S_{11}$: 5090.0; Found: 5091.3.

[00254] **Example 35. Synthesis of Oligonucleotide 141 ((*S*p, *S*p, *S*p, *R*p, *R*p, *R*p, *R*p, *R*p, *S*p, *S*p, *S*p)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G] (3S-5R-3S))**

[00255] Oligonucleotide 141 was synthesized as described above. RT in RP-HPLC (HPLC method 1): 18.09 min. UPLC/ESI-MS: Calcd for $C_{186}H_{278}N_{51}O_{73}P_{11}S_{11}$: 5090.0; Found: 5090.9.

[00256] **Example 36. Synthesis of Oligonucleotide 142 ((*R*p, *R*p, *R*p, *S*p, *S*p, *S*p, *S*p, *S*p, *R*p, *R*p, *R*p)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G] (3R-5S-3R))**

[00257] Oligonucleotide 142 was synthesized as described above. RT in RP-HPLC: 18.35 min. UPLC/ESI-MS (HPLC method 1): Calcd for $C_{186}H_{278}N_{51}O_{73}P_{11}S_{11}$: 5090.0; Found: 5088.9.

[00258] **Example 37. Synthesis of Oligonucleotide 143 ((*S*p, *S*p, *R*p, *S*p, *S*p, *R*p, *S*p, *S*p, *R*p, *S*p, *S*p)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G] ((SSR)$_3$-SS))**

[00259] Oligonucleotide 143 was synthesized as described above. RT in RP-HPLC (HPLC method 1): 18.48 min. UPLC/ESI-MS: Calcd for $C_{186}H_{278}N_{51}O_{73}P_{11}S_{11}$: 5090.0; Found: 5092.0.

[00260] **Example 38. Synthesis of Oligonucleotide 144 ((*R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p, *S*p, *R*p, *R*p)-d[5mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1G] ((RRS)$_3$-RR))**

[00261] Oligonucleotide 144 was synthesized as described above. RT in RP-HPLC (HPLC method 1): 18.02 min. UPLC/ESI-MS: Calcd for $C_{186}H_{278}N_{51}O_{73}P_{11}S_{11}$: 5090.0; Found: 5091.4.

[00262] **Example 39. Synthesis of Oligonucleotide 145 (All-(*R*p)-d[5mCs1Ts15mCs1As1Gs1Ts15mCs1Ts1Gs15mCs1Ts1Ts15mCs1Gs15mC])**

[00263] Oligonucleotide 145 was synthesized as described above. RT in RP-HPLC (HPLC method 1): 17.30 min. UPLC/ESI-MS: Calcd for $C_{234}H_{352}N_{62}O_{92}P_{14}S_{14}$: 6388.3; Found: 6390.6.

[00264] **Example 40. Synthesis of Oligonucleotide 146 (All-(*R*p)-d[Gs15mCs1Ts1G])**

[00265] Oligonucleotide 146 was synthesized as described above. RT in RP-HPLC (HPLC method 2): 15.89 min. UPLC/ESI-MS: Calcd for $C_{58}H_{85}N_{18}O_{23}P_3S_3$: 1591.5; Found: 1590.8.

[00266] **Example 41. Synthesis of Oligonucleotide 147 (All-(*R*p)-d[5mCs1As1Gs1T])**

[00267] Oligonucleotide 147 was synthesized as described above. RT in RP-HPLC (HPLC method 2): 16.30 min. UPLC/ESI-MS: Calcd for $C_{58}H_{85}N_{18}O_{22}P_3S_3$: 1575.5; Found: 1575.2.

[00268] **Example 42. Synthesis of Oligonucleotide 148 (All-(*R*p)-d[5mCs2As2Gs2Ts25mCs2Ts2Gs25mCs2Ts2Ts25mCs2G])**

[00269] Oligonucleotide 148 was synthesized as described above, using the sulfurization reagent in Example 22. RT in RP-HPLC (HPLC method 3): 16.51 min. UPLC/ESI-MS: Calcd for $C_{153}H_{223}N_{40}O_{73}P_{11}S_{11}$: 4484.1; Found: 4483.0.

[00270] **Example 43. Synthesis of Oligonucleotide 149 (All-(*R*p)-d[5mCs4As4Gs4Ts45mCs4Ts4Gs45mCs4Ts4Ts45mCs4G])**

[00271] Oligonucleotide 149 was synthesized as described above, using the sulfurization reagent in Example 24. RT in RP-HPLC (HPLC method 4): 17.87 min. UPLC/ESI-MS: Calcd for $C_{252}H_{388}N_{51}O_{95}P_{11}S_{11}$: 6345.6; Found: 6346.5.

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Synthesis of chirally controlled chimeric oligonucleotides comprising different internucleotidic linkages

[00272] As generally described above and herein, in some embodiments, the present invention provides chirally controlled oligonucleotide comprising different internucleotidic linkages. The non-limiting examples below illustrate such chirally controlled oligonucleotides, and the methods of synthesizing the same.

[00273] Examples 44-45 illustrate the synthesis of chirally controlled chimeric oligonucleotide comprising different internucleotidic linkages. The oligonucleotides containing mixed diastereomerically pure morpholinoethyl phosphorothioate triester/phosphorothioate diester internucleotidic linkages were synthesized on an ABI-394 DNA/RNA synthesizer according to the cycle summarized in Table E-12 using 1 μmol synthesis column and 1.5 μmol of oxalyl linked dT on HCP. As each iterative cycle allows for a different sulfurization reagent to be reacted, two different thiosulfonates were used. The synthesis was performed with removal of the terminal 5′-*O*-DMTr group (DMT Off). The solid support was washed with dry ACN and dried under a flux of argon. The dry solid support was then treated with 5 mL of anhydrous 1 M solution of 1,5-diazabicyclo(4.3.0)non-5-ene (DBN) in dry ACN-trimethylsilyl chloride – 16:1 (v/v) for 10 min at r.t. The DBN solution was slowly pushed through the column by means of a plastic luer syringe fixed to the outlet of the column. The support was then washed with dry ACN and dried under vacuum. The dry HCP was placed in a plastic vial and was treated with 1 mL of dry propylamine in dry pyridine (in a 1:4 ratio) for a period of 18 h at r.t. The solvents were then evaporated and the residue was re-suspended with ~pH 1.5 aqueous solution containing 10% DMSO and the HCP support was filtered off. The crude product is purified by reverse phase preparative HPLC (Gradient of 5 to 65% ACN in 20 mM sodium phosphate buffer, pH = 6.0). The fractions having purity above 95% are pooled, concentrated and desalted by reverse-phase HPLC (Gradient of 0 to 80% ACN). The final desalted product is lyophilized from water.

Table E-12. Summary for Oligonucleotide Synthesis on a DNA/RNA Synthesizer ABI-394 Used for the Synthesis of Examples 44-45.

step	reaction	reagent	delivery time (sec)	wait time (sec)

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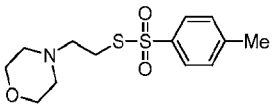
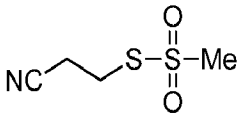
1	detritylation	3% TCA in DCM	3 + 60 + 10	N.A.
2	coupling	0.15 M phosphoramidite in ACN + 1.2 M CMPT in ACN	5 + 4	30 + 600
3	capping 1	5% Pac_2O in THF/2,6-lutidine	20	60
4	capping 2	5% Pac_2O in THF/2,6-lutidine + 16% NMI in THF	20	60
5	sulfurization 1	0.3 M S-Morpholinoethyl Toluylthiosulfonate  in ACN/BSTFA	10 + 4×2	300 + 3×150 + 600
6	sulfurization 2	0.3 M S-cyanoethyl Methylthiosulfonate  in ACN/BSTFA	10 + 4×2	300 + 3×150 + 600

[00274] **Example 44. Synthesis of Oligonucleotide 150 (All-(*Rp*)-d[TsCs1AsT])**

[00275] Oligonucleotide 150 was synthesized as described above. RT in RP-HPLC (HPLC method 2): 12.72 min. UPLC/ESI-MS: Calcd for $C_{45}H_{62}N_{13}O_{21}P_3S_3$: 1310.2; Found: 1309.2

[00276] **Example 45. Synthesis of Oligonucleotide 151 (All-(*Sp*)-d[Cs1AsGs1T])**

[00277] Oligonucleotide 151 was synthesized as described above. RT in RP-HPLC (HPLC method 2): 14.71 min. UPLC/ESI-MS: Calcd for $C_{51}H_{72}N_{17}O_{21}P_3S_3$: 1448.4; Found: 1446.9.

[00278] Example 46 describes the synthesis of chirally controlled chimeric oligonucleotide comprising both phosphate diester internucleotidic linkage and modified

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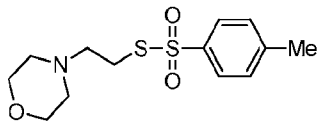
internucleotidic linkages. The exemplary oligonucleotides containing mixed morpholinoethyl phosphorothioate triester/phosphodiester internucleotidic linkages were synthesized on an ABI-394 DNA/RNA synthesizer according to the cycle summarized in Table E-13 using 1 μmol synthesis column and 1.5 μmol of oxalyl linked dT on HCP. As each iterative cycle allows for different sulfurization or oxidation reagents to be reacted, one thiosulfonate reagent was used for sulfurization in one cycle and iodine-promoted oxidation was used in another cycle. The synthesis was performed with removal of the terminal 5′-*O*-DMTr group (DMT Off). The solid support was washed with dry ACN and dried under a flux of argon. The dry HCP was placed in a plastic vial and was treated with 1 mL of dry propylamine in dry pyridine (in a 1:4 ratio) for a period of 18 h at r.t. The solvents were then evaporated and the residue was re-suspended with ~pH 1.5 aqueous solution containing 10% DMSO and the HCP support was filtered off. The crude product was purified by reverse phase preparative HPLC (Gradient of 5 to 65% ACN in 20 mM sodium phosphate buffer, pH = 6.0). The fractions having purity above 95% were pooled, concentrated and desalted by reverse-phase HPLC (Gradient of 0 to 80% ACN). The final desalted product was lyophilized from water.

Table E-13. Summary for Oligonucleotide Synthesis on a DNA/RNA Synthesizer ABI-394 Used for the Synthesis of Example 46.

step	reaction	reagent	delivery time (sec)	wait time (sec)
1	detritylation	3% TCA in DCM	3 + 60 + 10	N.A.
2	coupling	0.15 M phosphoramidite in ACN + 1.2 M CMPT in ACN	5 + 4	30 + 600
3	capping 1	5% Pac_2O in THF/2,6-lutidine	20	60
4	capping 2	5% Pac_2O in THF/2,6-lutidine + 16% NMI in THF	20	60

5	sulfurization	0.3 M S-Morpholinoethyl Toluylthiosulfonate  in ACN/BSTFA	10 + 4×2	300 + 3×150 + 600
6	oxidation	0.02 M I_2, Pyridine/Water	10	300

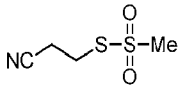
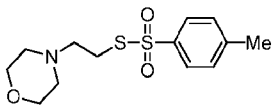
[00279] **Example 46. Synthesis of Oligonucleotide 152 (All-(*Sp*)-d[Cs1AGs1T])**

[00280] Oligonucleotide 152 was synthesized as described above. RT in RP-HPLC (HPLC method 2): 13.42 min. UPLC/ESI-MS: Calcd for $C_{51}H_{72}N_{17}O_{22}P_3S_2$: 1432.3; Found: 1431.7.

[00281] Example 47 describes the synthesis of chirally controlled chimeric oligonucleotide comprising a phosphodiester internucleotidic linkage and both modified chirally pure phosphotriester and phosphodiester internucleotidic linkages. The chirally controlled oligonucleotides containing mixed morpholinoethyl phosphorothioate triester/phosphodiester/phosphorothioate diester linkages were synthesized on an ABI-394 DNA/RNA synthesizer according to the cycle summarized in Table E-14 using 1 μmol synthesis column and 1.5 μmol of oxalyl linked dT on HCP. As each iterative cycle allows for different sulfurization or oxidation reagents to be reacted, two different thiosulfonate reagents were used for the two different sulfurization cycles and iodine-promoted oxidation was used for another cycle. The synthesis was performed with removal of the terminal 5′-*O*-DMTr group (DMT Off). The solid support was washed with dry ACN and dried under a flux of argon. The dry solid support was then treated with 5 mL of anhydrous 1 M solution of 1,5-diazabicyclo(4.3.0)non-5-ene (DBN) in dry ACN-trimethylsilyl chloride – 16:1 (v/v) for 10 min at r.t. The DBN solution was slowly pushed through the column by means of a plastic luer syringe fixed to the outlet of the column. The support was then washed with dry ACN and dried under vacuum. The dry HCP was placed in a plastic vial and was treated with 1 mL of dry propylamine in dry pyridine (in a 1:4 ratio) for a period of 18 h at r.t. The solvents were then evaporated and the residue was re-suspended with ~pH 1.5 aqueous solution containing 10% DMSO and the HCP support was filtered off. The crude product was purified by reverse phase preparative HPLC (Gradient of 5

to 65% ACN in 20 mM sodium phosphate buffer, pH = 6.0). The fractions having purity above 95% were pooled, concentrated and desalted by reverse-phase HPLC (Gradient of 0 to 80% ACN). The final desalted product was lyophilized from water.

[00282] Table E-14. Summary for Oligonucleotide Synthesis on a DNA/RNA Synthesizer ABI-394 Used for the Synthesis of Example 47.

step	reaction	reagent	delivery time (sec)	wait time (sec)
1	detritylation	3% TCA in DCM	3 + 60 + 10	N.A.
2	coupling	0.15 M phosphoramidite in ACN + 1.2 M CMPT in ACN	5 + 4	30 + 600
3	capping 1	5% Pac_2O in THF/2,6-lutidine	20	60
4	capping 2	5% Pac_2O in THF/2,6-lutidine + 16% NMI in THF	20	60
5	sulfurization 1	0.3 M S-cyanoethyl MethylThioSulfonate  in ACN/BSTFA	10 + 4×2	300 + 3×150 + 600
6	sulfurization 2	0.3 M S-Morpholinoethyl ToluylThioSulfonate  in ACN/BSTFA	10 + 4×2	300 + 3×150 + 600
7	oxidation	0.02 M I_2, Pyridine/Water	10	300

[00283] <u>Example 47. Synthesis of Oligonucleotide 153 (All-(*Sp*)-d[CAs1GsT]).</u>

Oligonucleotide 153 was synthesized as described above. RT in RP-HPLC (HPLC method 2): 11.48 min. UPLC/ESI-MS: Calcd for $C_{45}H_{61}N_{16}O_{21}P_3S_2$: 1318.1; Found: 1318.1.

[00284] As will be appreciated by a person skilled in the art, following examples 46 and 47, and other examples and methods described herein, longer chimeric chirally controlled oligonucleotides can be prepared.

Chirally Pure Oligonucleotides Have Different Properties than the Mixture of Diastereomers from Non-Stereospecific Synthesis

[00285] As described above and herein, in some embodiments, the present application provides chirally pure oligonucleotides that have different chemical and biological properties than the mixture of diastereomers with the same base sequence but synthesized through non-stereospecific oligonucleotide synthesis.

[00286] **Example 48. HPLC Profile of Chirally Pure Oligonucleotides and Mixture of Diastereomers from Non-Stereospecific Synthesis.**

[00287] Chirally pure phosphorothioate diester oligonucleotides A (Full R_P, Oligonucleotide 101) and B (Full S_P, Oligonucleotide 102) and non-stereospecific C (stereorandom phosphorothioate diester internucleotidic linkages), which was made by standard non-stereospecific oligonucleotide synthesis, were analyzed by the same RP-HPLC conditions and the corresponding HPLC traces are represented on Figure 2.

[00288] As clearly demonstrated in Figure 2, the stereochemistry of the phosphorothioate diester 20-mer oligonucleotides affects their behavior as determined by RP-HPLC and IEX-HPLC. Without the intention to be limited by theory, a correlation is observed between the retention times (RT) obtained by RP-HPLC and IEX-HPLC, as RT trends are conserved. The full R_P stereoisomer (A) has a shorter retention than the full S_P stereoisomer (B), while the stereorandom phosphorothioate diester oligonucleotide (C), being a mixture of all 2^{19} stereoisomers, has a broad HPLC peak, eluting between the extreme full R_P and full S_P diastereoisomers.

[00289] As will be appreciated by those skilled in the art, the presented data herewith confirm that depicted analyses comparing different chirally controlled or uncontrolled (e.g., stereorandom) oligonucleotide compositions of the same sequence show that the exemplified chirally uncontrolled oligonucleotide composition (i.e., as prepared by non-chiral controlled

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oligonucleotide synthesis), includes only an extremely low level of certain oligonucleotide types, such as the full *Rp* or *Sp* type.

[00290] **Example 49. Thermal Denaturation Experiment (Tm).**

[00291] Each DNA strand was mixed with complementary RNA in equimolar concentration of 0.5 μM in 1×PBS. Total 2.5 mL solution was prepared for each duplex and the mixture was heated at 90 °C for 2 min and was allowed to cool down over the course of several hours. The mixtures were then stored at 4 °C for 2 hrs. Absorbance at 254 nm was recorded at an interval of 0.5 min starting the temperature gradient from 15 °C to 90 °C with rise of 0.5 °C/minute, using a Perkin Elmer UV-spectrophotometer equipped with a Peltier unit. The 254 nm absorbance was plotted against the temperature and the Tm values were calculated from the respective first derivative of each curve.

[00292] Figure 3 illustrates the difference in Tm between two stereopure diastereoisomer phosphorothioate oligonucleotides (full R_P 20-mer A and full S_P 20-mer B) and the stereorandom phosphorothioate oligonucleotide (C). Full R_P phosphorothioate DNA demonstrate higher affinity towards complementary RNA when compared to both the opposite full S_P diastereoisomer and the stereorandom 20-mer.

[00293] Table E-15 below summarizes the differences between chirally controlled oligonucleotides and stereorandom oligonucleotide.

Table E-15. Differences between chirally controlled and stereorandom oligonucleotides.

Oligonucleotide	RT–IEX (min)	Tm (°C) ±0.5	IC_{50} (nM) (rounded up to single digit)
101	14.70	68.3	4
102	15.49	60.5	6
103	15.10	63.4	6
104	15.04	63.5	4
105	14.75	66.5	3

106	15.43	61.8	4
107	15.02	64.5	4
108	15.10	64.8	3
109	14.91	65.8	2
110	15.24	62.9	7
111	15.69	NA	NA
113	15.72	NA	NA
114	14.14	65.2	5
115	14.30	65.2	3
116	14.17	64.8	2
118	15.04	64.5	3
119	6.90	73.4	Not detected
120	15.49	65.3	4

[00294] **Example 50. Biological Activity of Chirally Pure Oligonucleotides**

[00295] After checking OD all oligonucleotide candidate molecules were diluted to a starting concentration of 20μM. Multidose forward transfection experiment was setup in 96-well plates using Hep3B cells (ATCC®, Cat.HB-8064 ™) using Lipofectamine 2000 (Life Technologies ,Cat.11668-019) transfection reagent.

[00296] Transfection protocol:

[00297] $2X10^4$ Hep3B cells per well were seeded and incubated for 24 hours at 37°C in a CO_2 incubator in 100 µl of antibiotic free MEM medium (Life Technologies, Cat.11095098) containing 10% FBS and 1% Glutamax I (Life Technologies, Cat.35050061). Twelve dilutions were setup in nuclease free water (Table E-16).

Table E-16. Concentrations of oligonucleotide transfection stock plate.

Dilution	1	2	3	4	5	6
In µM	20	10	3.33333	1.11111	0.37037	0.12346
Dilution	7	8	9	10	11	12
In µM	0.04115	0.01372	0.00457	0.00152	0.00051	0.00017

[00298] For each well, 0.5μL of Lipofectamine 2000 were mixed with 9.5μL of Opti MEM I reduced serum medium (Life Technologies, Cat. 31985062) and incubated for 5 minutes. Following incubation, 10μL of each of the candidates at the reported concentrations was mixed with the diluted Lipofectamine 2000 by gentle pipetting. The mixture was then incubated for 20 minutes at room temperature to allow for complex formation. During this time the cell growth medium was replaced with 80 μL of fresh antibiotic free MEM as previously described. 20 μL of lipid-oligo complex was gently mixed into each well bringing the total volume to 100 μL. The cells were then incubated for 24 hours at 37 °C in a CO_2 incubator.

[00299] RNA Extraction:

[00300] After 24 hour incubation the cell growth medium was removed and RNA was extracted using a Dynabeads mRNA Direct kit (Life Technologies, Cat. 61012) per the instructions provided in the kit manual without modifications. This magnetic Oligo $(dT)^{25}$ bead system allows for cost-effective and robust high-throughput poly (A) RNA extraction circumventing DNAse treatment. RNA was eluted in nuclease-free water and stored at -80 °C.

[00301] cDNA synthesis:

[00302] 10 μL of RNA was used in a 20 μL cDNA synthesis reaction using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Cat. 4374967) using the kit protocol with RNAse Inhibitor. The reverse transcription was performed in a 96-well format on a C1000 Touch thermal cycler (Biorad, Cat. 185-1196EDU).

[00303] Gene expression analysis:

[00304] Gene expression was measured by quantitative PCR using a LightCycler® 480 SYBR Green I Master (Roche, Cat. 04707516001) on LightCycler 480 Real-Time PCR instrument. Primers for the *Homo Sapiens* sequences of the target Apolipoprotein B (Apo B) (NM_000384) and endogenous control Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (NM_002046) were designed and ordered from IDT (Table E-17)..

[00305] **Table E-17. Sequences of HPLC purified primers used for gene expression quantification.**

Primer Name	Target	Sequence	Position
Apo B Forward primer	*H.sapiens* Apo B	AGCCTTGGTGGATACCCTGAAGTT	3209-3232
Apo B Reverse primer	*H.sapiens* Apo B	TGGACAAGGTCATACTCTGCCGAT	3289-3312
GAPDH Forward primer	*H.sapiens* GAPDH	CTCTGCTCCTCCTGTTCGAC	30-49
GAPDH Reverse primer	*H.sapiens* GAPDH	ACGACCAAATCCGTTGACTC	122-141

[00306] Measurement of the target and endogenous control was performed in separate wells using material from the same cDNA template. SYBR green assay PCR conditions were setup as described in the SYBR Green I Master protocol (Table E-18).

Table E-18: Real-time PCR conditions for SYBR green assay.

Step	Number of Cycles
Pre-incubation	1
Amplification	45
Melting curve	1
Cooling	1

Degrees °C	Time	Acquisition Mode	Ramp rate °C/s	Acquisition per °C
Pre-incubation				
95	5min	None	4.4	None
Amplification				
95	10 sec	None	4.4	None
60	10 sec	None	2.2	None
72	10 sec	Single	4.4	None
Melting Curve				
95	5 sec	None	4.4	None
65	1 min	None	2.2	None
97		Continuous	0.11	5
Cooling				
40	30 sec	None	2.2	None

[00307] Melt Curve Analysis show single amplicon peaks for each primer pair (Figure 4).

[00308] The data is a result of 3 biological replicates. All samples were compared to Naïve untransfected control. The following results were observed when evaluating gene expression by real-time PCR using SYBR green (Table E-19, Figure 5).

Table E-19. Complete IC_{50} data evaluated by SYBR green.

Oligonucleotide	IC_{50} (nM)
101 All-R	4.368
102 All-S	6.345
103 5R-9S-5R	5.727
104 5S-9R-5S	4.291
105 1S-17R-1S	2.877
106 1R-17S-1R	~ 3.855
107 $(R/S)_9R$	~ 4.180

108 $(S/R)_9S$	3.189
118 Stereorandom	3.087
109 3S-13R-3S	1.722
110 3R-13S-3R	7.437
114 3R-13S-3R	5.234
115 $S-(RRS)_6$	2.689
116 $RS-(RRS)_5-RR$	1.919
120 Mipomersen	4.386

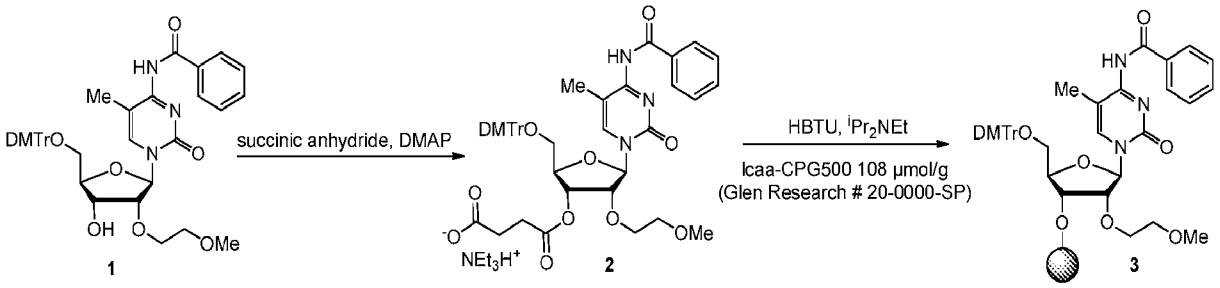
[00309] In some embodiments, provided chirally controlled oligonucleotides show an IC_{50} below 8 nM. In some embodiments, provided chirally controlled oligonucleotides show an IC_{50} below 7 nM. In some embodiments, provided chirally controlled oligonucleotides show an IC_{50} below 6 nM. In some embodiments, provided chirally controlled oligonucleotides show an IC_{50} below 5 nM. In some embodiments, provided chirally controlled oligonucleotides show an IC_{50} below 4 nM. In some embodiments, provided chirally controlled oligonucleotides show an IC_{50} below 3 nM. In some embodiments, provided chirally controlled oligonucleotides show an IC_{50} below 2 nM. In some embodiments, provided chirally controlled oligonucleotides show an IC_{50} within a range of 0.5–8 nM. In some embodiments, provided chirally controlled oligonucleotides show an IC_{50} within a range of 1–4 nM. In some embodiments, provided chirally controlled oligonucleotides show an IC_{50} within a range of 1.5-3 nM. In some embodiments, provided chirally controlled oligonucleotides show an IC_{50} within a range of 1.5-2 nM. In some embodiments, provided chirally controlled oligonucleotides show an IC_{50} within a range of 0.5-2 nM. In some embodiments, provided chirally controlled oligonucleotides show an IC_{50} within a range of 1-2.5 nM. In some embodiments, provided chirally controlled oligonucleotides show an IC_{50} within a range of 1.5-3 nM. In some embodiments, provided chirally controlled oligonucleotides show an IC_{50} within a range of 2.5-5 nM. In some embodiments, provided chirally controlled oligonucleotides show an IC_{50} within a range of 3-6 nM. In some embodiments, provided chirally controlled oligonucleotides show an IC_{50} within a

range of 5-8 nM. In some embodiments, provided chirally controlled oligonucleotides show an IC_{50} within a range between an upper boundary and a lower boundary. In some such embodiments, the upper boundary is 8, 5, 4, or 3 nM. In some such embodiments, the lower boundary is 1, 1.5, 2, or 2.5 nM. As can be seen with reference to the Examples, the present disclosure specifically exemplifies various chirally controlled oligonucleotides or chirally controlled oligonucleotide compositions showing representative such IC_{50} values.

[00310] Table E-19 illustrates that when test in chirally controlled form, certain chirally controlled oligonucleotides/oligonucleotide compositions are more potent than the corresponding stereorandom oligonucleotide mixture (Oligonucleotides 105, 109, 115 and 116 compared to Oligonucleotide 118); they are also more active than Mipomersen, which is a stereorandom oligonucleotide mixture (Oligonucleotides 104, 105, 106, 107, 109, 115 and 116 compared to 120).

Additional Chirally Controlled Preparations

Example 51. Preparation of Chirally Controlled Preparations of Oligonucleotides



[00311] The present Example describes preparation of a variety of particular chirally controlled composition of certain oligonucleotides as described herein. In particular, the present Example describes oligonucleotide preparation on utilizing loaded lcaa-CPG-500.

[00312] N^4-Bz-5'-*O*-DMTr-2'-*O*-MOE-5mC (**1**) (4.0 g, 5.5 mmol) was dissolved in anhydrous DCM (20 mL) and mixed with 2 equiv of succinic anhydride (1.1 g, 11.1 mmol) and 3 equiv of 4-*N,N*-dimethylaminopyridine (2.0 g, 16.6 mmol). The reaction was stirred under Argon at room temperature. After complete consumption of the starting material as determined by TLC (1 hour), the solvents were evaporated to dryness, the crude residue was dissolved in DCM containing 1% triethylamine then purified by flash silica gel chromatography using a gradient of

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0 – 2% of MeOH in DCM containing 2% triethylamine. Yield of pure compound (**2**) after evaporation was 4.5 g, 88%. The resulting 3'-*O*-succinate (**2**) (0.92 g, 1.0 mmol), *N,N*-diisopropylethylamine (0.82 ml, 5.0 mmol) and CPG-500 (10g) were taken up in DMF (50mL) then HBTU (0.42 g, 1.1 mmol) was added. The mixture was shaken for 2 h then filtered. The support was washed with DMF, MeOH and finally, DCM then dried *in vacuo*. Trityl cation analysis (monitoring at 504 nm) showed that the loading of the nucleoside on the support (**3**) was 63 μmol/g.

[00313] Chirally controlled compositions of oligonucleotides containing stereodefined chimeric deoxy and 2'-*O*-MOE phosphorothioate triester internucleotidic linkages was synthesized on MerMade-12 DNA/RNA synthesizer (BioAutomation) according to the cycle summarized in Table E-20 using a MM-6-200 synthesis column (BioAutomation) loaded with 2.0 g (126 μmol) of uncapped succinyl linked N^4-Bz-5'-*O*-DMTr-2'-*O*-MOE-5mC (63 μmol/g, lcaa CPG-500 from Glen Research. The oligonucleotide synthesis cycle was performed with a preliminary capping step (capping 2) and without removal of the final terminal 5'-*O*-DMTr oligonucleotide group (DMT On). The stereospecific sulfurization steps were performed using the 0.3 M *S*-(2-cyanoethyl)methylthiosulfonate reagent following the coupling of the corresponding chiral phosphoramide and the two-step capping process (Table E-20).

[00314] Once the automated oligonucleotide synthesis cycle was completed with the final 5'-*O*-DMTr group kept On, the synthesis column was taken off the DNA/RNA synthesizer and dried under vacuum. The dried support was transferred onto an empty glass manual peptide synthesizer and 40 mL solution of 0.5 M 1,5-diazabicyclo[4.3.0]non-5-ene (DBN), 0.25 M *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) in ACN was continuously passed through the support for 5 min without stopping the flow in the manual peptide synthesizer. The support was washed by 50%Py/ACN and dried with Argon flow for 1 sec. Then, the support was transferred into an empty screw-cap plastic vial and treated with 5% EtOH/conc NH_3 (20 mL) at 60 °C for 6 h, and left at room temperature for 12 h. The support was removed by filteration and washed with conc. NH_3. The filtrate was dried *in vacuo* then the resultant was dissolved in 50 mM TEAA (120 mL). Further filtration was performed when there was a suspension in the solution. The solution was loaded on a Sep-Pak cartridge (Waters, Sep-Pak Vac 35cc (10g) C_{18} Cartridges). The cartridge was washed with 20% ACN/50 mM TEAA (70 mL) to remove all capped truncated sequences and 50% ACN/water containing 0.5% of conc NH_3 (50 mL) to elute

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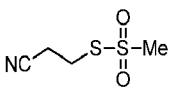
the full length DMT On oligonucleotide. The solution containing the DMT On oligonucleotide was dried *in vacuo* then diluted with 50 mM TEAA (120 mL) and loaded on another Sep-Pak cartridge (Waters, Sep-Pak Vac 35cc (10g) C_{18} Cartridges). The cartridge was washed with milli Q water (50 mL), 2%TFA/water (50 mL), then water (50 mL). The DMT Off oligonucleotide was eluted with 50%ACN/water containing 0.5% of conc NH_3 (50 mL).

Table E-20. Summary for Oligonucleotide Synthesis on a DNA/RNA Synthesizer MerMade 12 Used for Chirally Controlled Synthesis.

step	reaction	reagent	delivery volume (mL)	wait time (sec)
1	detritylation	3% TCA in DCM	3 × 10	3 × 24
2	coupling	0.15 M chiral phosphoramidite in ACN + 1.2 M CMPT in ACN	2 × 3.4	2 x 450 (MOE) 2 x 300 (DNA)
3	capping 1	5% Pac_2O in THF/2,6-lutidine	8	60
4	capping 2	5% Pac_2O in THF/2,6-lutidine + 16% NMI in THF	6.8	60
5	sulfurization	0.3 M *S*-(2-cyanoethyl) methanethiosulfonate  in ACN/BSTFA	9	600

[00315] Synthesis of Oligonucleotide ONT-75 (All (*R*p))-Gs5mCs5mCsTs5mCsAs GsTs5mCsTsGs5mCsTsTs5mCsGs5mCsAs 5mCs5mC

[00316] Synthesis of Oligonucleotide ONT-80 (All (*S*p))-Gs5mCs5mCsTs5mCsAs GsTs5mCsTsGs5mCsTsTs5mCsGs5mCsAs 5mCs5mC

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[00317] Synthesis of Oligonucleotide ONT-77 (*Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*)-Gs5mCs5mCsTs5mCsAs GsTs5mCsTsGs5mCsTsTs5mCsGs5mCsAs 5mCs5mC (5R-10S-4R)

[00318] Synthesis of Oligonucleotide ONT-81 (*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-Gs5mCs5mCsTs5mCsAs GsTs5mCsTsGs5mCsTsTs5mCsGs5mCsAs 5mCs5mC (5S-10R-4S)

[00319] Synthesis of Oligonucleotide ONT-87 (*Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Rp*, *Sp*, *Sp*, *Rp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*)-Gs5mCs5mCsTs5mCsAs GsTs5mCsTsGs5mCsTsTs5mCsGs5mCsAs 5mCs5mC (5R-$(SSR)_3$-5R)

[00320] Synthesis of Oligonucleotide ONT-88 (*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*)-Gs5mCs5mCsTs5mCsAs GsTs5mCsTsGs5mCsTsTs5mCsGs5mCsAs 5mCs5mC (5S-$(RRS)_3$-5S)

[00321] Synthesis of Oligonucleotide ONT-89 (*Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*)-Gs5mCs5mCsTs5mCsAs GsTs5mCsTsGs5mCsTsTs5mCsGs5mCsAs 5mCs5mC ($(SR)_9S$)

[00322] Synthesis of Oligonucleotide ONT-82 (All (*Rp*))-GsTs5mCs5mCs5mCsTsGsAsAsGsAsTsGsTs5mCsAsAsTsGs5mC

[00323] Synthesis of Oligonucleotide ONT-84 (All (*Sp*))-GsTs5mCs5mCs5mCsTsGsAsAsGsAsTsGsTs5mCsAsAsTsGs5mC

[00324] Synthesis of Oligonucleotide ONT-85 (*Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*)-GsTs5mCs5mCs5mCsTsGsAsAsGsAsTsGsTs5mCsAsAsTsGs5mC (5R-10S-4R)

[00325] Synthesis of Oligonucleotide ONT-86 (*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-GsTs5mCs5mCs5mCsTsGsAsAsGsAsTsGsTs5mCsAsAsTsGs5mC (5S-10R-4S)

Example 52: General RP-HPLC method for the analysis of crude DMT On and purified DMT Off oligonucleotides

[00326] The present Example describes RP-HPLC analysis of crude and purified oligonucleotide compositions prepared by chirally controlled synthesis as described herein.

Buffer A: 50 mM TEAA, pH 7.0

Buffer B: ACN

Column: XBridge C_{18}, 3.5 μm, C_{18}, 4.6x50mm, Part #186003034

Column temperature = 50 °C

Signal monitored at 254 and 280 nm

Gradient used:

Time(min)	Flow (mL/min)	% A	% B	Curve
Initial		99	1	
2	1	99	1	6
52	1	50	50	6
55	1	5	95	6
55.5	1	5	95	6
56	1	99	1	6
60	1	99	1	1

RP-HPLC of Crude DMT on:	**Figure 35:**
ONT-75 (All (*Rp*))-Gs5mCs5mCsTs5mCsAs GsTs5mCsTsGs5mCsTsTs5mCsGs5mCsAs 5mCs5mC	(Panel A)
ONT-80 (All (*Sp*))-Gs5mCs5mCsTs5mCsAs GsTs5mCsTsGs5mCsTsTs5mCsGs5mCsAs 5mCs5mC	(Panel B)
ONT-77: (*Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*)-Gs5mCs5mCsTs5mCsAs GsTs5mCsTsGs5mCsTsTs5mCsGs5mCsAs 5mCs5mC (5R-10S-4R)	(Panel C)
ONT-81 (*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-Gs5mCs5mCsTs5mCsAs GsTs5mCsTsGs5mCsTsTs5mCsGs5mCsAs 5mCs5mC (5S-10R-4S)	(Panel D)
ONT-87 (*Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Rp*, *Sp*, *Sp*, *Rp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*)-Gs5mCs5mCsTs5mCsAs GsTs5mCsTsGs5mCsTsTs5mCsGs5mCsAs 5mCs5mC (5R-$(SSR)_3$-5R)	(Panel E)
ONT-88 (*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*)-Gs5mCs5mCsTs5mCsAs GsTs5mCsTsGs5mCsTsTs5mCsGs5mCsAs 5mCs5mC (5S-$(RRS)_3$-5S)	(Panel F)
ONT-89 (*Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*)-Gs5mCs5mCsTs5mCsAs GsTs5mCsTsGs5mCsTsTs5mCsGs5mCsAs 5mCs5mC ($(SR)_9S$)	(Panel G)
ONT-82 (All (*Rp*))-GsTs5mCs5mCs5mCsTsGsAsAsGsAsTsGsTs5mCsAsAsTsGs5mC	(Panel H)
ONT-84 (All (*Sp*))-GsTs5mCs5mCs5mCsTsGsAsAsGsAsTsGsTs5mCsAsAsTsGs5mC	(Panel I)
ONT-85 (*Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*,	(Panel J)

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Rp)- GsTs5mCs5mCs5mCsTsGsAsAsGsAsTsGsTs5mCsAsAsTsGs5mC (5R-10S-4R)	
ONT-86 (*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-GsTs5mCs5mCs5mCsTsGsAsAsGsAsTsGsTs5mCsAsAsTsGs5mC (5S-10R-4S)	(Panel K)

Example 53: General IEX-HPLC method for the analysis of crude DMT On and purified DMT Off oligonucleotides

[00327] The present Example describes IEX-HPLC analysis of crude and purified oligonucleotide compositions prepared by chirally controlled synthesis as described herein.

Buffer A: 10 mM TrisHCl, 50%ACN, pH 8.0

Buffer B: 10 mM TrisHCl, 800 mM $NaClO_4$, 50%ACN, pH 8.0

Column: DIONEX, DNAPac, PA-100, Analytical, 4.0x250mm, Part #063000

Column temperature = 60 °C

Signal monitored at 254 and 280 nm

Gradient used:

Time (min)	Flow (mL/min)	% A	% B	Curve
Initial		85	15	
3	1	85	15	1
23	1	60	40	6
25	1	5	95	6
25.5	1	85	15	6
30	1	85	15	1

Example 54: General UPLC-LCMS method for the analysis of purified DMT Off oligonucleotides

[00328] The present Example describes UPLC-LCMS analysis of purified oligonucleotide compositions prepared by chirally controlled synthesis as described herein.

Buffer A: 15 mM TEA, 400 mM HFIP, Water

Buffer B: 50:50 Buffer A/Methanol

Column: UPLC@OST C_{18} 1.7 μm, 2.1×500mm

Column temperature = 50 °C

Gradient used:

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.2	80	20	
2	0.2	80	20	1

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22	0.2	30	70	6
25	0.2	5	95	6
25.5	0.2	80	20	6
30	0.2	80	20	1

[00329] **Example 55: General IEX-HPLC method for the purification of crude DMT Off oligonucleotide**

[00330] The present Example describes IEX-HPLC purification of crude oligonucleotide compositions prepared by chirally controlled synthesis as described herein.

Buffer A: 20 mM NaOH, pH 11.0

Buffer B: 20 mM NaOH, 2.5 M NaCl, pH 11.0

Column: Empty column Waters AP-2 (Waters), custom in-house packed with Source 15Q support (GE Healthcare). Individual purification columns were packed and used for the different stereopure oligonucleotides.

Instrument: AKTA Purifier, equipped with the P-900 pump, the UPC-900 detector and the 50 mL injection SuperLoop (GE Healthcare)

Buffer heater temperature set = 70 °C

Signal monitored at 254 nm

Fractions volume: 5 mL

Gradient used:

Time (min)	Flow (mL/min)	% A	% B	Curve
Initial		100	0	
25	10	100	0	1
40	10	85	15	6
60	10	85	15	1
80	10	70	30	6
100	10	70	30	1
140	10	60	40	6
180	10	60	40	1
200	10	45	55	6
210	10	45	55	1
211	10	100	0	6
235	10	100	0	1

[00331] Collected fractions were individually analyzed by analytical IEX-HPLC using the analytical conditions and gradient described above. Pure fractions were pooled in order to

provide purified material of 95% and above purity as determined by the 254 nm UV absorbance profiles.

RP-HPLC of Purified DMT Off	**Figure 36:**
ONT-75 (All (*Rp*))-Gs5mCs5mCsTs5mCsAs GsTs5mCsTsGs5mCsTsTs5mCsGs5mCsAs 5mCs5mC	(Panel A)
ONT-80 (All (*Sp*))-Gs5mCs5mCsTs5mCsAs GsTs5mCsTsGs5mCsTsTs5mCsGs5mCsAs 5mCs5mC	(Panel B)
ONT-77 (*Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*)-Gs5mCs5mCsTs5mCsAs GsTs5mCsTsGs5mCsTsTs5mCsGs5mCsAs 5mCs5mC (5R-10S-4R)	(Panel C)
ONT-81 (*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-Gs5mCs5mCsTs5mCsAs GsTs5mCsTsGs5mCsTsTs5mCsGs5mCsAs 5mCs5mC (5S-10R-4S)	(Panel D)
ONT-87 (*Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Rp*, *Sp*, *Sp*, *Rp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*)-Gs5mCs5mCsTs5mCsAs GsTs5mCsTsGs5mCsTsTs5mCsGs5mCsAs 5mCs5mC (5R-$(SSR)_3$-5R)	(Panel E)
ONT-88 (*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*)-Gs5mCs5mCsTs5mCsAs GsTs5mCsTsGs5mCsTsTs5mCsGs5mCsAs 5mCs5mC (5S-$(RRS)_3$-5S)	(Panel F)
ONT-89 (*Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*)-Gs5mCs5mCsTs5mCsAs GsTs5mCsTsGs5mCsTsTs5mCsGs5mCsAs 5mCs5mC ($(SR)_9$S)	(Panel G)
ONT-82 (All (*Rp*))- GsTs5mCs5mCs5mCsTsGsAsAsGsAsTsGsTs5mCsAsAsTsGs5mC	(Panel H)
ONT-84 (All (*Sp*))- GsTs5mCs5mCs5mCsTsGsAsAsGsAsTsGsTs5mCsAsAsTsGs5mC	(Panel I)
ONT-85 (*Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*)- GsTs5mCs5mCs5mCsTsGsAsAsGsAsTsGsTs5mCsAsAsTsGs5mC (5R-	(Panel J)

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10S-4R)	
ONT-86 (*Sp*, *Sp*, *Sp*, *Sp*, *Sp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Rp*, *Sp*, *Sp*, *Sp*, *Sp*)-GsTs5mCs5mCs5mCsTsGsAsAsGsAsTsGsTs5mCsAsAsTsGs5mC (5S-10R-4S)	(Panel K)

[00332] Table E-21. Compilation of the physico-chemical properties for the stereopure phosphorothioate oligonucleotides synthesized as described in Example XX.

[00333] Sequence A: human ApoB sequence 5′-Gs5mCs5mCsTs5mCsAsGsTs5mCsTsGs5mCsTsTs5mCsGs5mCsAs5mCs5mC-3′.

[00334] Sequence B: mouse ApoB sequence 5′-GsTs5mCs5mCs5mCsTsGsAsAsGsAsTsGsTs5mCsAs AsTsGs5mC-3′.

[00335] Underlined nucleotides designate 2′-*O*-MOE. s = phosphorothioate linkage. 5mC = 5-methyl-2′-deoxycytidine. 5mC = 5-methyl-2′-*O*-MOE-cytidine. Stereo architecture describes the stereoisomer nature (*Rp*/*Sp*) of each phosphorus atom in a given phosphorothioate linkage of the oligonucleotide. Retention time (t_R) in IEX-HPLC and Found molecular weight (MW) values were obtained using the corresponding analytical methods for the purified compounds (described above).

sequence ID	sequence	stereo architecture	t_R IEX-HPLC (min)	Calc MW	Found MW
ONT-41	A	Stereorandom diastereomixture	17.34	7177.2	7175.7
ONT-75	A	All *Rp*	16.44	7177.2	7177.0
ONT-77	A	5R-10S-4R	17.44	7177.2	7177.5
ONT-80	A	All *Sp*	18.13	7177.2	7175.5
ONT-81	A	5S-10R-4S	17.67	7177.2	7175.8
ONT-87	A	5R-$(SSR)_3$-5R	13.64	7177.2	-
ONT-88	A	5S-$(RRS)_3$-5S	14.88	7177.2	7177.0
ONT-89	A	$(SR)_9S$	14.25	7177.2	-

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ONT-83	B	Stereorandom diastereomixture	17.02	7233.2	7231.8
ONT-82	B	All *Rp*	16.43	7233.2	7231.6
ONT-84	B	All *Sp*	19.51	7233.2	7232.1
ONT-85	B	5R-10S-4R	18.76	7233.2	7231.1
ONT-86	B	5S-10R-4S	18.75	7233.2	7230.9

Example 56: General RP-HPLC method for the overlay analyses of purified DMT Off oligonucleotides

[00336] The present Example describes RP-HPLC analysis of purified oligonucleotide compositions prepared by chirally controlled synthesis as described herein.

Buffer A: 50 mM TEAA, pH 7.0

Buffer B: ACN

Column: XBridge C_{18}, 3.5 μm, C_{18}, 4.6×50mm

Column temperature = 50 °C

Signal monitored at 254 and 280 nm

Gradient used:

Time(min)	Flow (mL/min)	% A	% B	Curve
Initial		95	5	
2	1	95	5	1
52	1	70	30	6
54	1	5	95	6
54.5	1	5	95	1
55	1	99	1	6
60	1	99	1	1

[00337] Panel A of Figure 37 is an overlay of RP-HPLC traces of Purified DMT Off Oligonucleotide ONT-75, ONT-77, ONT-80, ONT-81, ONT-87, ONT-88, ONT-89 and ONT-41 (*diastereomixture*)-Gs5mCs5mCsTs5mCsAsGsTs5mCsTsGs5mCsTsTs5mCsGs5mCsAs 5mCs5mC .

[00338] Panel B of Figure 37 shows an expanded view of Panel A, with each curve labeled as follows:

Curve #	Oligonucleotide
1	diastereomixture (ONT-41)
2	all-Rp (ONT-75)
3	5R-$(SSR)_3$-5R (ONT-87)
4	$(SR)_9$-S (ONT-89)
5	5R-10S-4R (ONT-77)
6	5S-10R-4S (ONT-81)
7	5S-$(RRS)_3$-5S (ONT-88)
8	all-Sp (ONT-80)

[00339] Panel A of Figure 38 is an overlay of RP-HPLC of Purified DMT Off Oligonucleotide ONT-82, ONT-84, ONT-85, ONT-86, and ONT-83 (*diastereomixture*)-GsTs5mCs5mCs5mCsTsGsAsAsGsAsTsGsTs5mCsAsAsTsGs5mC

[00340] Panel B of Figure 38 shows an expanded view of Panel A, with each curve labeled as follows:

Curve #	Oligonucleotide
1	diastereomixture (ONT-83)
2	all-Rp (ONT-82)
3	5S-10R-4S (ONT-86)
4	5R-10S-4R (ONT-85)
5	all-Sp (ONT-84)

[00341] **Example 57: Thermal Denaturation Experiment (Tm)**

[00342] The present Example describes characterization of chirally controlled oligonucleotide compositions using thermal denaturation.

[00343] Each DNA strand was mixed with its complementary RNA strand in equimolar concentration of 1 μM in 1×PBS. Total 3 mL solution was prepared for each duplex and the

mixture was heated at 90 °C for 2 min and was allowed to cool down over the course of several hours. The mixtures were then stored at 4 °C for 2 hrs. Absorbance at 254 nm was recorded at an interval of 0.5 min starting the temperature gradient from 15.0 °C to 95.0 °C with rise of 0.5 °C/minute, using Cary100 Series (Agilent Technologies). The 254 nm absorbance was plotted against the temperature and the Tm values were calculated from the respective first derivative of each curve.

[00344] Figure 39 presents a Tm overlay of chirally controlled oligonucleotides and stereorandom oligonucleotide.

[00345] Figure 39 illustrates the difference in Tm between four stereopure diastereoisomer phosphorothioate oligonucleotides (all-Rp 20-mer, all-Sp 20-mer, 5R-10S-4R and 5S-10R-4S gapmers) and the stereorandom phosphorothioate oligonucleotide. Full *R*p phosphorothioate DNA demonstrates the highest affinity towards complementary RNA (Tm = 85.1 °C) when compared to full *S*p 20-mer (Tm = 75.1 °C) which has the lowest affinity. 5R-10S-4R and 5S-10R-4S gapmers and the stereorandom phosphorothioate oligonucleotides all showed intermediates values (Tm range = 80.1-81.2 °C).

[00346] Table E-22, below, gives Tm values for the studied stereopure and stereorandom phosphorothioate oligonucleotides having the human ApoB sequence 5′-Gs5mCs5mCsTs5mCsAsGsTs5mCsTsGs5mCsTsTs5mCsGs5mCsAs5mCs5mC-3′ with various stereochemistry architectures on the phosphorothioate backbone.

Table E-22

sequence ID	stereo architecture	Tm (°C)
ONT-41	Stereorandom diastereomixture	81.0
ONT-75	All *R*p	85.1
ONT-77	5R-10S-4R	80.1
ONT-80	All *S*p	75.1
ONT-81	5S-10R-4S	81.2

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Example 58: Preparation of Exemplary Chirally Controlled siRNA Oligonucleotides Targeting PCSK9

[00347] Proprotein convertase subtilisin/kexin type 9 (PCSK9), is an enzyme involved in cholesterol metabolism. PCSK9 binds to the receptor for low density lipoprotein (LDL), triggering its destruction. Although LDL associated with the receptor is also eliminated when the receptor is destroyed, the net effect of PCSK9 binding in fact increases LDL levels, as the receptor would otherwise cycle back to the cell surface and remove more cholesterol.

[00348] Several companies are developing therapeutic agents that target PCSK9. Of particular relevance to the present disclosure, each of Isis Pharmaceuticals, Santaris Pharma, and Alnylam Pharmaceuticals is developing a nucleic acid agent that inhibits PCSK9. The Isis Pharmaceuticals product, an antisense oligonucleotide, has been shown to increase expression of the LDLR and decrease circulating total cholesterol levels in mice (Graham et al "Antisense inhibition of proprotein convertase subtilisin/kexin type 9 reduces serum LDL in hyperlipidemic mice". *J. Lipid Res.* **48** (4): 763–7, April 2007). Initial clinical trials with the Alnylam Pharmaceuticals product, ALN-PCS, reveal that RNA interference offers an effective mechanism for inhibiting PCSK9 (Frank-Kamenetsky et al "Therapeutic RNAi targeting PCSK9 acutely lowers plasma cholesterol in rodents and LDL cholesterol in nonhuman primates". *Proc. Natl. Acad. Sci. U.S.A.* **105** (33): 11915–20, August 2008).

[00349] The present Example describes preparation of a variety of chirally controlled or stereorandom siRNA agents directed to PCSK9. Specifically, this Example describes preparation of each of the following oligonucleotide compositions as illustrated in Table E-23 below:

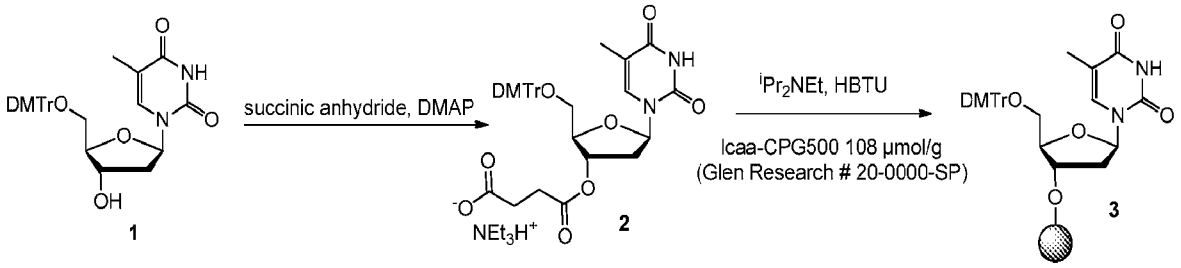
Table E-23:

ONT #	Sequence	Type
ONT-106	(*R*p)- uucuAGAccuGuuuuGcuudT**s**dT	PCSK9 sense
ONT-107	(*S*p)- uucuAGAccuGuuuuGcuudT**s**dT	PCSK9 sense
ONT-108	(*R*p)- AAGcAAAAcAGGUCuAGAAdT**s**dT	PCSK9 antisense
ONT-109	(*S*p)- AAGcAAAAcAGGUCuAGAAdT**s**dT	PCSK9 antisense
ONT-110	(*R*p, *R*p)- a**s**AGcAAAAcAGGUCuAGAAdT**s**dT	PCSK9 antisense
ONT-111	(*S*p, *R*p)- a**s**GcAAAAcAGGUCuAGAAdT**s**dT	PCSK9 antisense

ONT-112	(*S*p, *S*p)- a*s*GcAAAAcAGGUCuAGAAdT*s*dT	PCSK9 antisense
ONT-113	(*R*p, *S*p)- a*s*GcAAAAcAGGUCuAGAAdT*s*dT	PCSK9 antisense

NOTE: lower case letters represent 2'OMe RNA residues; capital letters represent 2'OH RNA residues; and bolded and italicized "***s***" indicates a phosphorothioate moiety.

[00350] Lcaa-CPG-500 used in the preparation of these oligonucleotides was prepared according to the following reaction:



[00351] In particular, 5'-*O*-DMTr-2'-deoxythymidine (**1**) (4.28 g, 7.86 mmol) was dissolved in anhydrous DCM (50 mL) and mixed with 2 equiv of succinic anhydride (1.57 g, 15.7 mmol) and 3 equiv of 4-*N*,*N*-dimethylaminopyridine (2.88 g, 23.6 mmol). The reaction was stirred under Argon at room temperature. After complete consumption of the starting material as determined by TLC (1 hour), the solvents were evaporated to dryness, the crude residue was dissolved in DCM containing 1% triethylamine then purified by flash silica gel chromatography using a gradient of 0 – 2% of MeOH in DCM containing 2% triethylamine. Yield of pure compound (**2**) after evaporation was 5.5 g, 94%. The resulting 5'-*O*-DMTr-2'-deoxythymidine-3'-*O*-succinate (**2**) (0.60 g, 0.81 mmol), *N*,*N*-diisopropylethylamine (0.71 mL, 4.02 mmol) and CPG-500 (10 g) were taken up in DMF (50 mL) then HBTU (0.37 g, 0.97 mmol) was added. The mixture was shaken for 2 h then filtered. The support was washed with DMF, MeOH and finally, DCM then dried *in vacuo*. Trityl cation analysis (monitoring at 504 nm) showed that the loading of nucleoside on the support (**3**) was 38 µmol/g.

[00352] Each oligonucleotide, containing 2'-OH and 2'-OMe phosphodiester and stereodefined 2'-deoxy and 2'-OMe phosphorothioate diester internucleotidic linkages as indicated above in Table E-23, was synthesized on an ABI 394 DNA/RNA synthesizer according to the cycles summarized in Table E-24 and Table E-25 respectively, using a 10 µmol capacity synthesis column loaded with 130 mg (4.9 µmol) of succinyl linked 5'-*O*-DMTr-2'-deoxythymidine (38 µmol/g). Prior to synthesis, a preliminary capping step (capping 2) was performed and the synthesis was terminated with removal of the terminal 5'-*O*-DMTr groups.

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The oxidation step was performed using a commercially available 5-6 M solution of *tert*-butyl hydroperoxide (TBHP) in decane which was then diluted with four parts dichloromethane. The stereospecific sulfurization step was performed using the 0.3 M *S*-cyanoethylmethylthiosulfonate reagent following the coupling of the corresponding chiral phosphoramide and the two-step capping process (Table E-25).

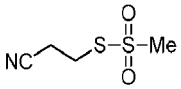
[00353] Once the automated oligonucleotide synthesis cycle was completed and the final 5'-*O*-DMTr group was removed, the synthesis column was taken off the DNA/RNA synthesizer and dried under vacuum. 10 mL solution of 0.5 M 1,5-diazabicyclo[4.3.0]non-5-ene (DBN), 0.25 M *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) in ACN was continuously added to the support through the synthesis column for 1 min without stopping the flow using a syringe attached to one end of the synthesis column. The support was then washed with anhydrous ACN and dried under vacuum. Then, the dried support was transferred into an empty screw-cap plastic vial and treated with 40% $MeNH_2$ aqueous solution (0.5 mL) at 60 °C for 10 min. After that, the vial was immediately cooled down and after dilution with DMSO (0.5 mL), the support was removed by filtration, washed again with DMSO (1 mL) and filtered. The filtrate was cooled down and then immediately treated with $3HF{\cdot}Et_3N$ (0.75 mL) at 60 °C for 10 min, then immediately frozen and stored at 4 °C prior to purification.

Table E-24. Summary for Oligonucleotide Synthesis on a DNA/RNA Synthesizer ABI 394 (2'-*O*-TBDMS and 2'-OMe substituted RNA cycle)

step	reaction	reagent	delivery time (sec)	wait time (sec)
1	detritylation	3% TCA in DCM	3 + 120 + 10	N. A.
2	coupling	0.15 M phosphoramidite in ACN + 2 M CMPT in ACN	7 + 6	30 + 600
3	capping	5% Pac_2O in THF/2,6-lutidine + 16% NMI in THF	10	20
4	oxidation	1.1 M *tert*-butyl hydroperoxide in 4:1	20	110

		dichloromethane:decane		

Table E-25. Summary for Oligonucleotide Synthesis on a DNA/RNA Synthesizer ABI 394 (stereodefined phosphorothioate 2'-deoxy and 2'-OMe RNA cycle)

step	reaction	reagent	delivery time (sec)	wait time (sec)
1	detritylation	3% TCA in DCM	3 + 120 + 10	N. A.
2	coupling	0.15 M chiral phosphoramidite in ACN + 2 M CMPT in ACN	8 + 6	30 + 900 (2'-OMe RNA) 30 + 600 (DNA)
3	capping 1	5% Pac_2O in THF/2,6-lutidine	30	60
4	capping 2	5% Pac_2O in THF/2,6-lutidine + 16% NMI in THF	30	60
5	sulfurization	0.3 M *S*-(2-cyanoethyl) methylthiosulfonate  in ACN/BSTFA	15 + 3×4	120 + 3×60 + 300

[00354] Example 59. General IEX-HPLC method for the analysis of crude and purified DMT Off RNA oligonucleotides.

Buffer A: 20 mM sodium phosphate, pH 11.0

Buffer B: 20 mM sodium phosphate, 1 M NaBr, pH 11.0

Column: DIONEX, DNAPac, PA-100, Analytical, 4.0×250mm

Column temperature = 60 °C

Signal monitored at 254 and 280 nm

Gradient used:

Time (min) Flow (mL/min) % A % B Curve

Initial		95	5	
3	1	95	5	1
23	1	20	80	6
25	1	5	95	6
25.5	1	95	5	6
30	1	95	5	1

[00355] **Example 60. General UPLC-LCMS method for the analysis of purified DMT Off RNA oligonucleotides.**

Buffer A: 15 mM TEA, 400 mM HFIP, Water

Buffer B: 50:50 Buffer A/Methanol

Column: UPLC@OST C_{18} 1.7 μm, 2.1×500mm

Column temperature = 50 °C

Gradient used:

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.2	95	5	
10	0.2	35	65	6
12	0.2	5	95	6
12.5	0.2	95	5	6
15	0.2	95	5	1

[00356] **Example 61. General IEX-HPLC method for the purification of crude DMTr Off RNA oligonucleotides.**

Buffer A: 20 mM sodium phosphate, pH 8.5

Buffer B: 20 mM sodium phosphate, 1 M NaBr, pH 8.5

Column: Empty column Waters AP-2 (Waters), custom in-house packed with Source 15Q support (GE Healthcare). The same purification column was used for the different stereopure oligonucleotides.

Instrument: Waters HPLC unit equipped with the 2525 binary gradient module, the 2487 dual absorbance detector and the 20 mL Flex injector (Waters).

Buffer heater temperature set = 70 °C

Signal monitored at 254 nm and 280 nm

Fractions volume: 4.5 mL

Gradient used:

Time (min) Flow (mL/min) % A % B Curve

Initial		100	0	
10	10	100	0	1
25	10	80	20	6
85	10	67.5	32.5	6
95	10	67.5	32.5	1
155	10	55	45	6
165	10	0	100	6
170	10	100	0	6
180	10	100	0	1

[00357] Collected fractions were individually analyzed by analytical IEX-HPLC using the analytical conditions and gradient described above. Pure fractions were pooled in order to provide purified material of 95% and above purity as determined by the 254 nm UV absorbance profiles.

[00358] **Example 62. General RP-Sep-Pak method for the desalting of purified RNA oligonucleotides.**

[00359] The solution of pooled pure fractions was loaded on a Sep-Pak cartridge (Waters, Sep-Pak Vac 35cc (10g) C_{18} Cartridges) pre-conditioned with water. After loading of the sample (100 mL), the cartridge was washed with milli Q water (50 mL) to remove all salt and then washed with 50% ACN/water to elute the full length desalted RNA oligonucleotide. The collected solution was concentrated *in vacuo* to a volume of 5 mL and lyophilized from water.

[00360] **Example 63: Preparation of Double Stranded siRNA Agents Using Chirally Controlled Oligonucleotide Strands**

[00361] The present Example describes preparation of double stranded siRNA agents by thermal annealing of chirally controlled oligonucleotide strands as described above.

[00362] Each RNA strand was mixed with its complementary RNA strand in equimolar concentration of 10 μM in 1×PBS. Total 0.5 mL solution was prepared for each duplex and the mixture was heated at 90 °C for 2 min and was allowed to cool down over the course of several hours. The mixtures were then stored at 4 °C. Physico-chemical properties of the utilized RNA strands are presented below in Table E-26.

Table E-26.

duplex ID	sequence	sequence ID	stereo architecture	Calc MW	Found MW

ONT D-1	S	ONT-116	Stereorandom diastereomixture	6735.4	6734.9
	AS1	ONT-114	Stereorandom diastereomixture	6805.4	6805.2
ONT D-2	S	ONT-107	*Sp*	6735.4	6736.6
	AS1	ONT-109	*Sp*	6805.4	6801.6
ONT D-3	S	ONT-106	*Rp*	6735.4	6731.8
	AS1	ONT-109	*Sp*	6805.4	6801.6
ONT D-4	S	ONT-107	*Sp*	6735.4	6736.6
	AS1	ONT-108	*Rp*	6805.4	6799.2
ONT D-5	S	ONT-106	*Rp*	6735.4	6731.8
	AS1	ONT-108	*Rp*	6805.4	6799.2
ONT D-7	S	ONT-116	Stereorandom diastereomixture	6735.4	6734.9
	AS2	ONT-115	Stereorandom diastereomixture	6835.5	6835.2
ONT D-8	S	ONT-106	*Rp*	6735.4	6731.8
	AS2	ONT-110	*Rp*, *Rp*	6835.5	6832.3
ONT D-9	S	ONT-107	*Sp*	6735.4	6736.6
	AS2	ONT-110	*Rp*, *Rp*	6835.5	6832.2
ONT D-10	S	ONT-106	*Rp*	6735.4	6731.8
	AS2	ONT-111	*Sp*, *Rp*	6835.5	6832.4
ONT D-11	S	ONT-107	*Sp*	6735.4	6736.6
	AS2	ONT-111	*Sp*, *Rp*	6835.5	6832.4
ONT D-12	S	ONT-106	*Rp*	6735.4	6731.8
	AS2	ONT-112	*Sp*, *Sp*	6835.5	6836.2
ONT D-13	S	ONT-107	*Sp*	6735.4	6736.6
	AS2	ONT-112	*Sp*, *Sp*	6835.5	6836.2
ONT D-14	S	ONT-106	*Rp*	6735.4	6731.8
	AS2	ONT-113	*Rp*, *Sp*	6835.5	6834.6

ONT D-15	S	ONT-107	*Sp*	6735.4	6736.6
	AS2	ONT-113	*Rp*, *Sp*	6835.5	6834.6

[00363] Oligonucleotide sequences used, human PCSK9 siRNA: Sense strand (S) 5′-uucuAGAccuGuuuuGcuudTsdT-3′; Antisense strand 1 (AS1) 5′-AAGcAAAAcAGGUCuAGAAdTsdT-3′; Antisense strand 2 (AS2) 5′-asAGcAAAAcAGGUCuAGAAdTsdT-3′. Upper case nucleotides: RNA, lower case nucleotides 2′-OMe, d = 2′-deoxy, s = phosphorothioate. Stereo architecture describes the stereoisomer nature (*R*p/*S*p absolute configuration) of each phosphorus atom in a given phosphorothioate linkage of the oligonucleotide. Found molecular weight (MW) values were obtained using the corresponding analytical methods for the purified compounds (described above).

[00364] Figures 51 shows overlays of IEX-HPLC profiles showing difference in retention times between stereopure RNA oligonucleotides.

Curve #	**Oligonucleotide**
1	ONT-109 (S*p*)
2	ONT-114 (diastereomixture)
3	ONT-108 (R*p*)

[00365] Figures 52 shows overlays of IEX-HPLC profiles showing difference in retention times between stereopure RNA oligonucleotides.

Curve #	**Oligonucleotide**
1	ONT-109 (R*p*)
2	ONT-114 (diastereomixture)
3	ONT-107 (S*p*)

[00366] In addition to the above specifically exemplified, prepared chirally controlled siRNA oligonucleotides, the present invention provides for preparation of siRNA duplexes

having several chiral phosphorothioate internucleotide linkages, and full chiral phosphorothioate internucleotide linkages.

[00367] For example, in accordance with the present invention, multiple chiral phosphorothioate linkages are introduced inside a RNA oligonucleotide by using the appropriate chiral RNA 3′-phosphoramidites, having suitable 2′-OH protecting groups, such as 2′-*O*-PivOM (Debart *et al.*, *Chem. Eur. J.*, **2008**, *14*, 9135), 2′-*O*-CEM (Ohgi *et al.*, *Org. Lett.*, **2005**, *7*, 7913; Wada *et al.*, *J. Org. Chem.*, **2012**, *77*, 7913), 2′-*O*-TOM (Pitsch *et al.*, *Helv. Chim. Acta*, **2001**, *84*, 3773) or 2′-*O*-TC (Dellinger *et al.*, *J. Am. Chem. Soc.*, **2011**, *133*, 11540). Each of the following Human PCSK9 siRNA Sense Strands having several chiral phosphorothioate internucleotide linkages and full chiral phosphorothioate internucleotide linkages can be prepared in accordance with the present invention:

	PCSK9 siRNA Sense Strands
PCSK9 (1)	(All (*Sp*))- ususcsusAsGsAscscsusGsususususGscsususdTsdT
PCSK9 (2)	(All (*Rp*))- ususcsusAsGsAscscsusGsususususGscsususdTsdT
PCSK9 (3)	(All (*Sp*))- usucuAsGsAsccuGsuuuuGscuusdTsdT
PCSK9 (4)	(All (*Rp*))- usucuAsGsAsccuGsuuuuGscuusdTsdT
PCSK9 (5)	(*Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*)-ususcsusAsGsAscscsusGsususususGscsususdTsdT
PCSK9 (6)	(*Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*)-ususcsusAsGsAscscsusGsususususGscsususdTsdT

NOTE: lower case letters represent 2'-OMe RNA residues; capital letters represent RNA residues; d = 2'-deoxy residues; and "s" indicates a phosphorothioate moiety.

[00368] Synthesis examples for Human PCSK9 siRNA Antisense Strands having several chiral phosphorothioate internucleotide linkages and full chiral phosphorothioate internucleotide linkages.

	Human PCSK9 siRNA Antisense Strands
PCSK9 (7)	(All (*Rp*))- AsAsGscsAsAsAsAscsAsGsGsUsCsusAsGsAsAsdTsdT
PCSK9 (8)	(All (*Sp*))- AsAsGscsAsAsAsAscsAsGsGsUsCsusAsGsAsAsdTsdT
PCSK9 (9)	(All (*Rp*))- AsAGcAAAAcsAsGsGsUsCsusAsGsAsAsdTsdT
PCSK9 (10)	(All (*Sp*))- AsAGcAAAAcsAsGsGsUsCsusAsGsAsAsdTsdT

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PCSK9 (11)	(All (*R*p))- AAsGscsAsAsAsAscAGGUCuAGAAdTsdT
PCSK9 (12)	(All (*S*p))- AAsGscsAsAsAsAscAGGUCuAGAAdTsdT
PCSK9 (13)	(All (*R*p))- AsAsGscAsAsAsAscAsGsGsUsCsuAsGsAsAsdTsdT
PCSK9 (14)	(All (*S*p))- AsAsGscAsAsAsAscAsGsGsUsCsuAsGsAsAsdTsdT
PCSK9 (15)	(All (*R*p))- AsAGcAAAsAscAsGsGsUsCsusAsGsAsAsdTsdT
PCSK9 (16)	(All (*S*p))- AsAGcAAAsAscAsGsGsUsCsusAsGsAsAsdTsdT
PCSK9 (17)	(*R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p, *S*p)-AsAGcAAAsAscAsGsGsUsCsusAsGsAsAsdTsdT
PCSK9 (18)	(*S*p, *R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p)-AsAGcAAAsAscAsGsGsUsCsusAsGsAsAsdTsdT

NOTE: lower case letters represent 2'-OMe RNA residues; capital letters represent RNA residues; d = 2'-deoxy residues; and "s" indicates a phosphorothioate moiety.

[00369] Alternatively or additionally, the present invention provides for preparation of siRNA duplexes having several chiral phosphorothioate internucleotide linkages and full chiral phosphorothioate internucleotide linkages and fully modified ribose moieties.

[00370] For example, in certain embodiments, multiple chiral phosphorothioate linkages are introduced inside a fully ribose-modified RNA oligonucleotide by using the appropriate chiral RNA 3′-phosphoramidites, having the corresponding desired ribose 2′-chemical modification, such as 2′-deoxy-2′-fluoro (2′-F) or 2′-*O*-methyl (2′-OMe). To give but a few examples, each of the following Human PCSK9 siRNA fully modified 2′-F/2′-OMe Sense Strands having several chiral phosphorothioate internucleotide linkages and full chiral phosphorothioate internucleotide linkages, can be prepared.

	Human PCSK9 siRNA fully modified 2'-F/2'-OMe Sense Strands
PCSK9 (19)	(All (*R*p))- UfsusCfsusAfsgsAfscsCfsusGfsusUfsusUfsgsCfsusUfsdTsdT
PCSK9 (20)	(All (*S*p))- UfsusCfsusAfsgsAfscsCfsusGfsusUfsusUfsgsCfsusUfsdTsdT
PCSK9 (21)	(All (*R*p))- UfsuCfsuAfsgAfscCfsuGfsuUfsuUfsgCfsuUfsdTsdT
PCSK9 (22)	(All (*S*p))- UfsuCfsuAfsgAfscCfsuGfsuUfsuUfsgCfsuUfsdTsdT
PCSK9 (23)	(*R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p,

	Sp)- UfsusCfsusAfsgsAfscsCfsusGfsusUfsusUfsgsCfsusUfsdTsdT
PCSK9 (24)	(*Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*)- UfsusCfsusAfsgsAfscsCfsusGfsusUfsusUfsgsCfsusUfsdTsdT

NOTE: lower case letters represent 2'-OMe RNA residues; capital letters represent 2'-F RNA residues; d = 2'-deoxy residues; and "s" indicates a phosphorothioate moiety.

[00371] Synthesis examples for Human PCSK9 siRNA fully modified 2'-F/2'-OMe Antisense Strands having several chiral phosphorothioate internucleotide linkages and full chiral phosphorothioate internucleotide linkages.

	Human PCSK9 siRNA fully modified 2'-F/2'-OMe Antisense Strands
PCSK9 (25)	(All (*Rp*))- asAfsgsCfsasAfsasAfscsAfsgsGfsusCfsusAfsgsAfsasdTsdT
PCSK9 (26)	(All (*Sp*))- asAfsgsCfsasAfsasAfscsAfsgsGfsusCfsusAfsgsAfsasdTsdT
PCSK9 (27)	(All (*Rp*))- asAfgCfaAfaAfcsAfsgsGfsusCfsusAfsgsAfsasdTsdT
PCSK9 (28)	(All (*Sp*))- asAfgCfaAfaAfcsAfsgsGfsusCfsusAfsgsAfsasdTsdT
PCSK9 (29)	(All (*Rp*))- asAfsgCfsaAfsaAfscAfsgGfsuCfsuAfsgAfsadTsdT
PCSK9 (30)	(All (*Sp*))- asAfsgCfsaAfsaAfscAfsgGfsuCfsuAfsgAfsadTsdT
PCSK9 (31)	(*Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*)-asAfgCfaAfasAfscAfsgsGfsusCfsusAfsgsAfsasdTsdT
PCSK9 (32)	(*Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*, *Sp*, *Rp*)-asAfgCfaAfasAfscAfsgsGfsusCfsusAfsgsAfsasdTsdT

NOTE: lower case letters represent 2'-OMe RNA residues; capital letters represent 2'-F RNA residues; d = 2'-deoxy residues; and "s" indicates a phosphorothioate moiety.

[00372] To assemble duplexes, RNA strand thermal annealing and preparation of siRNA duplexes is performed. Specifically, each RNA strand is mixed with its complementary RNA strand in equimolar concentration of 10 μM in 1×PBS. Total 0.5 mL solution is prepared for each duplex and the mixture is heated at 90 °C for 2 min and is allowed to cool down over the course of several hours. The mixtures are then stored at 4 °C.

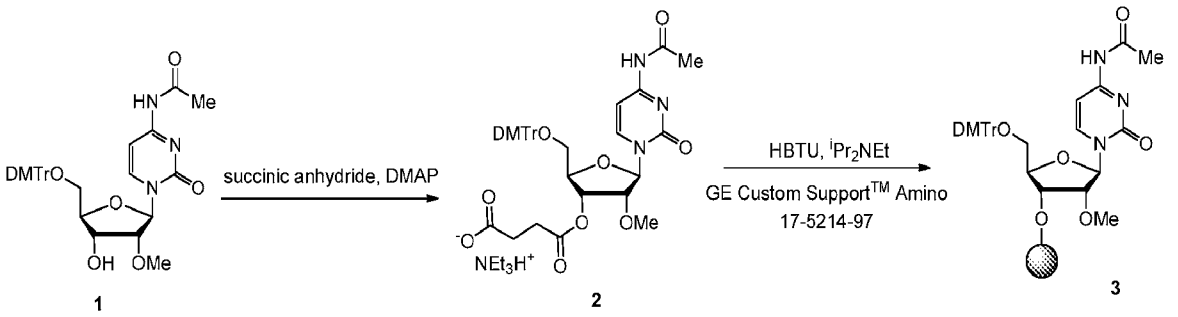
[00373] Following the thermal RNA strand annealing step, all the possible siRNA duplex combinations can be prepared by annealing any of the Sense strands with any possible complementary strand of the Antisense strands.

[00374] All prepared siRNA duplexes can be evaluated *in vitro* for their PCSK9 gene-silencing properties, following transfection in HeLa cells or Hep3B cells (e.g., as described

herein). According to the present invention, different potenties may be observed for different duplexes, e.g., that vary in number, position, and/or stereo architecture of the chiral phosphorothioate backbone linkages, and optionally also in presence, level, and or type of one or more other chemical modifications.

[00375] Any or all siRNA properties such as: nuclease resistance, cell penetration, endosomal escape, duplex thermodynamic stability, tridemnsional structure of the duplex, affinity towards the various mechanistic steps of enzyme interactions, affinity towards the target mRNA, specific off-target effects, immunestimulation, duration of action, pharmacokinetics, etc. may be modulated and influenced by the stereochemistry of the chiral phosphorothioate backbone linkages, as described herein.

[00376] **Example 64. Preparation of Chirally Controlled Preparations of Oligonucleotide**



[00377] The present Example describes preparation of a variety of particular chirally controlled compositions of certain oligonucleotides described therein.

[00378] N^4-Acetyl-5'-*O*-DMTr-2'-*O*-methylcytidine (**1**) (1.15 g, 1.91 mmol) was dissolved in anhydrous DCM (20 mL) and mixed with 2 equiv of succinic anhydride (0.383 g, 3.82 mmol) and 3 equiv of 4-*N*,*N*-dimethylaminopyridine (0.701 g, 5.73 mmol). The reaction was stirred under Argon at room temperature. After complete consumption of starting material as determined by TLC (1 hour), the solvents were evaporated to dryness, the crude residue was dissolved in DCM containing 1% triethylamine then purified by flash silica gel chromatography using a gradient of 0 – 2% of MeOH in DCM containing 2% of triethylamine. Yield of pure succinyl compound (**2**) after evaporation was 1.50 g, 98 %. MS (ESI +ve): calc $(M+H)^+$: 702.27, found: 702.34. The resulting N^4-acetyl-5'-*O*-DMTr-3'-*O*-succinyl-2'-*O*-methylcytidine (**2**) (0.18 g, 0.22 mmol), *N*,*N*-diisopropylethylamine (0.18 mL, 0.79 mmol) and GE Custom Support™ Amino (1 g) were taken up in DMF (5 mL) then HBTU (0.10 g, 0.26 mmol) was added. The

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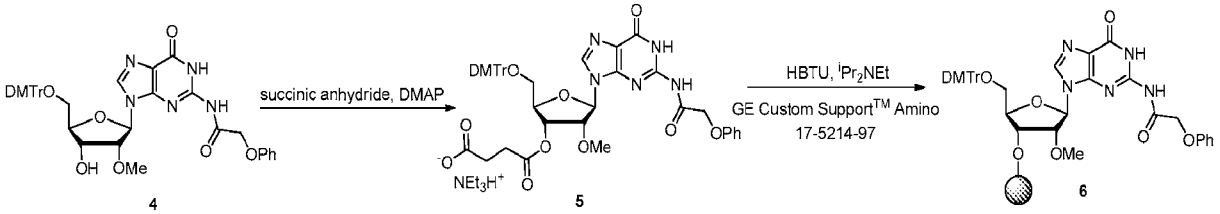
mixture was shaken for 2 h then filtered. The support was washed with DMF, MeOH and finally DCM then dried *in vacuo*. Trityl cation analysis (monitoring at 504 nm) showed that the loading of nucleoside on the support (**3**) was 180 μmol/g.

[00379] **Example 65. Preparation of Chirally Controlled Preparations of Oligonucleotide**



[00380] Using a procedure analogous to that described in Example 64, N^2-Phenoxyacetyl-5'-*O*-DMTr-3'-*O*-succinyl-2'-*O*-methylguanosine (**4**, MS (ESI +ve): calc $(M+H)^+$: 834.30, found: 834.32) was loaded onto GE Custom Support™ Amino. Trityl cation analysis (monitoring at 504 nm) showed that the loading of nucleoside on the support (**6**) was 140 μmol/g.

[00381] In some embodiments, oligonucleotides containing stereodefined 2'-OMe phosphorothioate triester internucleotidic linkages was synthesized on ABI 394DNA/RNA synthesizer according to the cycle summarized in Table E-27 using a 10 μmol capacity synthesis column loaded with 60 mg (10.8 and 8.4 μmol respectively) of either uncapped succinyl linked 5'-*O*-DMTr-2'-*O*-methyl-G^{Pac} (**6**, 140 μmol/g) or 5'-*O*-DMTr-2'-*O*-methyl-C^{Ac} (**3**, 180 μmol/g). The synthesis cycle was performed with a preliminary capping step (capping 2) and with removal of the terminal 5'-*O*-DMTr group. The stereospecific sulfurization steps were performed using the 0.3 M *S*-(2-cyanoethyl)methylthiosulfonate reagent in ACN containing BSTFA, following the coupling of the corresponding chiral phosphoramide and the two-step capping process (Table E-27).

[00382] Once the automated oligonucleotide synthesis cycle was complete, and the final 5'-*O*-DMTr group removed, the synthesis column was taken off the DNA/RNA synthesizer and dried under vacuum. The dried support was transferred onto an empty glass manual peptide synthesizer and 10 mL solution of 0.5 M 1,5-diazabicyclo[4.3.0]non-5-ene (DBN), 0.25 M *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) in ACN was continuously added to the support for 5 min without stopping the flow in the manual peptide synthesizer. The support was washed by ACN and dried *in vacuo*. Then, the support was treated with 5%EtOH/conc NH_3 (5 mL) at 60

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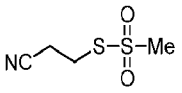
°C for 6 h, and left at room temperature for 12 h. The support was removed by filtration and washed with conc. NH_3. The filtrate was concentrated *in vacuo* then purified by IEX.

Table E-27. Summary for Oligonucleotide Synthesis .

step	reaction	reagent	delivery time (sec)	wait time (sec)
1	detritylation	3% TCA in DCM	3 + 120 + 10	N. A.
2	coupling	0.15 M phosphoramidite in ACN + 2 M CMPT in ACN	8 + 6	30 + 900
3	capping 1	5% Pac_2O in THF/2,6-lutidine	30	60
4	capping 2	5% Pac_2O in THF/2,6-lutidine + 16% NMI in THF	30	60
5	sulfurization	0.3 M (*S*-cyanoethyl)-methylthiosulfonate  in ACN/BSTFA	15 + 3×4	120 + 3×60 + 300

[00383] Synthesis of Oligonucleotide ONT-94: (All (*S*p))-gsgsusgsgsasasgsgsc. t_R (IEX-HPLC): 18.26 min. Calc MW: 3563.9; Found MW: 3562.6.

[00384] Synthesis of Oligonucleotide ONT-96: (All (*R*p))-gsgsusgsgsasasgsgsc. t_R (IEX-HPLC): 18.16 min. Calc MW: 3563.9; Found MW: 3561.7.

[00385] Synthesis of Oligonucleotide ONT-98: (*R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p) - gsgsusgsgsasasgsgsc. t_R (IEX-HPLC): 18.05 min. Calc MW: 3563.9; Found MW: 3562.5.

[00386] Synthesis of Oligonucleotide ONT-100: (*S*p, *R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p, *S*p) - gsgsusgsgsasasgsgsc. t_R (IEX-HPLC): 17.86 min. Calc MW: 3563.9; Found MW: 3561.1.

[00387] Synthesis of Oligonucleotide ONT-102: (*R*p, *R*p, *S*p, *S*p, *S*p, *S*p, *S*p, *R*p, *R*p) – gsgsusgsgsasasgsgsc. t_R (IEX-HPLC): 18.30 min. Calc MW: 3563.9; Found MW: 3561.3.

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[00388] Synthesis of Oligonucleotide ONT-104: (*S*p, *S*p, *R*p, *R*p, *R*p, *R*p, *R*p, *S*p, *S*p) – gsgsusgsgsasasgsgsc. t_R (IEX-HPLC): 17.95 min. Calc MW3563.9; Found MW: 3562.7.

[00389] Synthesis of Oligonucleotide ONT-95: (All (*S*p))- gscscsuscscsasg. t_R (IEX-HPLC): 14.78 min. Calc MW: 2709.2; Found MW: 2707.4.

[00390] Synthesis of Oligonucleotide ONT-97: (All (*R*p))- gscscsuscscsasg. t_R (IEX-HPLC): 15.60 min. Calc MW: 2709.2; Found MW: 2708.3.

[00391] Synthesis of Oligonucleotide ONT-99: (*R*p, *S*p, *R*p, *S*p, *R*p, *S*p, *R*p) - gscscsuscscsasg. t_R (IEX-HPLC): 16.10 min. Calc MW: 2709.2; Found MW: 2708.0.

[00392] Synthesis of Oligonucleotide ONT-101: (*S*p, *R*p, *S*p, *R*p, *S*p, *R*p, *S*p) - gscscsuscscsasg. t_R (IEX-HPLC): 16.23 min. Calc MW: 2709.2; Found MW: 2708.2.

[00393] Synthesis of Oligonucleotide ONT-103: (*R*p, *R*p, *S*p, *S*p, *S*p, *R*p, *R*p) – gscscsuscscsasg. t_R (IEX-HPLC): 16.26 min. Calc MW: 2709.2; Found MW: 2707.8.

[00394] Synthesis of Oligonucleotide ONT-105: (*S*p, *S*p, *R*p, *R*p, *R*p, *S*p, *S*p) – gscscsuscscsasg. t_R (IEX-HPLC): 16.22 min. Calc MW: 2709.2; Found MW: 2710.0.

[00395] Oligonucleotides containing stereodefined chimeric 2'-OMe phosphorothioate triester and 2'-*O*-MOE phosphorothioate triester internucleotidic linkages was synthesized on ABI 394DNA/RNA in an analogous fashion to those described in examples herein.

[00396] Synthesis of Oligonucleotide ONT-90: (All (*R*p)) – $G_{MOE}sG_{MOE}susG_{MOE}sG_{MOE}sasasG_{MOE}sG_{MOE}sc$. t_R (IEX-HPLC): 15.35 min. Calc MW: 3828.2; Found MW: 3826.5.

[00397] Synthesis of Oligonucleotide ONT-119: (All (*S*p)) – $G_{MOE}sG_{MOE}susG_{MOE}sG_{MOE}sasasG_{MOE}sG_{MOE}sc$. t_R (IEX-HPLC): 16.42 min. Calc MW: 3828.2; Found MW: 3827.2.

[00398] Synthesis of Oligonucleotide ONT-91: (All (*R*p)) –G_{MOE}scscsuscscsasg. t_R (IEX-HPLC): 15.69 min. Calc MW: 2753.3; Found MW: 2751.5.

[00399] Synthesis of Oligonucleotide ONT-120: (All (*S*p)) –G_{MOE}scscsuscscsasg. t_R (IEX-HPLC): 14.71 min. Calc MW: 2753.3; Found MW: 2751.4.

[00400] **Example 66. General IEX-HPLC method for the analysis of crude and purified DMT Off RIPtide oligonucleotides:**

Buffer A: 10 mM TrisHCl, 50%ACN, pH 8.0

Buffer B: 10 mM TrisHCl, 800 mM $NaClO_4$, 50%ACN, pH 8.0

Column: DIONEX, DNAPac, PA-200, Analytical, 4.0×250mm

Column temperature = 60 °C

Signal monitored at 254 and 280 nm

Gradient used:

Time (min)	Flow (mL/min)	% A	% B	Curve
Initial		95	5	
2	1	95	5	1
22	1	70	30	6
25	1	5	95	6
25.5	1	95	5	6
30	1	95	5	1

[00401] **Example 67. General UPLC-LCMS method for the analysis of purified DMT Off RIPtide oligonucleotides:**

Buffer A: 15 mM TEA, 400 mM HFIP, Water

Buffer B: 50:50 Buffer A/Methanol

Column: UPLC@OST C_{18} 1.7 μm, 2.1×500mm

Column temperature = 50 °C

Gradient used:

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.2	80	20	
2	0.2	80	20	1
22	0.2	30	70	6
25	0.2	5	95	6
25.5	0.2	80	20	6
30	0.2	80	20	1

[00402] **Example 68. General IEX-HPLC method for the purification of crude DMT Off RIPtide oligonucleotide:**

Buffer A: 20 mM NaOH, pH 11.0

Buffer B: 20 mM NaOH, 2.5 M NaCl, pH 11.0

Column: Empty column Waters AP-1 (Waters), custom in-house packed with Source 15Q support (GE Healthcare). The same purification column was used for the different stereopure RIPtide oligonucleotides.

Instrument: AKTA Purifier, equipped with the P-900 pump, the UPC-900 detector and a 50 mL injection SuperLoop (GE Healthcare)

Buffer heater temperature set = 70 °C

Column heater tape set = 70 °C

Signal monitored at 254 nm

Fractions volume: 5 mL

Gradient used:

Time (min)	Flow (mL/min)	% A	% B	Curve
Initial		100	0	
15	4	100	0	1
25	4	90	10	6
35	4	90	10	1
45	4	80	20	6
60	4	80	20	1
80	4	65	35	6
95	4	65	35	1
120	4	45	55	6
125	4	45	55	1
126	4	100	0	6
140	4	100	0	1

[00403] Collected fractions were individually analyzed by analytical IEX-HPLC using the analytical conditions and gradient described above. Pure fractions were pooled in order to provide purified material of 95% and above purity as determined by the 254 nm UV absorbance profiles.

[00404] General RP-Sep-Pak method for the desalting of purified RIPtides oligonucleotides. The solution of pooled pure fractions was loaded on a Sep-Pak cartridge (Waters, Sep-Pak Vac 35cc (10 g) C_{18} Cartridges) pre-conditioned with water. After loading of the sample (100 mL), the cartridge was washed with milli Q water (50 mL) to remove all salt and then washed with 50% ACN/water to elute the full length desalted RNA oligonucleotide. The collected solution was concentrated *in vacuo* to a volume of 5 mL and lyophilized from water.

[00405] A panel of stereocontrolled fully phosphorothioate modified RIPtides (8-mer or 10-mer) that bind naked hTR are investigated for the *in vitro* inhibition of the activity of the telomerase RNP complex. The Cy5-TRAP assay (Shay *et al.*, *Nat. Protoc.*, **2006**, *1*, 1583), a variation of the TRAP (Shay *et al.*, *Science*, **1994**, *266*, 2011), is used to the determine the IC_{50}

values for the stereocontrolled phosphorothioate RIPtides using HeLa cell extracts, according to previously reported protocols (Verdine *et al.*, *J. Biol. Chem.*, **2012**, *287*, 18843).

[00406] TRAP assays are performed following previously reported protocols that use fluorescence as a quantitation system, with some modifications. Briefly, extension of a fluorescent artificial substrate by telomerase is carried out for 30 minutes at 30 °C, followed by amplification with 30 PCR cycles (34 °C 30 s, 59 °C 30 s, 72 °C 1 min). The inhibitory potential of the RIPtides is initially assessed in HeLa cell extracts, in duplicate experiments, using a 600 pM-60 μM concentration range. Experiments with selected RIPtides are repeated for a concentration range of 0.06 pM-60 μM in HeLa, DU145 (prostate cancer) and HEK293 cell extracts. Several controls are used in these assays: a positive control (untreated cell lysate), negative controls (buffer only, heat inactivated and RNase treated cell extracts), and PCR amplification control (60 μM of RIPtide added after telomerase elongation and before PCR). In certain embodiments, chirally controlled RIPtide preparations show enhanced inhibition of hTR as compared with the positive control and/or enhanced inhibition of hTRas compared with the negative control; in some embodiments, some, but optionally not all RIPtides of a given sequence show such properties. In some embodiments, by "enhanced" in this context is meant enhanced by between about five-fold and about ten-fold. In some embodiments, by "enhanced" is meant at least about 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, or 10.0-fold.

[00407] Cell Culturing Conditions. The transformed embryonic kidney cell line HEK293 and the prostate cancer cell line DU145 are maintained in DMEM supplemented with 10% fetal bovine serum in 5% CO_2 at 37 °C. Soluble cell extracts for TRAP assays are prepared by detergent lysis of 106 cells with 200 μL 1X CHAPS Lysis Buffer (Chemicon) as described in the manufacturer's instructions.

[00408] According to the present invention, different potenties and/or different other properties may be observed for the various stereoarchitectures of fully stereocontrolled phosphorothioate RIPtides. For instance, RIPtide properties such as: nuclease resistance, tissue accumulation, cell penetration, endosomal escape, tridemnsional structure of the RIPtide, affinity towards the target folded hTR RNA, immunestimulation, duration of action, pharmacokinetics, etc. may vary for chirally controlled RIPtide preparations that share the same sequence but differ

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from one another with respect to different location and/or stereochemistry of one or more chiral phosphorothioate backbone linkages.

[00409] Example 69: Chirally controlled oligonucleotide compositions show different activity in vivo as compared with chirally uncontrolled compositions having the same sequence

[00410] The present Example compares *in vivo* pharmacological activity of chirally pure oligonucleotides with that observed for the "parent" stereorandom mixture (i.e., for a composition containing oligonucleotides of the same sequence as the chirally pure oligonucleotides but not displaying chiral purity, for example as a result of having been prepared via a stereorandom process). Four chirally pure oligonucleotides, each of which had a sequence complementary to that of a particular target transcript or gene encoding a protein of interest were synthesized, formulated, and administered twice per week for 5 weeks at two dose levels each, to animals expressing the target gene. Levels of encoded protein levels were quantified. In this Example, oligonucleotides having a sequence antisense to (and therefore targeting) human Apolipoprotein-B (ApoB) were used for proof-of-concept in transgenic mice expressing human ApoB.

Test Articles

[00411] Test articles were PBS alone (i.e., no oligonucleotide control) or the relevant oligonucleotide composition (i.e., Mipomersen (ONT-41), ONT-75, ONT-77, ONT-80, or ONT-81 oligonucleotides (see Example 52 for structures) were formulated in 1X PBS (diluted from 10X PBS (Life Technologies, AM9624) using nuclease-free water (Qiagen, 129115)) at 0.5 and 1 mg/mL for dosing at 5 and 10 mg/kg, respectively. Formulations were based on absolute mass with no adjustment for the active material. Oligonucleotide concentrations were confirmed by measurement with a Carry-100 UV-Vis (Agilent Technologies). Samples of all test articles were checked for endotoxin levels using a kinetic pyrochrome chromogenic endotoxin assay (Associates of Cape Cod, 1500-5, E005-5). All tested samples were found to have a lower amount of endotoxin than the 0.5 EU/ml acceptable limit.

Animals and In Vivo Procedures

[00412] Female transgenic mice (huApoB mice) expressing high plasma concentrations of human apolipoprotein B100 and lipoprotein(a) (J. Clin. Invest. 92: 3029-3037) were obtained

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from Taconic (strain B6.SJL-Tg(APOB)1 102Sgy N20+?, model #1004-F). All animals were genotyped prior to delivery.

[00413] Mice were delivered and allowed to acclimate for at least seven days prior to study start. All mice were given regular chow and water ad libitum, and were not fasted prior to compound administration. Mice were randomized to study groups, and dosed intraperitoneally (IP) at 10 ml/kg on Days 1, 4, 8, 11, 15, 18, 22, 25, 29 and 32, based on individual mouse body weight measured prior to dosing on each dosing day. Blood was collected during the course of the study on Days 0 (day before first dose), 17, 24, 31, 38, 45, 52 and 60 by submandibular (cheek) bleed and at sacrifice on Day 66 by cardiac puncture, and then processed to serum.

Apolipoprotein B Protein Assay

[00414] ApoB protein levels in serum were measured using an ApoB Human ELISA Kit (Abcam, ab108807). Serum samples were diluted 1:20,000 and assayed per the kit recommended protocol without modifications. Automated washes were done with 30 second soak periods using an Aquamax 4000 (Molecular Devices). Reactions were stopped after 12 minute incubations with Chromogen substrate and measurements were taken with a Spectramax M5 (Molecular Devices).

[00415] Standard curves were generated by plotting the standard concentration on the x-axis and the corresponding mean 450 nm absorbance on the y-axis. The best-fit line was determined by regression analysis using log-log curve-fit. Each assay plate included a negative (PBS treated) and a positive (Mipomersen-treated) control and each mouse serum sample was assayed in 4 technical replicates.

[00416] For each sample, the mean absolute level of ApoB was normalized to the mean value for the PBS treated group, to obtain the relative level of ApoB protein expression. In one instance, an animal was excluded from the analysis as measurements deviated from the cohort mean by 2 standard deviations. Relative levels of ApoB protein expression were used for statistical analysis (Graphpad Prism) by 2-way ANOVA followed by Newman-Keuls post-hoc test.

Results

[00417] The results are shown in Table E-28a and Figure 40. Relative to PBS, 5 mg/kg IP ONT-75 resulted in significant reductions of serum ApoB protein levels on day 24 of the study, to 87% ($p < 0.05$). Relative to PBS, 5 mg/kg IP ONT-77 resulted in significant reductions of

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serum ApoB protein levels on days 24,and 31 of the study, to 72% ($p < 0.0001$), 81% ($p < 0.05$) of control levels, respectively. Relative to PBS, 5 mg/kg IP ONT-80 resulted in significant reductions of serum ApoB protein levels on days 24 and 31 of the study, to 83% ($p < 0.05$), 80% ($p < 0.05$) of control levels, respectively. Relative to PBS, 5 mg/kg IP ONT-81 resulted in significant reductions of serum ApoB protein levels on days 17 and 24 of the study, to 85% ($p < 0.05$), 70% ($p < 0.0001$) of control levels, respectively.

Table E-28a: Serum Human Apolipoprotein B Levels Relative to PBS After Multiple IP Doses of 5 mg/kg Stereioisomer or Mipomersen in huApoB Mice (N = 4-5]

	Day				
Sample	17	24	31	38	45
PBS	100 ± 9	100 ± 9	100 ± 9	100 ± 11	100 ± 9
ONT-41	54 ± 11	49 ± 7	54 ± 9	64 ± 10	80 ± 9
ONT-75	103^{d} ± 11	87^{dw} ± 10	94^{d} ± 10	97^{d} ± 10	98^{a} ± 9
ONT-77	83^{d} ± 15	72^{cz} ± 10	81^{dw} ± 9	91^{d} ± 13	95^{a} ± 12
ONT-80	89^{d} ± 9	83^{dw} ± 8	80^{dw} ± 3	95^{d} ± 7	87 ± 11
ONT-81	85^{dw} ± 12	70^{cz} ± 5	88^{d} ± 15	101^{d} ± 10	102^{b} ± 7

a: Statistically different from the Mipomersen control group (ONT-41), with $p < 0.05$ (2-way ANOVA with Newman-Keuls post-hoc test)
b: Statistically different from the Mipomersen control group (ONT-41), with $p < 0.01$ (2-way ANOVA with Newman-Keuls post-hoc test)
c: Statistically different from the Mipomersen control group (ONT-41), with $p < 0.001$ (2-way ANOVA with Newman-Keuls post-hoc test)
d: Statistically different from the Mipomersen control group (ONT-41), with $p < 0.0001$ (2-way ANOVA with Newman-Keuls post-hoc test)
w: Statistically different from the PBS control group, with $p < 0.5$ (2-way ANOVA with Newman-Keuls post-hoc test)
x: Statistically different from the PBS control group, with $p < 0.01$ (2-way ANOVA with Newman-Keuls post-hoc test)
y: Statistically different from the PBS control group, with $p < 0.001$ (2-way ANOVA with Newman-Keuls post-hoc test)
z: Statistically different from the PBS control group, with $p < 0.0001$ (2-way ANOVA with Newman-Keuls post-hoc test)

[00418] Compared with 5 mg/kg IP Mipomersen, ONT-75 administered with the same dosing paradigm resulted in significantly less reduction of serum ApoB protein levels on days 17,24,31 and 38 ($p < 0.0001$), and 45 ($p < 0.05$) of the study. Compared with 5 mg/kg IP Mipomersen, ONT-77 administered with the same dosing paradigm resulted in significantly less reduction of serum ApoB protein levels on days 17,31 and 38 ($p < 0.0001$), 24 ($p < 0.001$) and 45 ($p < 0.05$) of the study. Compared with 5 mg/kg IP Mipomersen, ONT-80 administered with the same dosing paradigm resulted in significantly less reduction of serum ApoB protein levels

on days 17,24,31 and 38 ($p < 0.0001$), but was not different on day 45 ($p > 0.05$) of the study. Compared with 5 mg/kg IP Mipomersen, ONT-81 administered with the same dosing paradigm resulted in significantly less reduction of serum ApoB protein levels on days 17,31 and 38 ($p < 0.0001$), 24 ($p < 0.001$) and 45 ($p < 0.01$) of the study. **Figure 40** shows the timecourse of Serum Human Apolipoprotein B protein levels relative to PBS after 5 mg/kg stereoisomer or mipomersen IP dosing in huApoB mice. Downward arrows indicate dosing days. Group means normalized to the PBS control group are shown, where each groups comprised 4-5 animals. Error bars represent standard deviations.

[00419] The results of the 10mg/kg dosing paradigm are shown in Table E-28b and Figure 53. Relative to PBS, 10 mg/kg IP ONT-75 resulted in significant reductions of serum ApoB protein levels on days 17, 24 and 38 of the study, to 78% ($p<0.0001$), 76% ($p < 0.0001$) and 82% ($p<0.001$), respectively. Relative to PBS, 10 mg/kg IP ONT-77 resulted in significant reductions of serum ApoB protein levels on days 17, 24, 31, 38, 45 and 52 of the study, to 62% ($p < 0.0001$), 61% ($p < 0.0001$), 54% ($p < 0.0001$), 59% ($p < 0.0001$), 72% ($p < 0.0001$), and 73% ($p < 0.0001$) of control levels, respectively. Relative to PBS, 10 mg/kg IP ONT-80 resulted in significant reductions of serum ApoB protein levels on days 17, 24, 31, 38, 45, 52 and 66 of the study, to 55% ($p < 0.0001$), 59% ($p < 0.0001$), 73% ($p < 0.0001$), 67% ($p < 0.0001$), 76% ($p < 0.0001$), 76% ($p < 0.0001$), and 80% ($p<0.01$) of control levels, respectively. Relative to PBS, 10 mg/kg IP ONT-81 resulted in significant reductions of serum ApoB protein levels on days 17, 24, 31, 38 and 45 of the study, to 49% ($p < 0.0001$), 62% ($p < 0.0001$), 71% ($p < 0.0001$), 74% ($p < 0.0001$) and 79% ($p < 0.001$) of control levels, respectively.

[00420] Table E-28b: Serum Human Apolipoprotein B Levels Relative to PBS After Multiple IP Doses of 10 mg/kg Stereioisomer (ONT-75, -77, -80 or -81) or Mipomersen in huApoB Mice (N = 4-5)

	Day							
Sample	17	24	31	38	45	52	60	66
PBS	100 ± 11	100 ± 9	100 ± 17	100 ± 11	100 ± 7	100 ± 7	100 ± 9	100 ± 7
ONT-41	31 ± 13	24 ± 24	23 ± 6	29 ± 10	46 ± 10	64 ± 8	77 ± 8	64 ± 7
ONT-75	78^{dz} ± 5	76^{dz} ± 4	82^{dy} ± 7	95^{d} ± 9	97^{d} ± 3	94^{d} ± 13	108^{d} ± 6	87^{c} ± 12
ONT-77	62^{dz} ± 9	61^{dz} ± 4	54^{dz} ± 6	59^{dz} ± 10	72^{dz} ± 7	73^{z} ± 7	95^{a} ± 5	87^{b} ± 11
ONT-80	55^{cz} ± 4	59^{dz} ± 3	73^{dz} ± 4	67^{dz} ± 10	76^{dz} ± 10	76^{z} ± 11	87 ± 10	80^{bx} ± 7

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ONT-81	$49^{bz} \pm 7$	$62^{dz} \pm 19$	$71^{dz} \pm 7$	$74^{dz} \pm 7$	$79^{dy} \pm 11$	$88^{c} \pm 5$	90 ± 6	$89^{c} \pm 8$

a: Statistically different from the Mipomersen control group (ONT-41), with $p < 0.05$ (2-way ANOVA with Newman-Keuls post-hoc test)

b: Statistically different from the Mipomersen control group (ONT-41), with $p < 0.01$ (2-way ANOVA with Newman-Keuls post-hoc test)

c: Statistically different from the Mipomersen control group (ONT-41), with $p < 0.001$ (2-way ANOVA with Newman-Keuls post-hoc test)

d: Statistically different from the Mipomersen control group (ONT-41), with $p < 0.0001$ (2-way ANOVA with Newman-Keuls post-hoc test)

w: Statistically different from the PBS control group, with $p < 0.05$ (2-way ANOVA with Newman-Keuls post-hoc test)

x: Statistically different from the PBS control group, with $p < 0.01$ (2-way ANOVA with Newman-Keuls post-hoc test)

y: Statistically different from the PBS control group, with $p < 0.001$ (2-way ANOVA with Newman-Keuls post-hoc test)

z: Statistically different from the PBS control group, with $p < 0.0001$ (2-way ANOVA with Newman-Keuls post-hoc test)

[00421] Compared with 10 mg/kg IP Mipomersen, ONT-75 administered with the same dosing paradigm resulted in significantly lower reduction (knockdown) of serum ApoB protein levels on days 17, 24, 31,38, 45, 52, 60 ($p < 0.0001$), and 66 ($p < 0.001$) of the study. Compared with 10 mg/kg IP Mipomersen, ONT-77 administered with the same dosing paradigm resulted in significantly lower reduction (knockdown) of serum ApoB protein levels on days 17, 24, 31,38, and 45 ($p < 0.0001$), 60 ($p < 0.05$) and 66 ($p < 0.01$) of the study. Compared with 10 mg/kg IP Mipomersen, ONT-80 administered with the same dosing paradigm resulted in significantly lower reduction (knockdown) of serum ApoB protein levels on days 24,31,38 and 45 ($p < 0.0001$),17 ($p < 0.001$) and 66 ($p < 0.01$). Compared with 10 mg/kg IP Mipomersen, ONT-81 administered with the same dosing paradigm resulted in significantly lower reduction (knockdown) of serum ApoB protein levels on days 24, 31, 38 and 45 ($p < 0.0001$), 17 ($p < 0.01$), 52 and 66 ($p < 0.001$) of the study.

[00422] By day 38 (i.e. 6 days after the last dose), serum ApoB protein levels had returned to baseline after ONT-75. Considering the initial serum ApoB protein level reduction, the rate of return to baseline of serum ApoB protein was slower after ONT-77 and ONT-80 and the level of serum ApoB protein is similar to Mipomersen levels by day 52.

[00423] The results of the 5mg/kg dosing paradigm of additional stereopure architectures are shown in Table E-28c and Figure 54. Relative to PBS, 5 mg/kg IP ONT-87 resulted in significant reductions of serum ApoB protein levels on days 17, 24, 31 and 38 of the study, to 27%($p < 0.0001$), 40% ($p < 0.0001$), 55% ($p < 0.0001$) and 71% ($p < 0.0001$) of control levels, respectively. Relative to PBS, 5 mg/kg IP ONT-88 resulted in significant reductions of serum ApoB protein levels on days 17 , 24, 31 and 38 of the study, to 47% ($p < 0.0001$), 34% ($p < 0.0001$), 69% ($p < 0.0001$) and 85% ($p < 0.0001$) of control levels, respectively. Relative to PBS, 5

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mg/kg IP ONT-89 resulted in significant reductions of serum ApoB protein levels on days 17, 24 and 31 of the study, to 43% ($p<0.0001$), 48% ($p < 0.0001$) and 85% ($p<0.05$) of control levels, respectively.

[00424] Table E-28c: Serum Human Apolipoprotein B Levels Relative to PBS After Multiple IP Doses of 5 mg/kg Stereioisomer (ONT-87, -88 or -89) or Mipomersen in huApoB Mice (N = 3-4)

	Day			
SAMPLES	17	24	31	38
PBS	100 ± 5	100 ± 12	100 ± 4	100 ± 8
ONT-41	33^{z} ± 3	44^{z} ± 2	75^{y} ± 11	76^{y} ± 8
ONT-87	27^{z} ± 11	40^{z} ± 5	55^{bz} ± 8	71^{z} ± 7
ONT-88	47^{az} ± 5	34^{z} ± 8	69^{z} ± 13	85^{z} ± 7
ONT-89	43^{z} ± 9	48^{z} ± 6	85^{w} ± 12	93^{a} ± 7

a: Statistically different from the Mipomersen control group (ONT-41), with $p< 0.05$ (2-way ANOVA with Newman-Keuls post-hoc test)
b: Statistically different from the Mipomersen control group (ONT-41), with $p< 0.01$ (2-way ANOVA with Newman-Keuls post-hoc test)
c: Statistically different from the Mipomersen control group (ONT-41), with $p< 0.001$ (2-way ANOVA with Newman-Keuls post-hoc test)
d: Statistically different from the Mipomersen control group (ONT-41), with $p< 0.0001$ (2-way ANOVA with Newman-Keuls post-hoc test)
w: Statistically different from the PBS control group, with $p< 0.05$ (2-way ANOVA with Newman-Keuls post-hoc test)
x: Statistically different from the PBS control group, with $p< 0.01$ (2-way ANOVA with Newman-Keuls post-hoc test)
y: Statistically different from the PBS control group, with $p< 0.001$ (2-way ANOVA with Newman-Keuls post-hoc test)
z: Statistically different from the PBS control group, with $p< 0.0001$ (2-way ANOVA with Newman-Keuls post-hoc test)

[00425] Compared with 5 mg/kg IP Mipomersen, ONT-87 administered with the same dosing paradigm resulted in significantly higher reduction (knock-down) of serum ApoB protein levels on day 31, ($p < 0.01$) of the study. Compared with 5 mg/kg IP Mipomersen, ONT-88 administered with the same dosing paradigm resulted in significantly lower reduction (knock-down) of serum ApoB protein levels on day 17 ($p < 0.05$ of the study). Compared with 5 mg/kg IP Mipomersen, ONT-89 administered with the same dosing paradigm resulted in significantly lower reduction (knockdown) of serum ApoB protein levels on day 38 ($p < 0.05$) of the study.

[00426] The results of the 10mg/kg dosing paradigm of additional stereopure architectures are shown in Table E-28d and Figures 55 and 56. Relative to PBS, 10 mg/kg IP ONT-87 resulted in significant reductions of serum ApoB protein levels on days 17, and 24 of the study, to 27% ($p<0.0001$), 14% ($p < 0.0001$) of control levels, respectively. Relative to

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PBS, 10 mg/kg IP ONT-88 resulted in significant reductions of serum ApoB protein levels on days 17,and 24 of the study, to 23% ($p<0.0001$), and 17% ($p < 0.0001$) of control levels, respectively. Relative to PBS, 10 mg/kg IP ONT-89 resulted in significant reductions of serum ApoB protein levels on days 17 ,and 24 of the study, to 29%($p<0.0001$), and 23% ($p < 0.0001$) of control levels, respectively.

Table E-28d: Serum Human Apolipoprotein B Levels Relative to PBS After Multiple IP Doses of 10 mg/kg Stereioisomer (ONT-87, -88 or -89) or Mipomersen in huApoB Mice (N = 3-4)

	Day	
Sample	17	24
PBS	100 ± 5	100 ± 8
ONT-41	44^{z} ± 17	27^{z} ± 9
ONT-87	27^{az} ± 10	14^{z} ± 3
ONT-88	23^{az} ± 6	17^{z} ± 5
ONT-89	29^{az} ± 12	23^{z} ± 8

a: Statistically different from the Mipomersen control group (ONT-41), with $p< 0.05$ (2-way ANOVA with Newman-Keuls post-hoc test)

b: Statistically different from the Mipomersen control group (ONT-41), with $p< 0.01$ (2-way ANOVA with Newman-Keuls post-hoc test)

c: Statistically different from the Mipomersen control group (ONT-41), with $p< 0.001$ (2-way ANOVA with Newman-Keuls post-hoc test)

d: Statistically different from the Mipomersen control group (ONT-41), with $p< 0.0001$ (2-way ANOVA with Newman-Keuls post-hoc test)

w: Statistically different from the PBS control group, with $p< 0.05$ (2-way ANOVA with Newman-Keuls post-hoc test)

x: Statistically different from the PBS control group, with $p< 0.01$ (2-way ANOVA with Newman-Keuls post-hoc test)

y: Statistically different from the PBS control group, with $p< 0.001$ (2-way ANOVA with Newman-Keuls post-hoc test)

z: Statistically different from the PBS control group, with $p< 0.0001$ (2-way ANOVA with Newman-Keuls post-hoc test)

[00427] Compared with 10 mg/kg IP Mipomersen, ONT-87 administered with the same dosing paradigm resulted in significantly higher reduction (knockdown) of serum ApoB protein levels on day 17, ($p < 0.05$) of the study. Compared with 10 mg/kg IP Mipomersen, ONT-88 administered with the same dosing paradigm resulted in significantly higher reduction (knockdown) of serum ApoB protein levels on day 17 ($p < 0.05$) of the study. Compared with 10 mg/kg IP Mipomersen, ONT-89 administered with the same dosing paradigm resulted in significantly higher reduction (knockdown) of serum ApoB protein levels on day 17 ($p < 0.05$) of the study.

[00428] Thus, in at least some embodiments, the present invention provides chirally controlled oligonucleotide compositions that show a biological activity and are characterized by

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extended persistence and/or slower rate of decay of that activity over time as compared with an appropriate reference (e.g., a preparation of oligonucleotides of the same sequence but different chiral specificity, including particularly stereorandom preparations). In some embodiments where such extended persistence and/or slower rate of decay is observed, provided chirally controlled compositions may be administered according to a dosing regimen with fewer total doses and/or longer periods between two or more doses that is utilized with the "parent" stereorandom composition to achieve comparable biologic and/or therapeutic effect. For example, the slower return to baseline of serum ApoB protein following treatment with chirally pure oligonucleotide compared with Mipomersen as demonstrated in the present Example, suggests that chirally pure ApoB oligonucleotides can be dosed less frequently than the parental stereorandom mixture (e.g., than Mipomersen).

[00429] The results presented in this Example demonstrate, for instance, that chirally pure oligonucleotide compositions can have significantly different pharmacological activity *in vivo* as compared with an appropriate reference (e.g., a preparation of oligonucleotides of the same sequence but different chiral specificity, including particularly stereorandom preparations), and specifically as compared with a "parental" stereorandom preparation . Those skilled in the art, in light of this demonstration, will appreciate that chirally controlled oligonucleotide compositions provided by the present disclosure have unexpected activities and characteristics. Those skilled in the art, in light of the present disclosure, will further appreciate that various methodologies are provided, including therapeutic methods that utilize dosing regimens that differ from those utilized for non-chirally controlled compositions (e.g., of the same nucleotide sequence). In some embodiments, relatively larger individual doses of chirally controlled (e.g., chirally pure) oligonucleotides may be utilized as compared with a control (e.g., a stereorandom control). In some embodiments, relatively smaller individual doses of chirally controlled (e.g., chirally pure) oligonucleotides may be utilized as compared with a control (e.g., a stereorandom control). In some embodiments, relatively fewer doses of chirally controlled (e.g., chirally pure) oligonucleotides may be utilized as compared with a control (e.g., a stereorandom control) within a given period of time.

[00430] Example 70: siRNA Agents Comprising Chirally Controlled Oligonucleotides Inhibit PCSK-9

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[00431] The present Example demonstrates successful inhibition of target gene expression using siRNA agents comprised of chirally controlled oligonucleotides as described herein. Specifically, this Example describes hybridization of individual oligonucleotide strands prepared through chirally controlled synthesis as described herein, so that double-stranded chirally controlled siRNA oligonucleotide compositions are provided. This Example further demonstrates successful transfection of cells with such agents and, moreover, successful inhibition of target gene expression.

siRNA Transfection of Chiral siRNA Molecules

[00432] Hep3B, or HeLa cells were reverse transfected at a density of 2.0×10^4 cells/well in 96-well plates. Transfection of siRNA was carried out with lipofectamine RNAiMax (Life Technologies, cat. No. 13778-150) using the manufacturer's protocol, except with a decreased amount of Lipofectamine RNAiMax of 0.2ul per well. Twelve, 1:3 siRNA duplex dilutions were created starting at 1uM. 10ul of 10x siRNA duplex was then lipoplexed with a prepared mixture of 9.8ul of serum-free medium and 0.2ul of Lipofectamine RNAiMax per well. After a 10-15 minute incubation, 2.0×10^4 cells in 80ul of EMEM cell growing media (ATCC, 30-2003) were added to bring the final volume to 100ul per well. Two separate transfection events were performed for each dose.

[00433] 24 hours after transfection Hep3B or HeLa cells were lysed and PCSK9 mRNA was purified using MagMAX™-96 Total RNA Isolation Kit (Life Technologies, AM1830); 15ul of cDNA was synthesized with High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Life Technologies, 4374967). Gene expression was evaluated by Real-Time PCR on a Lightcycler 480(Roche) using a Probes Master Mix(Roche, 04 707 494 001) according to manufacturer's protocol using a FAM-labeled Taqman probe-set for PCSK9 (Life Technologies, Hs03037355_m1) and a VIC-labeled GAPDH primer-limited endogenous control (Life Technologies, NM_002046.3).

IC50s and Data Analysis

[00434] Delta delta Ct method was used to calculate values. Samples were normalized to hGAPDH and calibrated to mock transfected and untreated samples. A stereo-random molecule was used as positive control. The data are represented as a mean of 2 biological replicates using Graphpad Prism. A four-parameter linear regression curve was fitted to the data and the bottom

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and top were constrained to a 0 and 100 constants respectively in order to calculate a relative IC50.

Table E-29. Relative IC50 values [Hep3B Transfection]

	Bottom	Top	LogIC50		HillSlope		IC50 [nM]
			Mean	SEM	Mean	SEM	
ONT-D1	0	100	-0.39	0.12	-0.27	0.02	0.41
ONT-D2	0	100	-0.85	0.14	-0.24	0.03	0.14
ONT-D3	0	100	-0.62	0.14	-0.24	0.02	0.24
ONT-D4	0	100	-0.49	0.09	-0.28	0.02	0.33
ONT-D5	0	100	-0.32	0.09	-0.31	0.02	0.48

Table E-30. Relative IC50 values [HeLa Transfection]

	Bottom	Top	LogIC50		HillSlope		IC50
			Mean	SEM	Mean	SEM	
ONT-D1	0	100	-1.73	0.07	-0.61	0.05	0.02
ONT-D2	0	100	-1.94	0.07	-0.66	0.07	0.01
ONT-D3	0	100	-1.72	0.08	-0.62	0.07	0.02
ONT-D4	0	100	-1.26	0.06	-0.74	0.06	0.06
ONT-D5	0	100	-1.03	0.08	-0.86	0.12	0.09

Table E-31. Relative IC50 values of siRNAs with 3 Phosporothioate stereo-centers[HeLa Transfection]

	Bottom	Top	LogIC50		HillSlope		IC50
			Mean	SEM	Mean	SEM	
ONT-D7	0	100	0.75	0.15	-0.24	0.02	5.60
ONT-D8	0	100	0.39	0.09	-0.32	0.02	2.45
ONT-D9	0	100	0.43	0.13	-0.22	0.02	2.68
ONT-D10	0	100	1.57	0.20	-0.27	0.03	36.86
ONT-D11	0	100	2.92	0.33	-0.12	0.01	839.42
ONT-D12	0	100	-0.17	0.10	-0.38	0.03	0.68
ONT-D13	0	100	1.20	0.21	-0.39	0.08	15.97
ONT-D14	0	100	-0.22	0.18	-0.43	0.07	0.60
ONT-D15	0	100	-0.25	0.16	-0.41	0.06	0.57

[00435] Figures 45-50 present results of these studies. Figure 45 shows the % PCSK-9 mRNA remaining after Hep3B treatment with siRNA duplex. Figure 46 shows the % PCSK-9 mRNA remaining after Hep3B treatment with siRNA duplex curve fit. Figure 47 shows the % PCSK-9 mRNA remaining after HeLa treatment with siRNA duplex. Figure 48 shows the %

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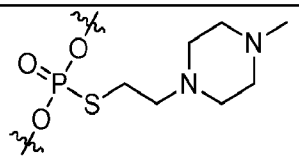
PCSK-9 mRNA remaining after HeLa treatment with siRNA duplex curve fit. Figure 49 shows the % PCSK-9 mRNA remaining after HeLa treatment with siRNA duplex containing 3 Phophorothiate stereo-centers. Figure 50 shows the % PCSK-9 mRNA remaining after HeLa treatment with siRNA duplex containing 3 Phophorothiate stereo-centers curve fit.

[00436] As can be seen, in molecules with a single sterochemically defined phosphorothioate in each strand, a slightly increased potency was observed with molecules with *Sp* stereo-chemistry at the phosphorothioate in both sense and antisense strands While the specifically exemplified siRNA agents contained only one chiral center per strand, those skilled in the art, reading the present disclosure, will recognize the significance of the demonstrated differential effect as proof of principle that presence of chirality can impact activity.

[00437] The impact of chirality is more pronounced in siRNAs with more than two stereo-centers. In siRNAs with 3 chiral phosphorothioates (one in the sense strand and two in the antisense strand), an *Sp* stereochemistry at the 5' end (between nucleotides n1 and n2) combined with a *Rp* stereochemistry at the 3' end (between nucleotides n20 and n21) of the antisense strand had deleterious effect on potency. However, an *Rp* stereochemistry at the 5' end (between nucleotides n1 and n2) combined with a *Sp* stereochemistry at the 3' end (between nucleotides n20 and n21) of the antisense strand showed a significant PCSK-9 mRNA knockdown improvement over stereo-random control siRNA. The results suggest that both strands are affected by the stereochemistry of the phosphorothioate. It is likely that the observed efficacy differential will increase for agents with larger numbers of chiral centers and, moreover that both location and type of chiral center can impact activity, for example through effects on stability, potency, and/or both. Those skilled in the art, reading the present disclosure, will therefore appreciate that it provides teachings of both single and double-stranded chirally controlled oligonucleotide compositions, and uses thereof that, in at least some embodiments, distinguish such chirally controlled oligonucleotides and agents from stereorandom agents of identical sequence.

Example 71. Additional Exemplary Oligonucleotides and Synthesis Thereof.

s16	

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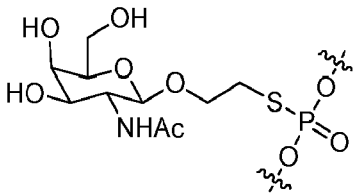
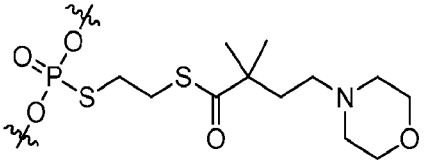
s17	
s18	

[00438] Oligonucleotides N101-N104 were synthesized using the automated synthesis on ABI-394 DNA/RNA synthesizer according to the synthetic cycle summarized on Table E-32, using 1 μmol synthesis column and 1.7 μmol of oxalyl linked $dG^{CE,Pac}$ on HCP. The synthesis cycle was performed with removal of the terminal 5′-*O*-DMTr group (DMT Off). After completion of the automated oligonucleotide synthesis, the HCP support was washed with dry ACN and dried under vacuum. The dry HCP was placed in a plastic vial and was treated with 1 mL of sat. NH_3/Py for 1 h at 55 °C then 1 mL of dry propylamine in dry pyridine (in a 1:4 ratio) for 18 h at RT. The solvents were then evaporated and the residue was re-suspended with ~pH 1.5 HCl aqueous solution containing 10% DMSO and the HCP support was filtered off. The crude product was purified by reverse phase preparative HPLC (According to the procedure described below). The fractions having purity above 95% were pooled, concentrated and desalted by reverse-phase HPLC (According to the procedure described below). The final desalted product was lyophilized from water.

Table E-32. Summary for Oligonucleotide Synthesis on a DNA/RNA Synthesizer ABI-394 Used for the Synthesis of oligonucleotides N101-N102.

step	reaction	reagent	delivery time (sec)	wait time (sec)
1	detritylation	3% TCA in DCM	3 + 60 + 10	N.A.
2	coupling	0.15 M phosphoramidite in ACN + 1.2 M CMPT in	5 + 4	30 + 600

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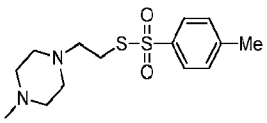
		ACN		
3	capping 1	5% Pac_2O in THF/2,6-lutidine	20	60
4	capping 2	5% Pac_2O in THF/2,6-lutidine + 16% NMI in THF	20	60
5	sulfurization	0.3 M S-(N-methylpiperazinoethyl-ToluylThioSulfonate  in ACN/BSTFA	7 + 1.5×4	360 + 3×180 + 900

[00439] **Example 72. HPLC method for N101 and N102:**

Buffer A: 50 mM TEAA, pH 9.6 (adjusted with TEA)

Buffer B: ACN

Column: XBridge C_8, 3.5 μm, 4.6x50mm, Part #186003034

Buffer heater set temperature = 35 °C

Signal monitored at 254 and 280 nm

Gradient used:

Time	Flow (ml/min)	% A	% B	Curve
Initial		99	1	
2	1	99	1	1
22	1	70	30	6
25	1	5	95	6
25.5	1	5	95	6
30	1	99	1	1

[00440] **Example 73. General UPLC-LCMS method for N101 and N102.**

Buffer A: 10 mM ammonium formate, pH 9.5 (adjusted with NH_3 aq.)

Buffer B: ACN

Column: UPLC@OST C_{18} 1.7 μm, 2.1×500mm

Column temperature = 35 °C

Gradient used:

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.5	99	1	
2	0.5	99	1	1
12	0.5	60	40	6
13	0.5	5	95	6
13.5	0.5	5	95	6
15	0.5	99	1	1

[00441] **Example 74. General Purification Method for N101 and N102:**

Buffer A: 50 mM TEAA pH=9.6 (adjusted with TEA)

Buffer B: ACN

Column: XBridge Prep C_{18}, 5 μm, C_{18}, 250×10mm, Part #186003256

Buffer heater set temperature = RT

Signal monitored at 254 and 280 nm

Gradient used:

Time	Flow (ml/min)	% A	% B	Curve
Initial		99	1	
5	4	99	1	1
10	4	95	5	6
30	4	74	26	6
35	4	5	95	6
36	4	5	95	6
38	4	99	1	6
45	4	99	1	1

100 μL of 49-51% phosphoric acid was added to each fraction (pH 3) and checked by HPLC, then stored at -80°C until frozen. The fractions were kept on lypholizer for a couple of hours until ½ volumes then stored at -80°C.

[00442] **Example 75. General Desalting Method for N101 and N102:**

Buffer A: 10 mM formic acid

Buffer B: ACN

Column: XBridge Prep C_{18}, 5 μm, C_{18}, 250×10mm, Part #186003256

Buffer heater set temperature = RT

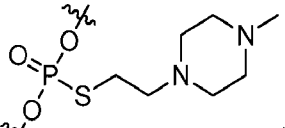
Signal monitored at 254 and 280 nm

Gradient used:

Time	Flow (ml/min)	% A	% B	Curve
Initial		100	0	
12	4	100	0	1
13	4	65	35	6
21	4	65	35	1
21.5	4	100	0	6
30	4	100	0	1

Collected fractions were stored in -80°C until frozen and kept on lypholizer until dryness and checked by HPLC.

[00443] **Example 76. Synthesis of Oligonucleotide N101 (All-(*Rp*)-**



d[5mCs16As16Gs16T] (s16 = _____))

[00444] Oligonucleotide N101 was synthesized as described above and purified. RT in RP-HPLC: (HPLC method N1): 15.9 min. UPLC/ESI-MS: Calcd: 1614.64; Found[+H]: 1615.06.

[00445] **Example 77. Synthesis of Oligonucleotide N102 (All-(*Sp*)-d[5mCs16As16Gs16T])**

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[00446] Oligonucleotide N102 was synthesized as described above and purified. RT in RP-HPLC (HPLC method N1): 16.4 min. UPLC/ESI-MS: Calcd: 1614.64; Found[+H]: 1614.97.

[00447] **Example 78. Synthesis of Oligonucleotide N103 (All-(*Rp*)-d[5mCs16As16Gs16Ts165mCs16Ts16Gs165mCs16Ts16Ts165mCs16G])**

[00448] Oligonucleotide N103 was synthesized as described above. RT in RP-HPLC (HPLC method N1): 18.4 min. UPLC/ESI-MS: Calcd for $C_{197}H_{311}N_{62}O_{62}P_{11}S_{11}$: 5233.38; Found: 5234.7.

[00449] **Example 79. Synthesis of Oligonucleotide N104 (All-(*Sp*)-d[5mCs16As16Gs16Ts165mCs16Ts16Gs165mCs16Ts16Ts165mCs16G])**

[00450] Oligonucleotide N104 was synthesized as described above. RT in RP-HPLC (HPLC method N1): 18.7 min. UPLC/ESI-MS: Calcd: 5233.38; Found: 5232.9.

[00451] Oligonucleotides N105-N106 were synthesized using the automated synthesis on ABI-394 DNA/RNA synthesizer according to the synthetic cycle summarized on Table E-33, using 1 μmol synthesis column and 1.7 μmol of oxalyl linked $dG^{CE,Pac}$ on HCP. The synthesis cycle was performed with removal of the terminal 5′-*O*-DMTr group (DMT Off). After completion of the automated oligonucleotide synthesis, the HCP support was washed with dry ACN and dried under vacuum. The dry HCP was placed in a plastic vial and was treated with 1 mL of sat. NH_3/i-PrOH for 16 h at 55 °C. The solvent was then evaporated and the residue was re-suspended with pH 7.0 aqueous solution containing 10% DMSO and the HCP support was filtered off. The crude product is purified by reverse phase preparative HPLC. The fractions having purity above 95% are pooled, concentrated and desalted by reverse-phase HPLC. The final desalted product is lyophilized from water.

Table E-33. Summary for Oligonucleotide Synthesis on a DNA/RNA Synthesizer ABI-394 Used for the Synthesis of oligonucleotides N105-N106.

step	reaction	reagent	delivery time (sec)	wait time (sec)
1	detritylation	3% TCA in DCM	3 + 60 + 10	N.A.
2	coupling	0.15 M phosphoramidite in ACN + 1.2 M CMPT in ACN	5 + 4	30 + 600

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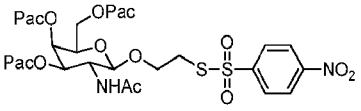
3	capping 1	5% Pac_2O in THF/2,6-lutidine	20	60
4	capping 2	5% Pac_2O in THF/2,6-lutidine + 16% NMI in THF	20	60
5	sulfurization	0.3 M  in ACN/BSTFA	7 + 1.5×4	360 + 3×180 + 900

[00452] **Example 80. HPLC method for N101 and N102:**

Buffer A: 50 mM TEAA, pH 7.0

Buffer B: ACN

Column: XBridge C_8, 3.5 μm, 4.6x150mm, Part #186003034

Buffer heater set temperature = 35 °C

Signal monitored at 254 and 280 nm

Gradient used:

Time	Flow (ml/min)	% A	% B	Curve
Initial		99	1	
2	1	99	1	1
22	1	80	20	6
25	1	5	95	6
25.5	1	5	95	6
30	1	99	1	1

[00453] **Example 81. General UPLC-LCMS method for N101 and N102.**

Buffer A: 10 mM ammonium formate, pH 7.0

Buffer B: ACN

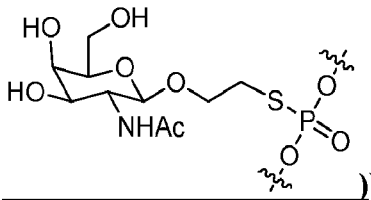
Column: UPLC@OST C_{18} 1.7 μm, 2.1×500mm

Column temperature = 35 °C

Gradient used:

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.5	99	1	
2	0.5	99	1	1
12	0.5	70	30	6
13	0.5	5	95	6
13.5	0.5	5	95	6
15	0.5	99	1	1

[00454] **Example 82. Synthesis of Oligonucleotide N105 (All-(*Rp*)-d[5mCs9As9Gs9T] (s9 =**



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[00455] Oligonucleotide N105 was synthesized as described above. RT in RP-HPLC: (HPLC method N2): 14.1 min. UPLC/ESI-MS: Calcd: 1977.78; Found[-H]: 1978.8.

[00456] **Example 83. Synthesis of Oligonucleotide N106 (All-(*Sp*)-d[5mCs9As9Gs9T])**

[00457] Oligonucleotide N106 was synthesized and purified as described above. RT in RP-HPLC (HPLC method N2): 14.7 min. UPLC/ESI-MS: Calcd: 1977.78; Found[-H]: 1977.37.

[00458] Oligonucleotides xxx were synthesized using the automated synthesis on ABI-394 DNA/RNA synthesizer according to the synthetic cycle summarized on Table E-33, using 1 μmol synthesis column and 1.7 μmol of oxalyl linked $dG^{CE,Pac}$ on HCP. The synthesis cycle was performed with removal of the terminal 5′-*O*-DMTr group (DMT Off). After completion of the automated oligonucleotide synthesis, the HCP support was washed with dry ACN and dried under vacuum. The dry HCP was placed in a plastic vial and was treated with 1 mL of sat. NH_3/i-PrOH for 16 h at 55 °C. The solvent was then evaporated and the residue was re-suspended with pH 7.0 aqueous solution containing 10% DMSO and the HCP support was filtered off. The crude product is purified by reverse phase preparative HPLC. The fractions having purity above 95% are pooled, concentrated and desalted by reverse-phase HPLC. The final desalted product is lyophilized from water.

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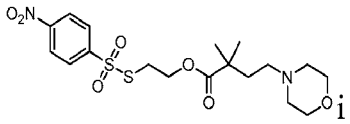
[00459] Table E-34. Summary for Oligonucleotide Synthesis on a DNA/RNA Synthesizer ABI-394.

step	reaction	reagent	delivery time (sec)	wait time (sec)
1	detritylation	3% TCA in DCM	3 + 60 + 10	N.A.
2	coupling	0.15 M phosphoramidite in ACN + 1.2 M CMPT in ACN	5 + 4	30 + 600
3	capping 1	5% Pac_2O in THF/2,6-lutidine	20	60
4	capping 2	5% Pac_2O in THF/2,6-lutidine + 16% NMI in THF	20	60
5	sulfurization	0.3 M  n ACN/BSTFA	10 + 4×2	300 + 3×150 + 600

[00460] Example 84. General Purification Method

Buffer A: 50 mM TEAA

Buffer B: ACN

Column: XBridge Prep C_{18}, 5 μm, C_{18}, 250×10mm, Part #186003256

Buffer heater set temperature = rt

Signal monitored at 254 and 280 nm

Gradient used:

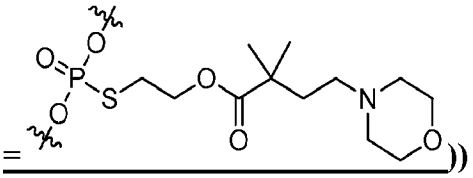
Time	Flow (ml/min)	% A	% B	Curve
Initial		99	1	
5	4	99	1	1
10	4	95	5	6

30	4	74	26	6
35	4	5	95	6
36	4	5	95	6
38	4	99	1	6
45	4	99	1	1

HPLC method 5:

Time	Flow (ml/min)	% A	% B	Curve
Initial		80	20	
2	1	80	20	1
22	1	45	55	6
25	1	5	95	6
25.5	1	5	95	6
26	1	85	20	6
30	1	85	20	1

[00461] **ONT-60: Oligonucleotide 149 All-(*Rp*)-d[5mCs8As8Gs8Ts85mCs8Ts8Gs85mCs8Ts8Ts85mCs8G] (s8**



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[00462] The oligonucleotide was synthesized as described above. RT in RP-HPLC (HPLC method 5): 15.80 min. UPLC/ESI-MS: Calcd: 6345.6; Found: 6340.7.

[00463] **ONT-69: All-(*Sp*)-d[5mCs16As8Gs8Ts85mCs8Ts8Gs85mCs8Ts8Ts85mCs8G]**

[00464] The oligonucleotide was synthesized as described above. RT in RP-HPLC (HPLC method 5): 18.61 min. UPLC/ESI-MS: Calcd: 6345.6; Found: 6340.6.

[00465] **Example 85.**

[00466] The P-modified blockmer and altmer oligonucleotides containing both stereodefined phosphorothioate diester and stereodefined morpholinoethyl phosphorothioate triester internucleotidic linkages were synthesized on an ABI-394 DNA/RNA synthesizer according to the cycle summarized in Table E-4 using 1 μmol synthesis column and 1.7 μmol of

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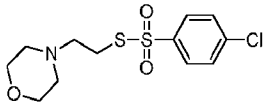
oxalyl linked $dG^{CE,Pac}$ on HCP. Either stereodefined P-modified phosphorothioate linkage was introduced at predetermined positions within the sequence by performing either sulfurization step (1) or (2). The synthesis cycle was performed with removal of the terminal 5′-*O*-DMTr group (DMT Off). The solid support was washed with dry ACN and dried under a flux of argon. The dry HCP was placed in a plastic vial and was treated with 1 mL of dry propylamine in dry pyridine (in a 1:4 ratio) for a period of 18 h at r.t. The solvents were then evaporated and the residue was re-suspended in DMSO and the HCP support was filtered off. The crude product was purified by reverse phase preparative HPLC. The fractions having purity above 95% were pooled, concentrated and desalted by reverse-phase HPLC (Gradient of 0 to 80% ACN). The final desalted product was lyophilized from water.

Table E-4. Summary for Oligonucleotide Synthesis on a DNA/RNA Synthesizer ABI-394 Used for the Synthesis of Example 85.

step	reaction	reagent	delivery time (sec)	wait time (sec)
1	detritylation	3% TCA in DCM	3 + 60 + 10	N.A.
2	coupling	0.15 M phosphoramidite in ACN + 1.2 M CMPT in ACN	5 + 4	30 + 600
3	capping 1	5% Pac_2O in THF/2,6-lutidine	20	60
4	capping 2	5% Pac_2O in THF/2,6-lutidine + 16% NMI in THF	20	60
5	sulfurization 1	0.3 M S-Morpholinoethyl *p*-Chlorophenyl thiosulfonate  in ACN/BSTFA	10 + 4×2	300 + 3×150 + 600

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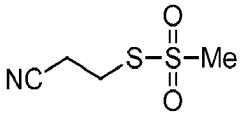
6	sulfurization 2	0.3 M S-cyanoethyl Methylthiosulfonate  in ACN/BSTFA	10 + 4×2	300 + 3×150 + 600

[00467] **Example 86. General Purification Method for Example 85:**

Buffer A: 20 mM Phosphate pH=6.0 (adjusted with phosphoric acid)

Buffer B: ACN

Column: XBridge Prep C_{18}, 5 μm, C_{18}, 250×10mm, Part #186003256

Buffer heater set temperature = 50 °C

Signal monitored at 254 and 280 nm

Gradient used:

Time	Flow (ml/min)	% A	% B	Curve
Initial		99	1	
5	4	99	1	1
10	4	77	23	6
60	4	70	30	6
65	4	20	80	6
70	4	20	80	6
71	4	99	1	6
80	4	99	1	1

[00468] HPLC method 6

Buffer A: 20 mM Ammonium acetate, pH 6.0

Buffer B: ACN

Column: XBridge C_8, 3.5 μm, 4.6x150mm, Part #186003034

Buffer heater set temperature = 60 °C

Signal monitored at 254 and 280 nm

Gradient used:

Time	Flow (ml/min)	% A	% B	Curve
Initial		90	10	
2	1	90	10	1
22	1	75	25	6
25	1	5	95	6
25	1	5	95	6
25.5	1	90	10	6
30	1	90	10	1

[00469] **ONT-71: All-(*Rp*)- d[5mCs1As1GsTs5mCsTsGs5mCsTsTs15mCs1G]**

[00470] The oligonucleotide was synthesized as described above. RT in RP-HPLC (HPLC method 6): 9.39 min. UPLC/ESI-MS: Calcd: 4297.9; Found: 4295.3

[00471] **ONT-72: All-(*Sp*)- d[5mCs1As1GsTs5mCsTsGs5mCsTsTs15mCs1G]**

[00472] The oligonucleotide was synthesized as described above. RT in RP-HPLC (HPLC method 6): 10.84 min. UPLC/ESI-MS: Calcd: 4297.9; Found: 4295.7

[00473] **ONT-73: All-(*Rp*)- d[5mCs1AsGs1Ts5mCs1TsGs15mCsTs1Ts5mCs1G]**

[00474] The oligonucleotide was synthesized as described above. RT in RP-HPLC (HPLC method 6): 13.54 min. UPLC/ESI-MS: Calcd: 4524.2; Found: 4522.6

[00475] **ONT-74: All-(*Sp*)- d[5mCs1AsGs1Ts5mCs1TsGs15mCsTs1Ts5mCs1G]**

[00476] The oligonucleotide was synthesized as described above. RT in RP-HPLC (HPLC method 6): 15.52 min. UPLC/ESI-MS: Calcd: 4524.2; Found: 4521.0

[00477] Prodrug oligonucleotide properties such as: nuclease resistance, tissue accumulation, cell penetration, endosomal escape, immunostimulation, duration of action, pharmacokinetics, etc. are all modulated and influenced by the stereochemistry of the chiral phosphorothioate backbone linkages.

[00478] RNA oligonucleotides containing 2'-OH, 2'-OMe, 2'-F, 2'-deoxy, internucleotidic phosphodiester or internucleotidic stereodefined phosphorothioate diester or internucleotidic stereodefined phosphorothioate triester (ProDrug) (either releasing a internucleotidic phosphodiester (PO) or a internucleotidic stereodefined phosphorothioate diester (PS)) linkages are synthesized on ABI 394 DNA/RNA synthesizer according to the cycles summarized in Table E-35, Table E-36, Table E-37 and Table E-38, using a 10 μmol capacity synthesis column loaded

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with 130 mg (4.9 μmol) of oxalyl linked 5'-*O*-DMTr-2'-deoxythymidine prepared as previously described. Prior to synthesis, a preliminary capping step (capping 2) is performed and the synthesis is terminated with removal of the terminal 5'-*O*-DMTr groups. The oxidation step is performed using a commercially available 5-6 M solution of *tert*-butyl hydroperoxide (TBHP) in decane which was then diluted with four parts dichloromethane. The stereospecific sulfurization step for internucleotidic stereodefined phosphorothioate diester linkage is performed using the 0.3 M *S*-cyanoethylmethylthiosulfonate reagent following the coupling of the corresponding chiral phosphoramide and the two-step capping process (Table E-36). The stereospecific sulfurization step for internucleotidic stereodefined phosphorothioate triester releasing a stereodefined internucleotidic phosphodiester (PS) linkage is performed using the 0.3 M *S*-(*N*-morpholinoethylthioester-ethyl)-*para*-nitro-toluylthiosulfonate reagent following the coupling of the corresponding chiral phosphoramide and the two-step capping process (Table E-37). The stereospecific sulfurization step for internucleotidic stereodefined phosphorothioate triester releasing an internucleotidic phosphodiester (PO) linkage is performed using the 0.3 M *S*-(*N*-morpholinoethyl)-toluylthiosulfonate reagent following the coupling of the corresponding chiral phosphoramide and the two-step capping process (Table E-38). For 2'-OH RNA nucleotides, 2'-*O*-base protecting groups are used, such as 2'-*O*-PivOM (Debart *et al.*, *Chem. Eur. J.*, **2008**, *14*, 9135) or 2'-*O*-TC (Dellinger *et al.*, *J. Am. Chem. Soc.*, **2011**, *133*, 11540). Ultra-mild (C-Ac, G-Pac, A-Pac) protecting groups are used for all of the nucleobases.

[00479] Once the automated oligonucleotide synthesis cycle is completed and the final 5'-*O*-DMTr group is removed, the synthesis column is taken off the DNA/RNA synthesizer and dried under vacuum. 10 mL solution of 0.5 M 1,5-diazabicyclo[4.3.0]non-5-ene (DBN), 0.25 M *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) in ACN is continuously added to the support through the synthesis column for 1 min without stopping the flow using a syringe attached to one end of the synthesis column. The support is then washed with anhydrous ACN and dried under vacuum. Then, the dried support is transferred into an empty screw-cap plastic vial and treated with 10% *n*-$PrNH_2$ solution in anhydrous pyridine (1.5 mL) at room temperature for 12 h. After that, the solvents are evaporated to dryness and the residue including the solid support is dissolved in 2 mL of water/DMSO (50/50) at pH 5, the support is filtered off and the filtrates are collected, immediately frozen and stored at -80 °C prior to purification.

Table E-35. Summary for Oligonucleotide Synthesis on a DNA/RNA Synthesizer ABI 394

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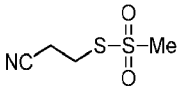
step	reaction	reagent	delivery time (sec)	wait time (sec)
1	detritylation	3% TCA in DCM	3 + 120 + 10	N. A.
2	coupling	0.15 M phosphoramidite in ACN + 2 M CMPT in ACN	7 + 6	30 + 600
3	capping	5% Pac_2O in THF/2,6-lutidine + 16% NMI in THF	10	20
4	oxidation	1.1 M *tert*-butyl hydroperoxide in 4:1 dichloromethane:decane	20	110

Table E-36. Summary for Oligonucleotide Synthesis on a DNA/RNA Synthesizer ABI 394

step	reaction	reagent	delivery time (sec)	wait time (sec)
1	detritylation	3% TCA in DCM	3 + 120 + 10	N. A.
2	coupling	0.15 M chiral phosphoramidite in ACN + 2 M CMPT in ACN	8 + 6	30 + 900 (2'-OMe RNA) 30 + 600 (DNA)
3	capping 1	5% Pac_2O in THF/2,6-lutidine	30	60
4	capping 2	5% Pac_2O in THF/2,6-lutidine + 16% NMI in THF	30	60
5	sulfurization	0.3 M *S*-(2-cyanoethyl) methylthiosulfonate 	15 + 3×4	120 + 3×60 + 300

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		in ACN/BSTFA		

Table E-37. Summary for Oligonucleotide Synthesis on a DNA/RNA Synthesizer ABI-394 .

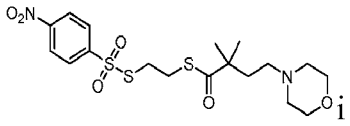
step	reaction	reagent	delivery time (sec)	wait time (sec)
1	detritylation	3% TCA in DCM	3 + 60 + 10	N.A.
2	coupling	0.15 M phosphoramidite in ACN + 1.2 M CMPT in ACN	5 + 4	30 + 600
3	capping 1	5% Pac_2O in THF/2,6-lutidine	20	60
4	capping 2	5% Pac_2O in THF/2,6-lutidine + 16% NMI in THF	20	60
5	sulfurization	0.3 M *S*-(*N*-morpholinothioester-ethyl-*p*-nitro-toluylthiosulfonate  n ACN/BSTFA	10 + 4×2	300 + 3×150 + 600

Table E-38. Summary for Oligonucleotide Synthesis on a DNA/RNA Synthesizer ABI-394 .

step	reaction	reagent	delivery time (sec)	wait time (sec)
1	detritylation	3% TCA in DCM	3 + 60 + 10	N.A.

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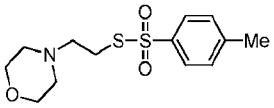
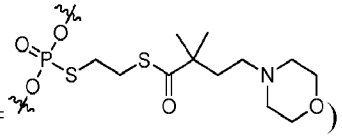
2	coupling	0.15 M phosphoramidite in ACN + 1.2 M CMPT in ACN	5 + 4	30 + 600
3	capping 1	5% Pac_2O in THF/2,6-lutidine	20	60
4	capping 2	5% Pac_2O in THF/2,6-lutidine + 16% NMI in THF	20	60
5	sulfurization	0.3 M *S*-Morpholinoethyl Toluylthiosulfonate  in ACN/BSTFA	10 + 4×2	300 + 3×150 + 600

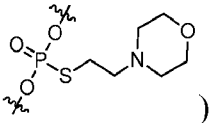
[00480] (s10 = )

[00481] Synthesis of Oligonucleotide: (*R*p)- uucuAGAccuGuuuuGcuudTs10dT

[00482] Synthesis of Oligonucleotide ONT-107: (*S*p)- uucuAGAccuGuuuuGcuudTs10dT

[00483] Synthesis of Oligonucleotide ONT-108: (*R*p)- AAGcAAAAcAGGUCuAGAAdTs10dT

[00484] Synthesis of Oligonucleotide ONT-109: (*S*p)- AAGcAAAAcAGGUCuAGAAdTs10dT

[00485] (s1 = )

[00486] Synthesis of Oligonucleotide: (*R*p, *R*p)- as1AGcAAAAcAGGUCuAGAAdTsdT

[00487] Synthesis of Oligonucleotide: (*S*p, *R*p)- as1GcAAAAcAGGUCuAGAAdTsdT

[00488] Synthesis of Oligonucleotide: (*S*p, *S*p)- as1GcAAAAcAGGUCuAGAAdTsdT

[00489] Synthesis of Oligonucleotide: (*R*p, *S*p)- as1GcAAAAcAGGUCuAGAAdTsdT

[00490] Synthesis of Oligonucleotide: (All (*S*p))- us1ucus1AGAccs1uGus1uus1uGcuudTs10dT

[00491] Synthesis of Oligonucleotide: (All (*R*p))-
As1OAGcAAAAcAGGs1UCuAs1GAs1AdTs1OdT

[00492] RNA strand thermal annealing and preparation of siRNA duplexes. Each RNA strand is mixed with its complementary RNA strand in equimolar concentration of 10 μM in 1×PBS. Total 0.5 mL solution is prepared for each duplex and the mixture is heated at 90 °C for 2 min and is allowed to cool down over the course of several hours. The mixtures are then stored at 4 °C.

[00493] Following the thermal RNA strand annealing step, all the possible siRNA duplex combinations are prepared by annealing any of the Sense strands with any possible complementary strand of the Antisense strands.

[00494] All prepared siRNA duplexes are evaluated *in vitro* for their PCSK9 gene-silencing properties, following transfection in HeLa cells or Hep3B cells.

[00495] All prepared siRNA duplexes with ProDrug groups are evaluated *in vitro* for their PCSK9 gene-silencing properties, following free uptake in Hep3B cells, Huh-7 cells or human primary hepatocytes.

[00496] Different potenties are observed, modulated by the number, the position and the stereo architecture of the chiral phosphorothioate backbone linkages, combined with the layers of additional chemical modifications and ProDrug groups explored.

[00497] siRNA properties such as: nuclease resistance, cell penetration, endosomal escape, duplex thermodynamic stability, tridemnsional structure of the duplex, affinity towards the various mechanistic steps of enzyme interactions, affinity towards the target mRNA, specific off-target effects, immunestimulation, duration of action, pharmacokinetics, etc. are all modulated and influenced by the stereochemistry of the chiral phosphorothioate backbone linkages as well as the presence or the absence of the ProDrug group.

[00498] The attachment of PO and PS releasing ProDrug groups to the siRNA duplexes enhances their intracellular delivery and free uptake in the absence of transfection reagent or targeting ligand.

[00499] **Example 87: Stability Studies of Diastereomerically pure Oligonucleotides**

[00500] The present Example compares the *in vitro* stability of chirally pure oligonucleotides with that observed for the "parent" stereorandom mixture(i.e., for a composition containing oligonucleotides of the same sequence as the chirally pure oligonucleotides but not

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displaying chiral purity, for example as a result of having been prepared via a stereorandom process). Seven chirally pure oligonucleotides, each of which had a sequence complementary to that of a particular target transcript or gene encoding a protein of interest were synthesized, formulated, and assessed for metabolic stability using three different biological matrices: snake venom phosphodiesterase (svPDE), nuclease P1 (nP1) and rat whole liver homogenates. Levels of full-length oligonucleotide were quantified by IEX-HPLC after different incubation periods. The results presented in this Example demonstrate, for instance, that chirally pure oligonucleotide compositions can have significantly different metabolic stability as compared with an appropriate reference (e.g., a preparation of oligonucleotides of the same sequence but different chiral specificity, including particularly stereorandom preparations), and specifically as compared with a "parental" stereorandom preparation. In this Example, oligonucleotides having a sequence antisense to (and therefore targeting) human Apolipoprotein-B (ApoB) were used for proof-of-concept in metabolic stability studies.

[00501] Snake venom phosphodiesterase (svPDE) digestion study for oligonucleotides ONT-75, ONT-77, ONT-80, ONT-81, ONT-87, ONT-88, ONT-89 and ONT-41

[00502] We used the protocol reported by Oka *et al. (J. Am. Chem. Soc.* **2008**, *130*, 16031–16037) with minor modifications. Purified oligonucleotide (10 nmoles) in water (50 μL) was added to the aqueous solution (450 μL, pH 8.6) containing svPDE (4×10^{-3} units), 100 mM Tris-HCl and 15 mM $MgCl_2$. The mixture was incubated at 37 °C with shaking at 400 rpm. A 50 μL aliquot was taken at each time point (0 h, 12 h, 1 d, 2 d, 3 d, 4 d, 5 d, 6 d and 7 d) and was quenched with 25 μL of 150 mM EDTA, 2 μL of Proteinase K solution (20 mg/mL) and 30 μL of Lysis buffer (Qiagen, #129115) and the mixture was heated at 60 °C for 20 min. 5μL of internal standard (5′-GCGTTTGCTCTTCTTCTTGCGTTTTTT-3′, a 27-mer oligonucleotide (underlined nucleotides are 2′-MOE modified), (200 μM) was added to the aliquot. Quantification analyses were performed by IEX-HPLC and metabolite identification was carried out by UPLC/MS. The results were illustrated in Figure 59.

[00503] IEX-HPLC analysis showed degradation of phosphorothioate 20-mer during incubation with svPDE with no significant difference between stereoisomers. LCMS analysis of metabolites revealed that the majority of the degradation products were formed as a result of desulfurization. As reported previously by Prakash *et al.* (*Biochemistry* **2002,** *41,* 11642-11648), Prhavc *et al.* (*Org.Lett.*, **2003**, *5*, 2017-2020) and others, we also observed that 2′-MOE

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modifications at 5′ and 3′-ends protect these oligomers from svPDE digestion which is a 3′→5′ exonuclease.

[00504] Nuclease P1 (nP1) digestion study for oligonucleotides ONT-75, ONT-77, ONT-80, ONT-81, ONT-87, ONT-88, ONT-89 and ONT-41

[00505] We employed the protocol reported by Oka et al. *(J. Am. Chem. Soc.* **2008**, *130*, 16031–16037) with minor modifications. Purified oligonucleotide (10 nmoles) in Water (50 μL) was added to an aqueous solution (500 μL, pH 7.2) containing nP1 (20 units), 100 mM Tris-HCl and 1 mM $ZnCl_2$. The mixture was incubated at 37 °C with shaking at 400 rpm. A 50 μL aliquot was taken at each time point (0 h, 1 h, 2 h, 4 h, 8 h, 12 h, 1 d, 2 d,) and was quenched with 25 μL of stop buffer (150 mM EDTA, 2 μL of Proteinase K solution (20 mg/mL) and 30 μL of Lysis buffer (Qiagen, #129115) and the mixture was heated at 60 °C for 20 min. 5 μL of internal standard (5′-GCGTTTGCTCTTCTTCTTGCGTTTTTT-3′), a 27-mer oligonucleotide (underlined nucleotides are 2′-MOE modified) (200 μM) was added to the aliquot. Quantification analyses were performed by IEX-HPLC and metabolite identification was carried out by UPLC/MS. The results were illustrated in Figures 60-67.

[00506] Nuclease nP1 has previously been reported to specifically cleave DNA phosphorothioate linkages having an (*S*p) absolute configuration at the phosphorus atoms (Porter *et al.*, *Biochemistry*, **1983**, *22*, 1369-1377; Oka *et al.*, *J. Am. Chem. Soc.*, **2008**, *130*, 16031-16037). We observed a similar pattern of cleavage for the 5-10-5 2′-MOE gapmer phosphorothioate oligonucleotides studied, where nP1 was found to digest efficiently the DNA core of the gapmer oligonucleotides at the (*S*p) phosphorothioate centers, without affecting the 2′-MOE wings, which were stable independent of stereochemistry. The complete digestion of the DNA core was observed in one hour for the stereorandom diastereomixture oligonucleotide ONT-41, as well as for the stereopure oligonucleotides containing (*S*p)-phosphorothioate internucleotidic linkages in the DNA core (ONT-77, ONT-80, ONT-87, ONT-88 and ONT-89). All products of cleavage were clearly identified by UPLC/MS. As previously reported in the literature, we found that (*R*p) phosphorothioate DNA was clearly not a substrate for nP1. The two chirally pure oligonucleotides having (*R*p) phosphorothioate DNA cores (ONT-75 and ONT-81) were completely stable to nP1 for an incubation period of 1 h at 37 °C. ONT-75 and ONT-81 showed c.a. 10-15% loss of the full length products during the course of incubation over several days. The results presented in this Example demonstrate, for instance, that chirally pure

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oligonucleotide compositions can have significantly different metabolic stability in an nP1 assay as compared with an appropriate reference (e.g., a preparation of oligonucleotides of the same sequence but different chiral specificity, including particularly stereorandom preparations), and specifically as compared with a "parental" stereorandom preparation.

[00507] General IEX-HPLC method for the analysis of enzyme digestion products

Buffer A: 10 mM TrisHCl, 50%ACN, pH 8.0

Buffer B: 10 mM TrisHCl, 800 mM $NaClO_4$, 50%ACN, pH 8.0

Column: DIONEX, DNAPac, PA-100, 4.0×250mm, Part #063000

Column temperature = 60 °C

Signal monitored at 254 and 280 nm

Gradient used:

Time (min)	Flow (mL/min)	% A	% B	Curve
Initial		95	5	
2	1	95	5	1
3	1	75	25	6
10	1	35	65	6
10.1	1	95	5	6
12.5	1	95	5	1

[00508] General UPLC-LCMS method for the analysis of enzyme digestion products.

Buffer A: 15 mM TEA, 400 mM HFIP, Water

Buffer B: 50:50 Buffer A/Methanol

Column: UPLC@OST C_{18} 1.7 μm, 2.1×500mm

Column temperature = 60 °C

Gradient used:

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.2	70	30	
2	0.2	70	30	1
27	0.2	35	65	6
27.5	0.2	5	95	6
28.5	0.2	5	95	6
29	0.2	70	30	6
30	0.2	70	30	1

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[00509] In Vitro Metabolic Stabilities of Human Diastereomerically pure Oligonucleotides in Preincubated Rat Whole Liver Homogenates

[00510] The metabolic stability of chirally pure oligonucleotides in *in vitro* rat whole liver homogenates was measured. The protocol employed here has previously been reported and used to evaluate the stability of oligonucleotide drugs.

[00511] Protocol: The protocol reported by Geary *et al* was employed in the current study (*Oligonucleotides*, **2010**, *20*, 309) with some modifications.

[00512] Test system: Two male Sprague-Dawley rats (*Rattus norvegicus*) were supplied by Charles River Laboratories, Inc., (Hollister, CA).

[00513] Tissue Collection: Animals were acclimated to the study room for two days prior to tissue collection. At the time of tissue collection, animals were anesthetized with an intraperitoneal (IP) injection of sodium pentobarbital solution. Liver perfusion was performed using 500 mL of chilled saline/animal, administered *via* the hepatic portal vein. After perfusion, the livers were dissected and maintained on ice. Livers were minced into small pieces then weighed.

[00514] Liver Homogenate Preparation: The minced pieces of liver tissues were transferred to tared 50 mL centrifuge tubes and weighed. Chilled homogenization buffer (100 mM Tris pH 8.0, 1 mM magnesium acetate, with antibiotic-antimycotic agents) was added to each tube, such that the tube(s) contained 5 mL of buffer per gram of tissue. Using a QIAGEN TissueRuptor tissue homogenizer, the liver/buffer mixture was homogenized while maintaining the tube on ice. The protein concentration of the liver homogenate was determined using a Pierce BCA protein assay. Liver homogenates were divided into 1 mL aliquots, transferred to cryovials and stored at -60 °C.

[00515] Incubation Conditions: 1 mL aliquots of frozen liver homogenate (protein concentration = 31.9 mg/mL) were thawed and incubated at 37 °C for 24 h. Six eppendorf tubes (2 mL) were taken and 450 μL of homogenate was added in each tube. 50 μL of test oligonucleotide (200 μM) was added to each tube. Immediately after mixing, 125 μL of (5×) stop buffer (2.5% IGEPAL, 0.5 M NaCl, 5 mM EDTA, 50 mM Tris, pH = 8.0) and 12.5 μL of 20 mg/mL Proteinase K (Ambion, # AM2546) was added to one tube for a 0 hour time point. The mixture was then heated at 60 °C for one hour. The remaining reaction mixtures were incubated at 37 °C with shaking at 400 rpm on a VWR Incubating Microplate shaker. After

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incubation for a designated period (1, 2, 3, 4, and 5 days), each mixture was treated with 125 μL of (5×) stop buffer (2.5% IGEPAL, 0.5 M NaCl, 5 mM EDTA, 50 mM Tris, pH = 8.0) and 12.5 μL of 20 mg/mL Proteinase K (Ambion, # AM2546).

[00516] **Work up and Bioanalysis:** (5′-GCGTTTGCTCTTCTTCTTGCGTTTTTT-3′), a 27-mer oligonucleotide (underlined nucleotides are 2′-MOE modified) was used as the internal standard for quantitation of diastereomerically pure oligonucleotides. 50 μL of internal standard (200 μM) was added to each tube followed by addition of 250 μL of 30% ammonium hydroxide, 800 μL of Phenol: Chloroform: isoamyl alcohol (25:24:1). After mixing and centrifugation at 600 rpm, the aqueous layer was evaporated on speed vac to 100 μL and loaded on a Sep Pak column (C18, 1g, WAT 036905). All the aqueous washings of the Sep pak column were tested with quick IEX-HPLC method to ensure that no product was found there. The acetonitrile eluate was concentrated to dryness and dissolved in 100 μL water and analyzed using RP-HPLC.

Eluant A = 10mM Tributylammonium acetate, pH=7.0

Eluant B = ACN

Coulmn: XTerra MS C_{18}, 3.5 μm, 4.6×150 mm, Part number: 186000432

Column Temperature = 60 °C

HPLC Gradient:

	Time	Flow	%A	%B	Curve
1		1.0	65	35	
2	5.0	1.0	65	35	1
3	30.0	1.0	40	60	6
4	35.0	1.0	5	90	6
5	36.0	1.0	65	35	6
6	40.0	1.0	65	35	1

[00517] Figure 68 shows that different chirally pure oligonucleotides have different metabolic stability profiles in rat whole liver homogenate. The experiment also demonstrates that the diastereoisomeric mixture ONT-41 has a different metabolic stability profile compared with diastereomerically pure stereodefined oligonucleotides with the same sequence and chemical composition (ONT-75 and ONT-77 are used here as examples, but this observation, as appreciated by a person of ordinary skill in the art, may be extrapolated to other possible diastereomerically pure stereodefined phosphorothioate diastereoisomers of this molecule).

[00518] The oligonucleotide ONT-75, having a stereocontrolled phosphorothioate backbone with full *R*p absolute configuration at all phosphorus atoms was degraded completely in one day in preincubated rat whole liver homogenates at 37 °C, demonstrating the lowest metabolic stability of the three used in the study.

[00519] The other diastereomerically pure stereodefined phosphorothioate oligonucleotide ONT-77 (having absolute configuration: 5R-10S-4R) was more than two-fold more stable than the stereorandom ONT-41 (Mipomersen). After incubation for 5 days 40% of full length oligonucleotide remained for the diastereomerically pure ONT-77 *vs.* approximately 15% of full length oligonucleotide for the stereorandom ONT-41 (Mipomersen). A direct comparison between ONT-77 and ONT-41 clearly demonstrated a significantly higher metabolic stability for the stereocontrolled isomer 5R-10S-4R (ONT-77), throughout all of the analyzed time points. The stereodefined architecture of the diastereomerically pure isomer ONT-77 clearly affects the rate of degradation and the overall metabolic stability of this oligonucleotide. These observations lead to the conclusion, without the intention to be limited by theory, that the *S*p stereochemistry applied to the DNA core of the 5-10-5 gapmer oligonucleotide provides enhanced endonuclease resistance and hence enhanced metabolic stability compared with the stereorandom diastereomixture. Some other stereo architectures used for other diastereomerically pure stereocontrolled phosphorothioate isomers of this sequence would be expected to show even higher metabolic stability in this experiment.

[00520] While not wishing to be limited by theory, the results presented in this Example demonstrate, for instance, that *R*p stereochemistry at the phosphorothioate linkage (ONT-75) is less metabolically stable in rat liver homogenates than ONT-77, which contains the *S*p DNA core. The results presented in this Example also demonstrate, for instance, that chirally pure oligonucleotide compositions can have significantly different metabolic stability in rat liver homogenate as compared with an appropriate reference (e.g., a preparation of oligonucleotides of the same sequence but different chiral specificity, including particularly stereorandom preparations), and specifically as compared with a "parental" stereorandom preparation. In this Example, oligonucleotides having a sequence antisense to (and therefore targeting) human Apolipoprotein-B (ApoB) were used for proof-of-concept in metabolic stability studies. A provided chirally controlled oligonucleotides may have increased or decreased stability towards endogenous enzymes. By providing chirally controlled oligonucleotide and compositions and

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methods thereof, the present invention provided oligonucleotides and compositions that have enhanced pharmacological properties than known chirally uncontrolled oligonucleotides and their compositions.

[00521] In Vitro Metabolic Stabilities of Human PCSK9 siRNA Duplexes Having Stereocontrolled Phosphorothioate Diester Linkages in Human Serum.

[00522] A protocol similar to previously reported procedures (Oka *et al.*, *J. Am. Chem. Soc.* **2008**, *130*, 16031–16037) is used. siRNA duplexes (2.5 μmoles) in water (50 μL) are added to human serum (450 μL). The mixture is incubated at 37 °C with shaking at 400 rpm. 50 μL aliquots are taken out at each time point (0 h, 0.5 h, 1 h, 2 h, 4 h, 6 h, 8 h and 24 h) and are quenched with 25 μL of 150 mM EDTA, 2 μL of Proteinase K solution (20 mg/mL) and 30 μL of Lysis buffer (Qiagen, #129115) and the mixture is heated at 60 °C for 20 min. 5μL of Internal standard (5′-GCGTTTGCTCTTCTTCTTGCGTTTTTT-3′, a 27-mer oligonucleotide (underlined nucleotides are 2′-MOE modified) (200 μM) is added to the aliquot. Quantification analyses are performed by IEX-HPLC and metabolite identification is carried out by UPLC/MS.

[00523] In some embodiments, the siRNA duplexes having a 3′ terminal diastereomerically pure phosphorothioate with the *S*p configuration show higher metabolic stability in human serum compared to the siRNAs having the phosphorothioate with the *R*p configuration, or the stereorandom diastereomixture, at the same position. In other embodiments, the siRNA duplexes having a 5′ terminal diastereomerically pure phosphorothioate with the *S*p configuration show higher metabolic stability in human serum compared to the siRNAs having the phosphorothioate with the *R*p configuration, or the stereorandom diastereomixture, at the same position. In other embodiments, siRNA duplexes having diastereomerically pure phosphorothioates with the *S*p configuration at both the 3′ and the 5′ extremities show higher metabolic stability in human serum compared to the siRNAs having the phosphorothioate with the *R*p configuration, or the stereorandom diastereomixtures, at the same positions. In other embodiments, siRNA duplexes having multiple diastereomerically pure phosphorothioates with the *S*p configuration show higher metabolic stability in human serum compared to the siRNAs having multiple phosphorothioates with the *R*p configuration, or the stereorandom diastereomixtures. In other embodiments, siRNA duplexes having full backbone of diastereomerically pure phosphorothioates with the *S*p configuration show higher metabolic

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stability in human serum compared to the siRNAs having the full backbone of phosphorothioates with the *R*p configuration, or the stereorandom diastereomixtures.

[00524] Example 88. Chirally controlled oligonucleotide compositions show different potency in vitro as compared with chirally uncontrolled compositions having the same sequence

[00525] The present Example compares in vitro pharmacological activity of chirally pure oligonucleotides with that observed for the "parent" stereorandom mixture (i.e., for a composition containing oligonucleotides of the same sequence as the chirally pure oligonucleotides but not displaying chiral purity, for example as a result of having been prepared via a stereorandom process). Four chirally pure oligonucleotides, each of which had a sequence complementary to that of a particular target transcript or gene encoding a protein of interest were synthesized, formulated, and transfected into primary mouse hepatocytes. mRNA levels were quantified to assess level of suppression. In this Example, oligonucleotides having a sequence antisense to (and therefore targeting) human Apolipoprotein-B (ApoB) were used for proof-of-concept in transgenic mice expressing human ApoB.

Transfection of Mouse Primary Hepatocytes with Oligonucleotides

[00526] C57BL6 male mice, 7 weeks of age were used to extract and plate mouse primary hepatocytes in 96-well plates (without overlay) (Celsis/Bioreclamation IVT). Transfection of primary hepatocytes was carried out with lipofectin (Life Technologies, cat. No. 18292-037) using the manufacturer's protocol, using 0.5ul of Lipofectin per 96-plate well. Twelve, 1:3 siRNA duplex dilutions were created starting at 6uM. 10ul of 10x oligo was then lipoplexed with a prepared mixture of 9.5ul of In vitro Gro HI Medium (Celsis Cat. Z99009) serum-free medium and 0.5ul of Lipofectin per well. After a 10-15 minute incubation, 20 ul of lipoplexed oligo was added to primary hepatocytes in 80ul of In vitro Gro HI Medium to bring the final volume to 100ul per well. 24 hours after transfection, cells were lysed.

Apolipoprotein B mRNA Assay

[00527] Total mRNA was purified from cell lysates using MagMAX™-96 Total RNA Isolation Kit (Life Technologies, AM1830); 15ul of cDNA was synthesized with High Capacity

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cDNA Reverse Transcription Kit with RNase Inhibitor (Life Technologies, 4374967). Gene expression was evaluated by Real-Time PCR on a Lightcycler 480(Roche) using a Probes Master Mix (Roche, 04 707 494 001) according to manufacturer's protocol using the following primers:

Mouse apolipoprotein B primers:

[Template: (GenBank accession number M35186)]

- forward primer: CGTGGGCTCCAGCATTCTA
- reverse primer: AGTCATTTCTGCCTTTGCGTC
- PCR probe: FAM-CCAATGGTCGGGCACTGCTCAA-TAMRA

Mouse GAPDH primers:

- forward primer: GGCAAATTCAACGGCACAGT
- reverse primer: GGGTCTCGCTCCTGGAAGAT
- PCR probe: 5′ JOE-AAGGCCGAGAATGGGAAGCTTGTCATC-TAMRA 3′

Data Analysis

[00528] The delta delta Ct method was used to calculate values. For each sample, the mean ApoB signal was normalized to the mean GAPDH signal, and then normalized to the mean corresponding ratio for mock transfected and untreated samples, to obtain the relative level of ApoB protein expression. The "parent" stereorandom mixture was used for reference. A four-parameter linear regression curve was fitted to the data, and the bottom and top were constrained to a 0% and 100% constants respectively in order to calculate a relative IC50 using Graphpad Prism.

[00529] The in vitro dose-responses (Figures 71 and 72) show that the chirally pure oligonucleotides have significantly different potencies, with ONT-83 being the most potent, and ONT-84 and ONT-85 being the least potent, with a 8.6-fold difference in IC50s (Table E-39).

Table E-39. IC50 values of Stereoisomers (ONT-83, -84, -85 or -86) for Suppression of Mouse Apolipoprotein B/GAPDH mRNA Levels in Primary Mouse Hepatocytes

				Hill Slope	IC50

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	Bottom	Top	LogIC50		
ONT-83	0	100	1.6	-0.4	35.8
ONT-82	0	100	1.8	-0.3	64.4
ONT-85	0	100	2.5	-0.3	308.0
ONT-84	0	100	2.5	-0.8	307.8
ONT-86	0	100	1.7	-0.6	51.2

[00530] The results presented in this Example demonstrate, for instance, that chirally pure oligonucleotide compositions can have significantly different pharmacological activity in vitro as compared with an appropriate reference (e.g., a preparation of oligonucleotides of the same sequence but different chiral specificity, including particularly stereorandom preparations), and specifically as compared with a "parental" stereorandom preparation. Those skilled in the art, in light of this demonstration, will appreciate that chirally controlled oligonucleotide compositions provided by the present disclosure have unexpected activities and characteristics.

[00531]

Equivalents

[00532] Having described some illustrative embodiments of the invention, it should be apparent to those skilled in the art that the foregoing is merely illustrative and not limiting, having been presented by way of example only. Numerous modifications and other illustrative embodiments are within the scope of one of ordinary skill in the art and are contemplated as falling within the scope of the invention. In particular, although many of the examples presented herein involve specific combinations of method acts or system elements, it should be understood that those acts and those elements may be combined in other ways to accomplish the same objectives. Acts, elements, and features discussed only in connection with one embodiment are not intended to be excluded from a similar role in other embodiments. Further, for the one or more means-plus-function limitations recited in the following claims, the means are not intended to be limited to the means disclosed herein for performing the recited function, but are intended to cover in scope any means, known now or later developed, for performing the recited function.

[00533] Use of ordinal terms such as "first", "second", "third", *etc.*, in the claims to modify a claim element does not by itself connote any priority, precedence, or order of one claim element over another or the temporal order in which acts of a method are performed, but are used merely as labels to distinguish one claim element having a certain name from another element

having a same name (but for use of the ordinal term) to distinguish the claim elements. Similarly, use of a), b), *etc.*, or i), ii), *etc.* does not by itself connote any priority, precedence, or order of steps in the claims. Similarly, the use of these terms in the specification does not by itself connote any required priority, precedence, or order.

[00534] The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by examples provided, since the examples are intended as a single illustration of one aspect of the invention and other functionally equivalent embodiments are within the scope of the invention. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims. The advantages and objects of the invention are not necessarily encompassed by each embodiment of the invention.

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APPENDIX (A)

Accession #	Unigene #	Gene Symbol	Name	Sequence
NM_003467	Hs.421986	CXCR4	Chemokine (C-X-C motif) receptor 4	5'-UAAAAUCUUCCUGCCCACCdTdT-3'
NM_003467	Hs.421986	CXCR4	Chemokine (C-X-C motif) receptor 4	5'-GGAAGCUGUUGGCUGAAAAdTdT-3'
NM_006799.2	Hs.72026	PRSS21	Protease, serine, 21 (testisin)	5'-CACAUCCAGCCCAUCUGUC(dTdT)-3'
NM_000117.1	Hs.522823	EMD	Emerin	5'-CCGUGCUCCUGGGGCUGGG(dTdT)-3'
NM_001350.3	Hs.336916	DAXX	Death-associated protein 6	5'-GGAGUUGGAUCUCUCAGAA(dTdT)-3'
NM_003014.2	Hs.105700	SFRP4	Secreted frizzled-related protein 4	5'-AAGTCCCGCTCATTACAAATT-3'
NM_015062.3	Hs.533551	PPRC1	Peroxisome proliferative activated receptor, gamma, coactivator-related 1	5'-AAGACCAGCCUCUUUGCCCAG-3'
NM_001005360.1	Hs.211463	DNM2	Dynamin 2	5'-GGACCAGGCAGAAAACGAG-3
NM_001904.2	Hs.476018	CTNNB1	Catenin (cadherin-associated protein), beta 1, 88kDa	5'-CUAUCAGGAUGACGCGG-3'
NM_153831.2	Hs.395482	PTK2	PTK2 protein tyrosine kinase 2	5'-AACCACCUGGGCCAGUAUUAU-dTT-3'
NM_001429.2	Hs.517517	EP300	E1A binding protein p300	5'-UGACACAGGCAGGCUUGACUU-3'
NM_005904.2	Hs.465087	SMAD7	SMAD, mothers against DPP homolog 7 (Drosophila)	5' AA GCU CAA UUC GGA CAA CAA G 3'
NM_001904.2	Hs.476018	CTNNB1	Catenin (cadherin-associated protein), beta 1, 88kDa	5' AAG UCC UGU AUG AGU GGG AAC 3'
NM_175847.1	Hs.172550	PTBP1	Polypyrimidine tract binding protein 1	5'-TCG ACG AAC ATC TAC AAC GCC TGC TTC AAG AGA GCA GGC GTT GTA GAT GTT CTT TTT TT-3'
NM_175847.1	Hs.172550	PTBP1	Polypyrimidine tract binding protein 1	5'-TCG ACC AAT GAC AAG AGC CGT GAC TTC AAG AGA GTC ACG GCT CTT GTC ATT GTT TTT TT-3'

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NM_002659.2	Hs.466871	PLAUR	Plasminogen activator, urokinase receptor	5'-GGTGAAGAAGGGCGTCCAA-3'
NM_033360.2	Hs.505033	KRAS2	V-Ki-ras2 Kirsten rat sarcoma 2 viral oncogene homolog	5'-GATCCGTTGGAGCTGTTGGCGTAG TTCAAGAGACTACGCCA ACAGCTCCAACTTTTTGGAAA-3'
NM_002959.4	Hs.485195	SORT1	Sortilin 1	5'-AGGTGGTGTTAACAGCAGAG-3'
NM_002959.4	Hs.485195	SORT1	Sortilin 1	5'-AATGTTCCAATGCCCCACTC-3'
NM_000743.2	Hs.89605	CHRNA3	Cholinergic receptor, nicotinic, alpha polypeptide 3	5'-AACUGCCAGUGGCCAGGGCCU-3'
NM_004859.2	Hs.491351	CLTC	Clathrin, heavy polypeptide (Hc)	5'-AACCUGCGGUCUGGAGUCAAC-3'
NM_004859.2	Hs.491351	CLTC	Clathrin, heavy polypeptide (Hc)	5'-UAAUCCAAUUCGAAGACCAAU-3'
NM_000038.3	Hs.158932	APC	Adenomatosis polyposis coli	5'-AGGGGCAGCAACTGATGAAAA-3'
NM_004850.3	Hs.58617	ROCK2	Rho-associated, coiled-coil containing protein kinase 2	5'-AAGGCATCGCAGAAGGTTTAT-3'
NM_001274.2	Hs.24529	CHEK1	CHK1 checkpoint homolog (S. pombe)	5'-UCGAAGUACUCAGCGUAAG-3'
NM_007194.3	Hs.291363	CHEK2	CHK2 checkpoint homolog (S. pombe)	5'-GAACCUGAGGACCAAGAAC-3'
NM_001901.1	Hs.410037	CTGF	Connective tissue growth factor	5'-AATGTTCTCTTCCAGGTCAGCCCTGTCTC-3'
NM_001619.2	Hs.83636	ADRBK1	Adrenergic, beta, receptor kinase 1	5'-AAGAAGUACGAGAAGCUGGAG-3'
NM_005160.2	Hs.517493	ADRBK2	Adrenergic, beta, receptor kinase 2	5'-AAGCAAGCUGUAGAACACGUA-3'
NM_005308.2	Hs.524625	GRK5	G protein-coupled receptor kinase 5	5'-AAGCCGUGCAAAGAACUCUUU-3'
NM_001004106.1	Hs.235116	GRK6	G protein-coupled receptor kinase 6	5-AACAGUAGGUUUGUAGUGAGC-3'
NM_017556.1	Hs.530101	FBLP-1	Filamin-binding LIM protein-1	5'-AAAGGGGCAUCCACAGACAUC-3'

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NM_005857.2	Hs.132642	ZMPSTE24	Zinc metallopeptidase (STE24 homolog, yeast)	5'- TTAT TCTTCTCTTT GGAGGA -3'
NM_005572	Hs.491359	LMNA	Lamin A/C	5'- ACTGGACTTC CAGAAGAAC -3'
NM_015878.3	Hs.459106	OAZIN	Ornithine decarboxylase antizyme inhibitor	5'-AATTGCACGTAATCACCCAAA-3'
NM_015878.3	Hs.459106	OAZIN	Ornithine decarboxylase antizyme inhibitor	5'-AAGAAATACAAGGAAGATGAG-3'
NM_001664.2	Hs.247077	RHOA	Ras homolog gene family, member A	5'-GACAUGCUUGCUCAUAGUCTT-3'
NM_175744.3	Hs.502659	RHOC	Ras homolog gene family, member C	5'-GACCUGCCUCCUCAUCGUCTT-3'
NM_000041.2	Hs.515465	APOE	Apolipoprotein E	5'- AAGGTGGAGCAAGCGGTGGAG -3'
NM_000041.2	Hs.515465	APOE	Apolipoprotein E	5'-AAGGAGTTGAAGGCCTACAAA-3'
AF520590.1	Hs.536600	BAK1	BCL2-antagonist/killer 1	5'-UGCCUACGAACUCUUCACCdTdT-3'
NM_138761.2	Hs.159428	BAX	BCL2-associated X protein	5'-UAUGGAGCUGCAGAGGAUGdTdT-3'
NM_005733.1	Hs.73625	KIF20A	Kinesin family member 20A	5'-TTGGCCAAGCCACACACAG-3'
NM_005733.1	Hs.73625	KIF20A	Kinesin family member 20A	5'-GTTCTCAGCCATTGCTAGC-3'
NM_005733.1	Hs.73625	KIF20A	Kinesin family member 20A	5'-GGCAGCATGTATTGCTGAG-3'
NM_014034.1	Hs.292316	ASF1A	ASF1 anti-silencing function 1 homolog A (S. cerevisiae)	5'-AAUC CAGGACUCAUUCCAGAU-3'
NM_014034.1	Hs.292316	ASF1A	ASF1 anti-silencing function 1 homolog A (S. cerevisiae)	5'-AAGUGAAGAAUACGAUCAAGU-3'
NM_018154.1	Hs.26516	ASF1B	ASF1 anti-silencing function 1 homolog B (S. cerevisiae)	5'-AACAACGAGUACCUCAACCCU-3'
NM_022110.3	Hs.520042	WISp39	FK506 binding protein like	5' AACGCUUGAGCUGGAAGUAAG 3'
NM_022110.3	Hs.520042	WISp39	FK506 binding protein like	5'-CCUUCAAGCUUCUGAUCUC-3'
NM_000389.2	Hs.370771	CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	5'-AACUUCGACUUUGUCACCGAG-3'
NM_004064.2	Hs.238990	CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	5'-AAGCACUGCAGAGACAUGGAAG-3'

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NM_033084.2	Hs.208388	FANCD2	Fanconi anemia, complementation group D2	5'- AACAGCCATGGATACACTTGA -3'
NM_001641.2	Hs.73722	APEX1	APEX nuclease (multifunctional DNA repair enzyme) 1	5'-AATGACAAAGAGGCAGCAGG-3-
NM_001641.2	Hs.73722	APEX1	APEX nuclease (multifunctional DNA repair enzyme) 1	5'-AACCTGCCACACTCAAGATC-3'
NM_001641.2	Hs.73722	APEX1	APEX nuclease (multifunctional DNA repair enzyme) 1	5'-AGCTGAACTTCAGGAGCTGCC-3'
NM_001641.2	Hs.73722	APEX1	APEX nuclease (multifunctional DNA repair enzyme) 1	5'-AAGCCTTTCGCAAGTTCCTGA-3'
NM_001641.2	Hs.73722	APEX1	APEX nuclease (multifunctional DNA repair enzyme) 1	5'-ACGGCATAGGCGATGAGGAG-3'
NM_001641.2	Hs.73722	APEX1	APEX nuclease (multifunctional DNA repair enzyme) 1	5'-AGGAAGGCCGGGTGATTGTG-3'
NM_001641.2	Hs.73722	APEX1	APEX nuclease (multifunctional DNA repair enzyme) 1	5'-GTCTGGTACGACTGGAGTA-3'
NM_001641.2	Hs.73722	APEX1	APEX nuclease (multifunctional DNA repair enzyme) 1	5'-GACAGCTTTAGGCACCTCTA-3'
NM_015641.2	Hs.533391	TES	Testis derived transcript (3 LIM domains)	5'-GGAUUCGAACUGCACUUCU-3'
NM_015641.2	Hs.533391	TES	Testis derived transcript (3 LIM domains)	5'-ACUGUGGCACCCAGCUUGU-3'
NM_003461.3	Hs.490415	ZYX	Zyxin	5'-GCCCAAAGUGAAUCCCUUC-3'

NM_002880.2	Hs.159130	RAF1	V-raf-1 murine leukemia viral oncogene homolog 1	5'-TTTGAATATCTGTGCTGAGAACACA GTTCTCAGCACAGATATTCTTTTT-3'
NM_002880.2	Hs.159130	RAF1	V-raf-1 murine leukemia viral oncogene homolog 1	5'-TTTGTCAATTAGCTGGAACATCACAG ATGTTCCAGCTAATTGACTTTTT-3'
NM_004506.2	Hs.158195	HSF2	Heat shock transcription factor 2	5'-AATGAGAAAAGCAAAAGGTGCCCTGTCTC-3'
NM_005356.2	Hs.470627	LCK	Lymphocyte-specific protein tyrosine kinase	5'-CAUCGAUGUGUGUGAGAACUGC-3'
NM_005546.3	Hs.483938	ITK	IL2-inducible T-cell kinase	5'-CUGUUCUCAGCUGGAGAAGCUU-3'
NM_005546.3	Hs.483938	ITK	IL2-inducible T-cell kinase	5'-GGAGCCUUCAUGGUAAGGGAUU-3'
NM_002133.1	Hs.517581	HMOX1	Heme oxygenase (decycling) 1	5'-GGCACCATGAAGGCG-3'
NM_000639.1	Hs.2007	FASLG	Tumor necrosis factor (ligand) superfamily, member 6	5'-CUGGGCU GUACU UUGUA UATT-3'
NM_018417.2	Hs.320892	SAC	Testicular soluble adenylyl cyclase	5'-AUGUAGCCUGGAGAUCCAUUU-3'
NM_003743.3	Hs.412293	NCOA1	Nuclear receptor coactivator 1	5'-CCUCAGGGCAGAGAACCAUCUdTdT-3'
NM_005572.2	Hs.491359	LMNA	Lamin A/C	5'-CUGGACUUCCAGAAGAACAUCdTdT-3'
NM_176871.2	Hs.521444	PDLIM2	PDZ and LIM domain 2 (mystique)	5'-AAGAUCCGCCAGAGCCCCUCG-3'
NM_014188.2	Hs.30026	HSPC182	HSPC182 protein	5'-AACAGGGACTCACGTGAAGCT-3'
NM_014188.2	Hs.30026	HSPC182	HSPC182 protein	5'-AAGACCTGTTTGATCTGATCC-3'
AF263744.1	Hs.519346	ERBB2IP	Erbb2 interacting protein	5'-UAGACUGACCCAGCUGGAAdTdT-3'
NM_002583.2	Hs.406074	PAWR	PRKC, apoptosis, WT1, regulator	5'-GAUGCAAUUACACAACAGAdTdT-3'
NM_003766.2	Hs.12272	BECN1	Beclin 1 (coiled-coil, myosin-like BCL2 interacting protein)	5'-CUCAGGAGAGGAGCCAUUU-3'
NM_003766.2	Hs.12272	BECN1	Beclin 1 (coiled-coil, myosin-like BCL2 interacting protein)	5'-GAUUGAAGACACAGGAGGC-3'
NM_004849.1	Hs.486063	APG5L	APG5 autophagy 5-like (S.	5'-GCAACUCUGGAUGGGAUUG-3'

			cerevisiae)	
NM_031482.3	Hs.527193	APG10L	APG10 autophagy 10-like (S. cerevisiae)	5'-GGAGUUCAUGAGUGCUAUA-3'
NM_004707.2	Hs.264482	APG12L	APG12 autophagy 12-like (S. cerevisiae)	5'-CAGAGGAACCUGCUGGCGA-3'
NM_002613.2	Hs.459691	PDPK1	3-phosphoinositide dependent protein kinase-1	5'-AACTGGCAACCTCCAGAGAAT-3'
NM_002613.2	Hs.459691	PDPK1	3-phosphoinositide dependent protein kinase-1	5'-AAGAGACCTCGTGGAGAAACT-3'
NM_000314.2	Hs.500466	PTEN	Phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	5'-AACAGTAGAGGAGCCGTCAAA-3'
NM_006092.1	Hs.405153	CARD4	Caspase recruitment domain family, member 4	5'-GGGUGAGACCAUCUUCAUCTT-3'
NM_006092.1	Hs.405153	CARD4	Caspase recruitment domain family, member 4	5'-GGCCAAAGUCUAUGAAGAUTT-3'
NM_000598.3	Hs.450230	IGFBP3	Insulin-like growth factor binding protein 3	5'-AAUCAUCAUCAAGAAAGGGCA-3'
NM_006839.1	Hs.148559	IMMT	Inner membrane protein, mitochondrial (mitofilin)	5'-AAUUGCUGGAGCUGGCCUUTT-3'
NM_016485.3	Hs.431367	C6ORF55	Chromosome 6 open reading frame 55	5'-GAATGAAGATCGATAGTAA-3'
NM_016485.3	Hs.431367	C6ORF55	Chromosome 6 open reading frame 55	5'-GCACAGGTGTAGCAAGTAA-3'
NM_016485.3	Hs.431367	C6ORF55	Chromosome 6 open reading frame 55	5'-GGAGAATTATGCTTTGAAA-3'
NM_016485.3	Hs.431367	C6ORF55	Chromosome 6 open reading frame 55	5'-GCAGTGCTTTGCAGTATGA-3'
NM_016410.2	Hs.415534	SNF7DC2	SNF7 domain containing 2	5'-CAGAAAGCCTTGCGAGTTT-3'
NM_016410.2	Hs.415534	SNF7DC2	SNF7 domain containing 2	5'-GAATTTGGATTGCCACAGA-3'
NM_016410.2	Hs.415534	SNF7DC2	SNF7 domain containing 2	5'-GAAGGTGTTCCCACTGATA-3'

NM_016410.2	Hs.415534	SNF7DC2	SNF7 domain containing 2	5'-GAGAGGGTCCTGCAAAGAA-3'
NM_199185.1	Hs.519452	NPM1	Nucleophosmin (nucleolar phosphoprotein B23, numatrin)	5'-UGAUGAAAAUGAGCACCAG-3'
NM_003118.2	Hs.111779	SPARC	Secreted protein, acidic, cysteine-rich	5-AAAATCCCTGCCAGAACCACC-3'
NM_003118.2	Hs.111779	SPARC	Secreted protein, acidic, cysteine-rich	5-AACAAGACCTTCGACTCTTCC-3'
NM_003183.3	Hs.404914	ADAM17	A disintegrin and metalloproteinase domain 17 (tumor necrosis factor, alpha, converting enzyme)	5'-AAACGAAAGCGAGTACACT-3'
NM_012164.2	Hs.494985	FBXW2	F-box and WD-40 domain protein 2	5'-AGATGGACTTCTCTGTACAGG-3'
NM_012164.2	Hs.494985	FBXW2	F-box and WD-40 domain protein 2	5'-GACATTGTCTGTCTCTGAGGA-3'
NM_175940.1	Hs.272813	DUOX1	Dual oxidase 1	5'-GGACUUAUCCUGGCUAGAGtt-3'
NM_004503.2	Hs.820	HOXC6	Homeo box C6	5' CCGGAUCUACUCGACUCCCTT 3'
NM_004503.2	Hs.820	HOXC6	Homeo box C6	5' CCUAAUCACACACUCUGUATT 3'
NM_004503.2	Hs.820	HOXC6	Homeo box C6	5' ACUGCAGACAAAACACCUUTT 3'
NM_004503.2	Hs.820	HOXC6	Homeo box C6	5' UCCAACCUCUGGGUCCGUUTT 3'
NM_004503.2	Hs.820	HOXC6	Homeo box C6	5' ACUGUGACCGUUUCUGUGUTT 3'
NM_004503.2	Hs.820	HOXC6	Homeo box C6	5' CUCAGACUCUACAGAUUGCTT 3'
NM_182965.1	Hs.68061	SPHK1	Sphingosine kinase 1	5'-GGG CAA GGC CUU GCA GCU C-3'
NM_003329.2	Hs.435136	TXN	Thioredoxin	5'-AUGACUGUCAGGAUGUUGCdTT-3'
NM_003329.2	Hs.435136	TXN	Thioredoxin	5'-GCAACAUCCUGACAGUCAUdCC-3'
NM_203500.1	Hs.465870	KEAP1	Kelch-like ECH-associated protein 1	5'-UGAACGGUGCUGUCAUGUAdTdT-3'
NM_005239.4	Hs.517296	ETS2	V-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	5'-GCAGAGGUUCGGCAUGAAUdTdT-3'

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NM_002067.1	Hs.515056	GNA11	Guanine nucleotide binding protein (G protein), alpha 11 (Gq class)	5'-AAGATGTTCGTGGACCTGAAC-3'
NM_004827.1	Hs.480218	ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2	5'-AAGATGATTGTTCGTCCCTGCTAT AGTGAGTCGTATTA-3'
NM_000610.3	Hs.502328	CD44	CD44 antigen (homing function and Indian blood group system)	5'-GAACGAAUCCUGAAGACAUCU-3'
NM_003489.1	Hs.155017	NRIP1	Nuclear receptor interacting protein 1	5'-GAAGGAAGCUUUGCUAGCU-3'
NM_004995.2	Hs.2399	MMP14	Matrix metalloproteinase 14	5'-AAGCCTGGCTACAGCAATATGCCTGTCTC-3'
NM_022045.2	Hs.546363	MTBP	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse) binding protein, 104kDa	5' GGCUCAUUUGCACUCAAUU 3'
NM_002392.2	Hs.369849	MDM2	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse)	5' GCCACAAAUCUGAUAGUAU 3'
NM_170707.1	Hs.491359	LMNA	Lamin A/C	5' CUGGACUUCCAGAAGAACA 3'
NM_004759.3	Hs.519276	MAPKAPK2	Mitogen-activated protein kinase-activated protein kinase 2	5'-UGACCAUCACCGAGUUUAUdTdT-3'
NM_001948.2	Hs.527980	DUT	DUTP pyrophosphatase	5'-GATTATAGGAAATGTTG-3'
NM_016022.1	Hs.108408	APH-1A	Likely ortholog of C. elegans anterior pharynx defective 1A	5'-AAGAAGGCAGATGAGGGGTTA-3'
NM_031301.2	Hs.511703	PSFL	Anterior pharynx defective 1B-like	5'-AACAAAGATGGACCAACACAG-3'
BC007496.1	Hs.36915	SMAD3	SMAD, mothers against DPP homolog 3 (Drosophila)	5'-GGACGAGGUCUGCGUGAAUdTdT-3'
NM_182763.1	Hs.532826	MCL1	Myeloid cell leukemia sequence 1 (BCL2-related)	5'-AAGAAACGCGGUAAUCGGACU-3'

NM_001022.3	Hs.438429	RPS19	Ribosomal protein S19	5'-GCACAAAGAGCTTGCTCCCttcaagaga GGGAGCAAGCTCTTTGTGC-3'
NM_001022.3	Hs.438429	RPS19	Ribosomal protein S19	5'-GTCCGGGAAGCTGAAAGTCttcaagaga GACTTTCAGCTTCCCGGAC-3'
NM_001022.3	Hs.438429	RPS19	Ribosomal protein S19	5'-GAGATCTGGACAGAATCGCttcaagaga GCGATTCTGTCCAGATCTC-3'
NM_001400.2	Hs.154210	EDG1	Endothelial differentiation, sphingolipid G-protein-coupled receptor, 1	5'-GGAGAACAGCATTAAACTG-3'
NM_001001938.1	Hs.546252	C9orf47	Chromosome 9 open reading frame 47	5'-GGTCAACATTCTGATGTCT-3'
NM_021972.2	Hs.68061	SPHK1	Sphingosine kinase 1	5'-GGGCAAGGCCTTGCAGCTC-3'
NM_016068.1	Hs.423968	TTC11	Tetratricopeptide repeat domain 11	5'-GTACAATGATGACATCCGTAA-3'
NM_016068.1	Hs.423968	TTC11	Tetratricopeptide repeat domain 11	5'-GTACGTCCGCGGGTTGCTGCA-3'
NM_153831.2	Hs.395482	PTK2	PTK2 protein tyrosine kinase 2	5'-AAGCAUGUGGCCUGCUAUGGA-3'
NM_003749.2	Hs.442344	IRS2	Insulin receptor substrate 2	5'-GATCCCGCCTCAACAACAACAACAACTTCAAG AGAGTTGTTGTTGTTGAGGTTTTTTGGAAA -3'
NM_000691.3	Hs.531682	ALDH3A1	Aldehyde dehydrogenase 3 family, memberA1	5'-AAG AAG AGC UUC GAG ACU UUC-3'
NM_000689.3	Hs.76392	ALDH1A1	Aldehyde dehydrogenase 1 family, member A1	5'-AAC TGG GAG AGT ACG GTT TCC-3'
NM_000604.2	Hs.549034	FGFR1	Fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)	5'-AAGTCGGACGCAACAGAGAAA-3'

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NM_006006.3	Hs.171299	ZBTB16	Zinc finger and BTB domain containing 16	5'-GGCCAACCAGAUGCGGCUGUU-3'
NM_006006.3	Hs.171299	ZBTB16	Zinc finger and BTB domain containing 16	5'-GAUGUUUGACAUCCUCUUCUU-3'
NM_004348.1	Hs.122116	RUNX2	Runt-related transcription factor 2	5'-GGCUGCAAGCAGUAUUUACUU-3'
NM_004348.1	Hs.122116	RUNX2	Runt-related transcription factor 2	5'-GGACAGAGUCAGAUUACAGUU-3'
NM_014382.2	Hs.546361	ATP2C1	ATPase, Ca++ transporting, type 2C, member 1	5'- AGCCACTGTGGAAGAAGTATATT-3'
NM_002083.2	Hs.2704	GPX2	Glutathione peroxidase 2 (gastrointestinal)	5'- CCCUCUGGUUGGUGAUUCAdTdT-3'
NM_002083.2	Hs.2704	GPX2	Glutathione peroxidase 2 (gastrointestinal)	5'- GGAUGAUGGCACCUUCCUAdTdT-3'
NM_000942.4	Hs.434937	PPIB	Peptidylprolyl isomerase B (cyclophilin B)	5'-AATTGGAGATGAAGATGTAGG-3'
NM_003153.3	Hs.524518	STAT6	Signal transducer and activator of transcription 6, interleukin-4 induced	5'-CAGUUCCGCCACUUGCCAAdTdT-3'
NM_003153.3	Hs.524518	STAT6	Signal transducer and activator of transcription 6, interleukin-4 induced	5'-AGCCUGGUGGACAUUUAUUdTdT-3'
NM_003153.3	Hs.524518	STAT6	Signal transducer and activator of transcription 6, interleukin-4 induced	5'-GAUGUGUGAAACUCUGAACdTdT-3'
NM_003153.3	Hs.524518	STAT6	Signal transducer and activator of transcription 6, interleukin-4 induced	5'-CAGAUGGGUAAGGAUGGCAdTdT-3'
NM_002945.2	Hs.461925	RPA1	Replication protein A1, 70kDa	5'-AAGCACUAUCAUUGCGAAUCC-3'
NM_003169.2	Hs.437056	SUPT5H	Suppressor of Ty 5 homolog	5'-AACTGGGCGAGTATTACATGA-3'
NM_003318.3	Hs.169840	TTK	TTK protein kinase	5'- TGAACAAAGTGAGAGACAT -3'
NM_007194.3	Hs.291363	CHEK2	CHK2 checkpoint homolog (S.	5'- AATGTGTGAATGACAACTACT -3'

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			pombe)	
NM_002358.2	Hs.533185	MAD2L1	MAD2 mitotic arrest deficient-like 1 (yeast)	5'- AATACGGACTCACCTTGCTTG -3'
NM_001401.3	Hs.126667	EDG2	Endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2	5'-r(CCGCCGCUUCCAUUUUUCCU)d(TT)-3'
NM_001401.3	Hs.126667	EDG2	Endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2	5'-r(AGGAAAAAUGGAAGCGGCGGG)d(TT)-3')
NM_004448.2	Hs.446352	ERBB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2	5'-CCUGGAACUCACCUACCUGdTdT-3'
NM_004448.2	Hs.446352	ERBB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2	5'-CUACCUUUCUACGGACGUGdTdT-3'
NM_004448.2	Hs.446352	ERBB2	V-crb-b2 crythroblastic leukemia viral oncogene homolog 2	5'-GAUCCGGAAGUACACGAUGdTdT-3'
NM_014812.1	Hs.533635	KAB	KARP-1-binding protein	5'-GAAGGAAUCCUCCAAGUCA-3'
NM_002737.2	Hs.531704	PRKCA	Protein kinase C, alpha	5'-AAGCTCCATGTCACAGTACGA-3'
NM_212535.1	Hs.460355	PRKCB1	Protein kinase C, beta 1	5'-AAGCGCTGCGTCATGAATGTT-3'
NM_138578.1	Hs.516966	BCL2L1	BCL2-like 1	5'-CTG CCT AAG GCG GAT TTG AAT-3'
NM_138578.1	Hs.516966	BCL2L1	BCL2-like 1	5'-GGC AGG CGA CGA GTT TGA ACT-3'
NM_138578.1	Hs.516966	BCL2L1	BCL2-like 1	5'-GTG CGT GGA AAG CGT AGA CAA-3'
NM_004050.2	Hs.410026	BCL2L2	BCL2-like 2	5'-GGC GGA GTT CAC AGC TCT ATA-3'
NM_004050.2	Hs.410026	BCL2L2	BCL2-like 2	5'-GTG GGC ATA AGT GCT GAT CTA-3'
NM_004050.2	Hs.410026	BCL2L2	BCL2-like 2	5'-CTC GGT CCT GCG ATT ATT AAT-3'
NM_003443.1	Hs.433764	ZBTB17	Zinc finger and BTB domain containing 17	5'- AAGGCCGAGATCAGCAAAGTTCAAGAGACT TTGCTGATCTCGGCCTTTTTTTT -3'
NM_003345.3	Hs.302903	UBE2I	Ubiquitin-conjugating cnzymc	5'-GGC CAG CCA UCA CAA UCA ATT-3'

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			E2I (UBC9 homolog, yeast)	
NM_003345.3	Hs.302903	UBE2I	Ubiquitin-conjugating enzyme E2I (UBC9 homolog, yeast)	5'-GGA ACU UCU AAA UGA ACC ATT-3'
NM_016166.1	Hs.162458	PIAS1	Protein inhibitor of activated STAT, 1	5'-GGU CCA GUU AAG GUU UUG UTT-3'
NM_016166.1	Hs.162458	PIAS1	Protein inhibitor of activated STAT, 1	5'-GGU UAC CUU CCA CCU ACA ATT-3'
NM_004068.2	Hs.518460	AP2M1	Adaptor-related protein complex 2, mu 1 subunit	5'-AAGUGGAUGCCUUUCGGGUCA-3'
NM_004068.2	Hs.518460	AP2M1	Adaptor-related protein complex 2, mu 1 subunit	5'-AAGGAGAACAGUUCUUGCGGC-3'
NM_004068.2	Hs.518460	AP2M1	Adaptor-related protein complex 2, mu 1 subunit	5'-AAGGUCCAGU-CAUUCCAAAUG-3'
NM_001278.2	Hs.198998	CHUK	Conserved helix-loop-helix ubiquitous kinase	5'-AGGAAGGACCUGUUGACCUUTT-3'
NM_001556.1	Hs.413513	IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	5'-UGGUGAGCUUAAUGAAUGATT-3'
NM_021975.2	Hs.502875	RELA	V-rel reticuloendotheliosis viral oncogene homolog A	5'-AGAGGACAUUGAGGUGUAUTT-3'
NM_000963.1	Hs.196384	PTGS2	Prostaglandin-endoperoxide synthase 2	5'-AACTGCTCAACACCGGAATTTTT-3'
NM_005427.1	Hs.192132	TP73	Tumor protein p73	5'-CCAUCCUGUACAACUUCAUGU G-3'
NM_005157.2	Hs.431048	ABL1	V-abl Abelson murine leukemia viral oncogene homolog 1	5'-CAAUAAGGAAGAAGCCCUUTT-3'
NM_005157.2	Hs.431048	ABL1	V-abl Abelson murine leukemia viral oncogene homolog 1	5'-TTAUUCCUUCUUCGGGAAGUC-3'
NM_001168.1	Hs.514527	BIRC5	Baculoviral IAP repeat-containing 5 (survivin)	5'-GGCUGGCUUCAUCCACUGCTT-3'

NM_002940.1	Hs.12013	ABCE1	ATP-binding cassette, sub-family E (OABP), member 1	5'-CGAAGATGTTGACCTGGTC-3'
NM_002940.1	Hs.12013	ABCE1	ATP-binding cassette, sub-family E (OABP), member 1	5'-AGAGTTGTCCTGTAGTTCG-3'
NM_004208.2	Hs.424932	PDCD8	Programmed cell death 8 (apoptosis-inducing factor)	5'-GGAAAUAUGGGAAAGAUCCdTdT
NM_000115.1	Hs.82002	EDNRB	Endothelin receptor type B	5'-GGAGACUUUCAAAUACAUCTTtt-3'
NM_001712.2	Hs.512682	CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1	5'-AACCTTCTGGAACCCGCCCAC-3'
NM_001712.2	Hs.512682	CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1	5'-AATGTTGCAGAGGGGAAGGAG-3'
NM_033284.1	Hs.436900	TBL1Y	Transducin (beta)-like 1Y-linked	5'-AAGAGAATGGAGCACATGAAA-3'
NM_033284.1	Hs.436900	TBL1Y	Transducin (beta)-like 1Y-linked	5'-AAGATGAGCATAACCAGTGAC-3'
NM_024665.3	Hs.438970	TBL1XR1	Transducin (beta)-like 1X-linked receptor 1	5'-AAGGCCCTATATTTGCATTAA-3'
NM_173174.1	Hs.491322	PTK2B	PTK2B protein tyrosine kinase 2 beta	5'-GTTGGCTGAGTGCTATGGGCTGA-3'
NM_006311.2	Hs.462323	NCOR1	Nuclear receptor co-repressor 1	5'-GGGCTTATGGAGGACCCTATGA-3'
NM_002211.2	Hs.429052	ITGB1	Integrin, beta 1	5'-GGAACAGCAGAGAAGCTCATTCAAGAG ATGAGCTTCTCTGCTGTTCCTTTTT-3'
NM_139176.2	Hs.351118	NALP7	NACHT, leucine rich repeat and PYD containing 7	5'-CACCGAAGCAGCACGACTTCTTCTTCAAG AGAGAAGAAGTCGTGCTGCTTC-3'
NM_004422.2	Hs.118640	DVL2	Dishevelled, dsh homolog 2 (Drosophila)	5'-AGGUUCAGCAGCUCCACGGAdTdT-3'
NM_001228.2	Hs.369736	CASP8	Caspase 8, apoptosis-related cysteine protease	5'-gatccccCCTCGGGGATACTGTCTGAttcaagaga TCAGACAGTATCCCCGAGGtttttggaaa-3'

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NM_001769.2	Hs.114286	CD9	CD9 antigen (p24)	5'-GAGCATCTTCGAGCAAGAA-3'
NM_004357.3	Hs.512857	CD151	CD151 antigen	5'-CATGTGGCACCGTTTGCCT-3'
NM_003188.2	Hs.485968	MAP3K7	Mitogen-activated protein kinase kinase kinase 7	5'-UGGCUUAUCUUACACUGGA-3'
NM_006116.2	Hs.507681	MAP3K7IP1	Mitogen-activated protein kinase kinase kinase 7 interacting protein 1	5'-GGCUCAAGUUCAGGAGUGAGAACAA-3'
NM_015093.2	Hs.269775	MAP3K7IP2	Mitogen-activated protein kinase kinase kinase 7 interacting protein 2	5'-GGAACGACUUCAAAGAGAACUUGAG-3'
NM_001315.1	Hs.485233	MAPK14	Mitogen-activated protein kinase 14	5'-GCAUUACAACCAGACAGUUGAUAUU-3'
NM_006502.1	Hs.439153	POLH	Polymerase (DNA directed), eta	5'-GUG GAG CAG CGG CAA AAU CTT-3'
NM_006502.1	Hs.439153	POLH	Polymerase (DNA directed), eta	5'-UCC UCA UUU GAG GAA UAA ATT-3'
NM_006502.1	Hs.439153	POLH	Polymerase (DNA directed), eta	5'-GGA AUA AAC CUU GUG CAG UTT-3'
NM_006502.1	Hs.439153	POLH	Polymerase (DNA directed), eta	5'-UAA ACC UUG UGC AGU UGU ATT-3'
NM_006502.1	Hs.439153	POLH	Polymerase (DNA directed), eta	5'-CCU UGU GCA GUU GUA CAG UTT-3'
NM_015321.1	Hs.371096	MECT1	Mucoepidermoid carcinoma translocated 1	5'- CCGGCAACCUCGCGGCCAAUU -3'
NM_181715.1	Hs.406392	TORC2	Transducer of regulated cAMP response element-binding protein (CREB) 2	5'- CGACUACCAUCUGCACUUAUU -3'
NM_001079.3	Hs.234569	ZAP70	Zeta-chain (TCR) associated protein kinase 70kDa	5'-AACCGGCTCTCCATTGGCATT-3'
NM_004834.3	Hs.431550	MAP4K4	Mitogen-activated protein kinase kinase kinase kinase 4	5'-GTGGTTGGAAATGGCACCTTT-3'
NM_006191.1	Hs.524498	PA2G4	Proliferation-associated 2G4,	5'-AAGCGACCAGGAUUAUAUUCU-3'

			38kDa	
NM_006191.1	Hs.524498	PA2G4	Proliferation-associated 2G4, 38kDa	5'-AAGUGAGGUGGAAAGGCGUUU-3'
NM_005940.3	Hs.143751	MMP11	Matrix metalloproteinase 11 (stromelysin 3)	5'-TCCCATGTCCACTTCGACTATGATGTCA AGAGCATCATAGTCGAAGTGGACATTT-3'
NM_005940.3	Hs.143751	MMP11	Matrix metalloproteinase 11 (stromelysin 3)	5'-TCCCAGATCTACTTCTTCCGAGGTCAAG AGCCTCGGAAGAAGTAGATCTTT-3'
NM_005940.3	Hs.143751	MMP11	Matrix metalloproteinase 11 (stromelysin 3)	5'-TCCCAGGATGCTGATGGCTATGCCTTCA AGAGAGGCATAGCCATCAGCATCCTTT-3'
NM_003684.3	Hs.371594	MKNK1	MAP kinase interacting serine/threonine kinase 1	5'-AATGCCCATCTCTATAGGTTT-3'
NM_003668.2	Hs.413901	MAPKAPK5	Mitogen-activated protein kinase-activated protein kinase 5	5'- GGAUAUGCGAAGAAAGAUCTT -3'
NM_004604.3	Hs.83734	STX4A	Syntaxin 4A (placental)	5'-AAGGAGGAAGCTGATGAGAAC-3'
NM_004177.3	Hs.530733	STX3A	Syntaxin 3A	5'-AACGTCCGGAACAAACTGAAG-3'
NM_001009567.1	Hs.461247	MRC1L1	Mannose receptor, C type 1-like 1	5'-AAGTGGTACGCAGATTGCACG-3'
NM_002576.3	Hs.435714	PAK1	P21/Cdc42/Rac1-activated kinase 1	5'-AAGGAGAAGAAAAAGAAGGAC-3'
NM_001664.2	Hs.247077	RHOA	Ras homolog gene family, member A	5'-GCAGGTAGAGTTGGCTTTG-3'
NM_175744.3	Hs.502659	RHOC	Ras homolog gene family, member C	5'-GACTATGATCGACTGCGGC-3'
NM_080491.1	Hs.429434	GAB2	GRB2-associated binding protein 2	5'-GTGAGAACGATGAGAAATA-3'
NM_080491.1	Hs.429434	GAB2	GRB2-associated binding protein 2	5'-GTTGGTGCCTAATCACTTA-3'
NM_005225.1	Hs.96055	E2F1	E2F transcription factor 1	5'-GACGTGTCAGGACCTTCGT-3'

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NM_005225.1	Hs.96055	E2F1	E2F transcription factor 1	5'-CTTAACTGGTGTACATTAA-3'
NM_006392.2	Hs.376064	NOL5A	Nucleolar protein 5A (56kDa with KKE/D repeat)	5'-CAAUAUGAUCAUCCAGUCCAUUA-3'
NM_015934	Hs.471104	NOP5/NOP58	Nucleolar protein NOP5/NOP58	5'-CAAGCAUGCAGCUUCUACCGUUC-3'
NM_001436	Hs.299002	FBL	Fibrillarin	5'-CAGUCGAGUUCUCCCACCGCUCU-3'
NM_006666	Hs.515846	RUVBL2	RuvB-like 2 (E. coli)	5'-GAGACCAUCUACGACCUGGGCAC-3'
NM_006666	Hs.515846	RUVBL2	RuvB-like 2 (E. coli)	5'-GAGAGUGACAUGGCGCCUGUCCU-3'
NM_003707.1	Hs.272822	RUVBL1	RuvB-like 1 (E. coli)	5'-AAGGAACCAAACAGUUGAAACUG-3'
NM_003707.1	Hs.272822	RUVBL1	RuvB-like 1 (E. coli)	5'-GAGUCUUCUAUCGCUCCCAUCGU-3'
NM_004741	Hs.523238	NOLC1	Nucleolar and coiled-body phosphoprotein 1	5'-AAAUUGAGGUGGAUUCACGAGUU-3'
NM_032177	Hs.546453	PHAX	RNA U, small nuclear RNA export adaptor (phosphorylation regulated)	5'-UAGUAUCAGCGAGGAACAAAUUA-3'
NM_032177	Hs.546453	PHAX	RNA U, small nuclear RNA export adaptor (phosphorylation regulated)	5'-AAGAGUAUAUAGCACAGGAUUUA-3'
NM_024831	Hs.335068	NCOA6IP	Nuclear receptor coactivator 6 interacting protein	5'-AAGAUUGCCCUUGCUCGCAAUAA-3'
NM_024831	Hs.335068	NCOA6IP	Nuclear receptor coactivator 6 interacting protein	5'-UAUCACCGUAUGAAAUGGAAACU-3'
NM_022874.1	Hs.202179	SMN2	Survival of motor neuron 1, telomeric	5'-AAGUGGAAUGGGUAACUCUUCUU-3'
NM_012321.2	Hs.515255	LSM4	LSM4 homolog, U6 small nuclear RNA associated	5'-AACGGCCGUCCCAAAGCUGGCUG-3'
NM_016200.2	Hs.446179	LSM8	LSM8 homolog, U6 small nuclear RNA associated	5'-AAGAAACAGAUUCUGCGCUUGAU-3'
NM_003142	Hs.546301	SSB	Sjogren syndrome antigen B (autoantigen La)	5'-GAAUUAGGUCCACUUCAAUGUCC-3'
NM_003142	Hs.546301	SSB	Sjogren syndrome antigen B	5'-AAGAUUCUUCCAUUAAAUUGCCU-3'

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			(autoantigen La)	
NM_001228	Hs.369736	CASP8	Caspase 8, apoptosis-related cysteine protease	5'-AACTACCAGAAAGGTATACCT-3'
NM_003842.3	Hs.521456	TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b	5'-AAGACCCTTGTGCTCGTTGTC-3'
NM_017672.2	Hs.512894	TRPM7	Transient receptor potential cation channel, subfamily M, member 7	5'-AAGCAGAGTGACCTGGTAGAT-3'
NM_007294.1	Hs.194143	BRCA1	Breast cancer 1, early onset	5'-UCACAGUGUCCUUUAUGUAdTdT-3'
NM_033238.1	Hs.526464	PML	Promyelocytic leukemia	5'-AUGGCUUCGACGAGUUCAATT-3'
NM_000546.2	Hs.408312	TP53	Tumor protein p53 (Li-Fraumeni syndrome)	5'-GCAUGAACCGGAGGCCCAUTT-3'
NM_002198.1	Hs.436061	IRF1	Interferon regulatory factor 1	5'-AGACCAGAGCAGGAACAAGTT-3'
NM_024790.3	Hs.370147	FLJ22490	Hypothetical protein FLJ22490	5'-GAAGATTTGCGCAGTGGAC-3'
NM_000546.2	Hs.408312	TP53	Tumor protein p53 (Li-Fraumeni syndrome)	5'-UGGUUCACUGAAGACCCAGUU-3'
NM_002880.2	Hs.159130	RAF1	V-raf-1 murine leukemia viral oncogene homolog 1	5'-AUUCCUGCUCAAUGGAUUUdTdT-3'
NM_198400.1	Hs.1565	NEDD4	Neural precursor cell expressed, developmentally down-regulated 4	5'-TAGAGCCTGGCTGGGTTGTTTTG-3'
NM_015277.2	Hs.185677	NEDD4L	Neural precursor cell expressed, developmentally down-regulated 4-like	5'-AACCACAACACAAAGTCACAG-3'
NM_016931.2	Hs.371036	NOX4	NADPH oxidase 4	5'-AAACCGGCAGGAGUUUACCCAG-3'
NM_005975.2	Hs.51133	PTK6	PTK6 protein tyrosine kinase 6	5'-AAGGUGGCCAUUAAGGUGAUU-3'
NM_005531.1	Hs.380250	IFI16	Interferon, gamma-inducible protein 16	5'-UCAGAAGACCACAAUCUAC-3'
NM_000633.1	Hs.150749	BCL2	B-cell CLL/lymphoma 2	5'-GUGAAGUCAACAUGCCUGC-dTdT-3'
NM_182981.1	Hs.528383	OKL38	Pregnancy-induced growth inhibitor	5'-CACCCUACACGAAGCCAGATT-3'

NM_002961.2	Hs.81256	S100A4	S100 calcium binding protein A4	5'-GGA CAG AUG AAG CUG CUU UTT-3'
NM_014585.3	Hs.529285	SLC40A1	Solute carrier family 40 (iron-regulated transporter), member 1	5'-GGTGGACAAGAATGCTAGAC-3'
NM_014585.3	Hs.529285	SLC40A1	Solute carrier family 40 (iron-regulated transporter), member 1	5'-GAAGGATTGACCAGTTAACC-3'
NM_014585.3	Hs.529285	SLC40A1	Solute carrier family 40 (iron-regulated transporter), member 1	5'-GCTTGAACATGAGCAAGAGC-3'
NM_021127.1	Hs.96	PMAIP1	Phorbol-12-myristate-13-acetate-induced protein 1	5'-AACTTCCGGCAGAAACTTCTG-3'
NM_002467.2	Hs.202453	MYC	V-myc myelocytomatosis viral oncogene homolog (avian)	5'-GCCACAGCAUACAUCCUGUdTdT-3'
NM_002187.2	Hs.674	IL12B	Interleukin 12B	5'-CGCACGCUAAUGCUGGCAUdTdT-3'
NM_019887.3	Hs.169611	DIABLO	Diablo homolog (Drosophila)	5'-AAGCGGUGUUUCUCAGAATTGtt-3'
NM_017563	Hs.150725	IL17RD	Interleukin 17 receptor D	5'-GUCGG AGGGA AGACA GUGCT T-3'
NM_017563	Hs.150725	IL17RD	Interleukin 17 receptor D	5'-GCAUG UGAUU GCUGA CGCCT T-3'
NM_003142.2	Hs.546301	SSB	Sjogren syndrome antigen B (autoantigen La)	5Õ-AAGGCTTCCC AACTGATGC AA-3Õ
NM_003142.2	Hs.546301	SSB	Sjogren syndrome antigen B (autoantigen La)	5Õ-AAGCCAAG GAAGCAT TGGGTA-3Õ
NM_003142.2	Hs.546301	SSB	Sjogren syndrome antigen B (autoantigen La)	5Õ-AAGTACTAGAA GGAGAGGTGG-3Õ
NM_006101	Hs.414407	KNTC2	Kinetochore associated 2	5'-GTTCAAAAGCTGGATGATCTT-3'
NM_145697	Hs.234545	CDCA1	Cell division cycle associated 1	5'- AAGATACGGTCCAGAAGCTTA -3'
NM_003550	Hs.209128	MAD1L1	MAD1 mitotic arrest deficient-like 1	5'- CCAGCGGCTCAAGGAGGTTTT -3'
NM_002358	Hs.533185	MAD2L1	MAD2 mitotic arrest deficient-like 1	5'- GAGTCGGGACCACAGTTTATT -3'

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NM_004336	Hs.469649	BUB1	BUB1 budding uninhibited by benzimidazoles 1 homolog	5'- TAGGCTAATTGTACTGCTCTT -3'
NM_001211.4	Hs.36708	BUB1B	BUB1 budding uninhibited by benzimidazoles 1 homolog beta	5'- GGAGATCCTCTACAAAGGGTT -3'
NM_016343.3	Hs.497741	CENPF	Centromere protein F, 350/400ka (mitosin)	5'- AAGAGATGCTAATAGCAGTTT -3'
NM_001813	Hs.75573	CENPE	Centromere protein E, 312kDa	5'- ACTCTTACTGCTCTCCAGTTT -3'
NM_004217	Hs.442658	AURKB	Aurora kinase B	5'- CGAGACCTATCGCCGCATCGT -3'
NM_005030	Hs.329989	PLK1	Polo-like kinase 1	5'- GGGCGGCTTTGCCAAGTGCTT -3'
NM_004104	Hs.83190	FASN	Fatty acid synthase	5'-CCCUGAGAUCCCAGCGCUGdTdT-3'
NM_021975.2	Hs.502875	RELA	V-rel reticuloendotheliosis viral oncogene homolog A	5'-GATCAATGGCTACACAGGA-3'
NM_033256	Hs.348037	PPP1R14A	Protein phosphatase 1, regulatory (inhibitor) subunit 14A	5'-ACCUGUCGAGGACUUCAUCdTdT-3'
NM_177966.3	Hs.151293	2'-PDE	2'-phosphodiesterase	5'-GUACAAGGUGGAGCGCAACdTdT-3'
NM_015355	Hs.462732	SUZ12	Suppressor of zeste 12 homolog	5'-CCCGGAAATTTCCCGTCCC-3'
NM_015355	Hs.462732	SUZ12	Suppressor of zeste 12 homolog	5'-GAGATGACCTGCATTGCCC-3'
NM_016179.1	Hs.262960	TRPC4	Transient receptor potential cation channel, subfamily C, member 4	5'-ACUCUUGGUUCAGAAAGGATT-3'
NM_000249	Hs.195364	MLH1	MutL homolog 1, colon cancer, nonpolyposis type 2	5'-GGTTCACTACTAGTAAACT-3'
NM_000534	Hs.111749	PMS1	PMS1 postmeiotic segregation increased 1	5'-GGAATCTACTCGTTTGTAT-3'
NM_002198	Hs.436061	IRF1	Interferon regulatory factor 1	5'-CCAAGAACCAGAGAAAAGATT-3'
NM_002199.2	Hs.374097	IRF2	Interferon regulatory factor 2	5'-CUCUUUAGAAACUGGGCAATT-3'
NM_000546.2	Hs.408312	TP53	Tumor protein p53 (Li-Fraumeni syndrome)	5'-AAGACTCCAGTGGTAATCTAC-3'

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NM_000051	Hs.435561	ATM	Ataxia telangiectasia mutated (includes complementation groups A, C and D)	5'-TAGAGCTACAGAACGAAAG-3'
NM_000051	Hs.435561	ATM	Ataxia telangiectasia mutated (includes complementation groups A, C and D)	5'-GAATGTGAACACCACCAAA-3'
NM_000051	Hs.435561	ATM	Ataxia telangiectasia mutated (includes complementation groups A, C and D)	5'-CTACACAAATATTGAGGAT-3'
NM_000051	Hs.435561	ATM	Ataxia telangiectasia mutated (includes complementation groups A, C and D)	5'-CTGTACTTCCATACTTGAT-3'
NM_001184	Hs.271791	ATR	Ataxia telangiectasia and Rad3 related	5'-AAGCCAAGACAAATTCTGTGT-3'
NM_001184	Hs.271791	ATR	Ataxia telangiectasia and Rad3 related	5'-AACCTCCGTGATGTTGCTTGA-3'
NM_001798.2	Hs.19192	CDK2	Cyclin-dependent kinase 2	5'-CAAAGCCAGAAACAAGTTG-3'
NM_001798.2	Hs.19192	CDK2	Cyclin-dependent kinase 2	5'-AAATAAACTCTACCTGGTT-3'
NM_001798.2	Hs.19192	CDK2	Cyclin-dependent kinase 2	5'-AAACCTCAGAATCTGCTTA-3'
NM_001798.2	Hs.19192	CDK2	Cyclin-dependent kinase 2	5'-GTTACTTCTATGCCTGATT-3'
NM_207003	Hs.469658	BCL2L11	BCL2-like 11 (apoptosis facilitator)	5'-(GACCGAGAAGGUAGACAAUUG)d(TT)-3'
NM_000166	Hs.333303	GJB1	Gap junction protein, beta 1, 32kDa	5'-AAGAGGCACAAGGTCCACATCdTdT-3'
NM_000359	Hs.508950	TGM1	Transglutaminase 1	5'-AUGCAGCUGGAGAUGGCACdTdT-3'
NM_024596	Hs.550532	MCPH1	Microccphaly, primary autosomal recessive 1	5'-AGGAAGUUGGAAGGAUCCAdTdT-3'
NM_024596	Hs.550532	MCPH1	Microcephaly, primary autosomal recessive 1	5'-GAACACUUAUCAAGCCUAAUU-3'
NM_024596	Hs.550532	MCPH1	Microcephaly, primary autosomal recessive 1	5'-GGAGAGAACAAGCAUAUUUUU-3'

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NM_024596	Hs.550532	MCPH1	Microcephaly, primary autosomal recessive 1	5'-UGAUGUACCUAUUCUCUUAUU-3'
NM_024596	Hs.550532	MCPH1	Microcephaly, primary autosomal recessive 1	5'-GAUAAGAGAUUUCAGAAGAUU-3'
NM_024596	Hs.550532	MCPH1	Microcephaly, primary autosomal recessive 1	5'-GUCACCACAGCGCAATGGAdTdT-3'
NM_000245	Hs.132966	MET	Met proto-oncogene (hepatocyte growth factor receptor)	5'-ACUCUAGAUGCUCAGACUUTT-3'
NM_205860.1	Hs.33446	NR5A2	Nuclear receptor subfamily 5, group A, member 2	5'-AGGATCCATCTTCCTGGTTAC-3'
NM_182763.1	Hs.532826	MCL1	Myeloid cell leukemia sequence 1 (BCL2-related)	5'-UAACACCAGTACGGACGGGdTdT-3'
NM_008765	Hs.444870	ORC2L	Origin recognition complex, subunit 2-like	5'-UGCUCCUCUCAUGUGGGAU-3'
NM_006190	Hs.444870	ORC2L	Origin recognition complex, subunit 2-like	5'-UCAUUGGUCAGUUGUCAUC-3'
NM_181837	Hs.410228	ORC3L	Origin recognition complex, subunit 3-like	5'-GAGACUUGGGCGGUCAAAU-3'
NM_002592.2	Hs.147433	PCNA	Proliferating cell nuclear antigen	5'-CGGUGACACUCAGUAUGUC-3'
NM_016526	Hs.414418	BET1L	Blocked early in transport 1 homolog (S. cerevisiae) like	5'-AAGCAUGACCAGCCUGCUUAC-3'
NM_001569	Hs.522819	IRAK1	Interleukin-1 receptor-associated kinase 1	5'-GGUUGUCCUUGAGUAAUAAtt-3'
NM_080649	Hs.73722	APEX1	APEX nuclease (multifunctional DNA repair enzyme) 1	5'-GUCUGGUACGACUGGAGUACC-3'
NM_002658	Hs.77274	PLAU	Plasminogen activator, urokinase	5'-AACATTCACTGGTGCAACTGC-3'
NM_001654	Hs.446641	ARAF	V-raf murine sarcoma 3611 viral oncogene homolog	5'-AACAACATCTTCCTACATGAG-3'

NM_004333	Hs.490366	BRAF	V-raf murine sarcoma viral oncogene homolog B1	5'-AAAGAATTGGATCTGGATCAT-3'
NM_002880	Hs.159130	RAF1	V-raf-1 murine leukemia viral oncogene homolog 1	5'-AAUAGUUCAGCAGUUUGGCUA-3'
NM_014314	Hs.190622	DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	5'-GAATTTAAAACCAGAATTATC-3'
NM_000927.3	Hs.489033	ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	5'-AAGCGAAGCAGTGGTTCAGGT-3'
NM_001753.3	Hs.74034	CAV1	Caveolin 1, caveolae protein, 22kDa	5'-AGACGAGCUGAGCGAGAAGCA-3'
NM_001753.3	Hs.74034	CAV1	Caveolin 1, caveolae protein, 22kDa	5'-CAUCUACAAGCCCAACAACTT-3'
NM_000389.2	Hs.370771	CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	5'-CUUCGACUUUGUCACCGAG-3'
NM_007294.1	Hs.194143	BRCA1	Breast cancer 1, early onset	5'-AACCTGTCTCCACAAAGTGTG-3'
NM_002105	Hs.477879	H2AFX	H2A histone family, member X	5'-CAA CAA GAA GAC GCG AAU CdTdT-3'
NM_020382	Hs.443735	SET8	PR/SET domain containing protein 8	5'-AAUCGCCUAGGAAGACUGAUC-3'
NM_012331	Hs.490981	MSRA	Methionine sulfoxide reductase A	5'-CCCCUGUAGCGGCCAAACAUU-3'
NM_012331	Hs.490981	MSRA	Methionine sulfoxide reductase A	5'-CAAAGUACAAAGGAAUUUAUU-3'
NM_012331	Hs.490981	MSRA	Methionine sulfoxide reductase A	5'-CGGGAGGGACAGACUUUCUUU-3'
NM_014554	Hs.371957	SENP1	SUMO1/sentrin specific protease 1	5'-GTGAACCACAACTCCGTATTC-3'
NM_002945	Hs.461925	RPA1	Replication protein A1, 70kDa	5'-AACUGGUUGACGAAAGUGGUG-3'
NM_001184	Hs.271791	ATR	Ataxia telangiectasia and Rad3 related	5'-AACCCGCGUUGGCGUGGUUGA-3'
NM_001430.3	Hs.468410	EPAS1	Endothelial PAS domain	5'-ACCAAUCCAGCACCCAUCCdTdT-3'

			protein 1	
NM_001530.2	Hs.509554	HIF1A	Hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	5'-CUGAUGACCAGCAACUUGAdTdT-3'
NM_021972	Hs.68061	SPHK1	Sphingosine kinase 1	5'-GAGCUGCAAGGCCUUGCCCdTdT-3
NM_002502	Hs.73090	NFKB2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	5'-CTCCTCCATTGTGGAACCCAAGGAGC-3'
NM_016829	Hs.380271	OGG1	8-oxoguanine DNA glycosylase	5'-GUAUGGACACUGACUCAGAUU-3'
NM_016829	Hs.380271	OGG1	8-oxoguanine DNA glycosylase	5'-GUACUUCCAGCUAGAUGUUUU-3'
NM_006142	Hs.523718	SFN	Stratifin	5'-GAGCGAAACCUGCUCUCAG-3'
NM_006142	Hs.523718	SFN	Stratifin	5'-GGGUGACUACUACCGCUAC-3'
NM_006142	Hs.523718	SFN	Stratifin	5'-AGACAGCACCCUCAUCAUG-3'
NM_00615	Hs.477693	NCK1	NCK adaptor protein 1	5'-GUCCUGGUGGCGAGUUCGATT-3'
NM_00615	Hs.477693	NCK1	NCK adaptor protein 1	5'-CGUCUCUAUGACCUCAACATT-3'
NM_002422	Hs.375129	MMP3	Matrix metalloproteinase 3 (stromelysin 1, progelatinase)	5'-AUGAAGAGUCUUCCAAUCCUU-3'
NM_000021.2	Hs.3260	PSEN1	Presenilin 1 (Alzheimer disease 3)	5'-AAGGTCCACTTCGTATGCTGG-3'
NM_015331	Hs.517249	NCSTN	Nicastrin	5'-AAGGGCAAGTTTCCCGTGCAG-3'
NM_016022	Hs.108408	APH-1A	Anterior pharynx defective 1 homolog A (C. elegans)	5'-AAGAAGGCAGATGAGGGGTTA-3'
NM_172341	Hs.534465	PEN2	Presenilin enhancer 2 homolog (C. elegans)	5'-AAUCAAAGGCUAUGUCUGGCG-3'
NM_020673	Hs.529044	RAB22A	RAB22A, member RAS oncogene family	5'-AAGGACUACGCCGACUCUAUU-3'
NM_001002814	Hs.191179	RAB11FIP1	RAB11 family interacting protein 1 (class I)	5'-CGCCTCTTTCCCAGTCCATGT-3'
NM_015470	Hs.24557	RAB11FIP5	RAB11 family interacting	5'-GAGCTGAGTGCTCAGGCTAAA-3'

			protein 5 (class I)	
NM_030791	Hs.24678	SGPP1	Sphingosine-1-phosphate phosphatase 1	5'-AGUGGCCCGUUUCCAGCGGdTT-3'
NM_005406	Hs.306307	ROCK1	Rho-associated, coiled-coil containing protein kinase 1	5'-AAGGTGATTGGTAGAGGTGCA-3'
NM_198437	Hs.250822	STK6	Serine/threonine kinase 6	5'-AAGCACAAAAGCTTGTCTCCA-3'
NM_006272	Hs.422181	S100B	S100 calcium binding protein, beta (neural)	5'-GGAAUUCAUGGCCUUUGUU-3'
NM_004219	Hs.350966	PTTG1	Pituitary tumor-transforming 1	5'-GAU CUC AAG UUU CAA CAC Ctt-3'
NM_004219	Hs.350966	PTTG1	Pituitary tumor-transforming 1	5'-GUC UGU AA A GAC CAA GG GAtt-3'
NM_001478.2	Hs.159481	GALGT	UDP-N-acetyl-alpha-D-galactosamine:(N-acetylneuraminyl)- galactosylglucosylceramide N-acetylgalactosaminyltransferase	5'-GGAGCAAGUAGUGGGGCUGdTdT-3'
NM_000657	Hs.150749	BCL2	B-cell CLL/lymphoma 2	5'-GUACAUCCAUUAUAAGCUGTT-3'
NM_032984	Hs.368982	CASP2	Caspase 2, apoptosis-related cysteine protease	5'-AACTTCCAGCTGGCATATAGGdTdT-3'
NM_001228	Hs.369736	CASP8	Caspase 8, apoptosis-related cysteine protease	5'-AAGGGUCAUGCUCUAUCAGAUdTdT-3'
NM_197967	Hs.474150	BID	BH3 interacting domain death agonist	5'-AAGAAGACAUCAUCCGGAAUAdTdT-3'
NM_001167	Hs.356076	BIRC4	Baculoviral IAP repeat-containing 4	5'-AAGGAGAUACCGUGCGGUGCUdTdT-3'
NM_002483	Hs.466814	CEACAM6	Carcinoembryonic antigen-related cell adhesion molecule 6	5'-CCGGACAGTTCCATGTATA-3'
NM_001008490	Hs.285313	KLF6	Kruppel-like factor 6	5'-GGAGAAAAGCCUUACAGAUTT-3'
NM_024309	Hs.368551	TNIP2	TNFAIP3 interacting protein 2	5'-GUAUUUGGCCGCCGACGCAd(TT)-3'
NM_001621	Hs.171189	AHR	Aryl hydrocarbon receptor	5'-AAGACTGGAGAAAGTGGCATG-3'

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NM_001005845	Hs.2442	ADAM9	A disintegrin and metalloproteinase domain 9 (meltrin gamma)	5'-AAUCACUGUGGAGACAUUUGCdTdT-3'
NM_001110	Hs.172028	ADAM10	A disintegrin and metalloproteinase domain 10	5'-AAUGAAGAGGGACACUUCCCUdTdT-3'
NM_021641	Hs.386283	ADAM12	A disintegrin and metalloproteinase domain 12	5'-AACCUCGCUGCAAAGAAUGUGdTdT-3'
NM_207196	Hs.312098	ADAM15	A disintegrin and metalloproteinase domain 15 (metargidin)	5'-AACUCCAUCUGUUCUCCUGACdTdT-3'
NM_021832	Hs.404914	ADAM17	A disintegrin and metalloproteinase domain 17	5'-AAAGUUUGCUUGGCACACCUUdTdT-3'
NM_000927.3	Hs.489033	ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	5'-AAG GCC TAA TGC CGA ACA CA-3'
NM_000927.3	Hs.489033	ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	5'-AAC TTT GGC TGC CAT CAT CCA-3'
NM_000572	Hs.193717	IL10	Interleukin 10	5'-UAAGCUCCAAGAGAAAGGCdTdT-3'
NM_021975	Hs.502875	RELA	V-rel reticuloendotheliosis viral oncogene homolog A	5'-GCCCUAUCCCUUUACGUCA-3'
NM_001331	Hs.166011	CTNND1	Catenin (cadherin-associated protein), delta 1	5'-GTGGACCATGCACTGCATGCCTAT AGTGAGTCGTATTAC-3'
NM_001211	Hs.36708	BUB1B	BUB1 budding uninhibited by benzimidazoles 1 homolog beta	5'-AGATCCTGGCTAACTGTTC-3'
NM_002358	Hs.533185	MAD2L1	MAD2 mitotic arrest deficient-like 1 (yeast)	5'-TACGGACTCACCTTGCTTG-3'
NM_001530.2	Hs.509554	HIF1A	Hypoxia-inducible factor 1, alpha subunit	5'-CUGGACACAGUGUGUUUGAdTdT-3'
NM_001530.2	Hs.509554	HIF1A	Hypoxia-inducible factor 1, alpha subunit	5'-CUGAUGACCAGCAACUUGAdTdT-3'

NM_001430	Hs.468410	EPAS1	Endothelial PAS domain protein 1	5'-GCUCUUCGCCAUGGACACAdTdT-3'
NM_001430	Hs.468410	EPAS1	Endothelial PAS domain protein 1	5'-GCGACAGCUGGAGUAUGAAdTdT-3'
NM_001379	Hs.202672	DNMT1	DNA (cytosine-5-)-methyltransferase 1	5'-CCAUGAGCACCGUUCUCCTT-3'
NM_031310	Hs.107125	PLVAP	Plasmalemma vesicle associated protein	5'-CUUGACCAAGGAGCUCAACTT-3'
NM_031310	Hs.107125	PLVAP	Plasmalemma vesicle associated protein	5'-GGAGCUCAACUUCACCACCTT-3'
NM_016734	Hs.126365	PAX5	Paired box gene 5 (B-cell lineage specific activator)	5'-CGGCCACUCGCUUCCGGGCTT-3'
NM_016734	Hs.126365	PAX5	Paired box gene 5 (B-cell lineage specific activator)	5'-GCUCCGUCGACUGCGCGCC-3'
NM_006257	Hs.498570	PRKCQ	Protein kinase C, theta	5'-AAACCACCGTGGAGCTCTACT-3'
NM_006257	Hs.498570	PRKCQ	Protein kinase C, theta	5'-AAGAGCCCGACCTTCTGTGAA-3'
NM_032430	Hs.182081	BRSK1	BR serine/threonine kinase 1	5'-GUU CUU CCG CCA GAU UGU GdTdT-3'
NM_015045	Hs.203099	KIAA0261	KIAA0261	5'-CGGACUACCCUUAGCACAAUU-3'
NM_015045	Hs.203099	KIAA0261	KIAA0261	5'-GAAUAGUCACCAUAUUCACUU-3'
NM_005430	Hs.248164	WNT1	Wingless-type MMTV integration site family, member 1	5'-GGTTCCATCGAATCCTGCA-3'
NM_004421.2	Hs.74375	DVL1	Dishevelled, dsh homolog 1 (Drosophila)	5'-AACAAGATCACCTTCTCCGAG-3'
NM_004422	Hs.118640	DVL2	Dishevelled, dsh homolog 2 (Drosophila)	5'-AACTTTGAGAACATGAGCAAC-3'
NM_139049	Hs.522924	MAPK8	Mitogen-activated protein kinase 8	5'-CGTGGATTTATGGTCTGTG-3'
NM_003376	Hs.73793	VEGF	Vascular endothelial growth factor	5'-UGGAUGUCUAUCAGCGCAGdTdT-3'
NM_003376	Hs.73793	VEGF	Vascular endothelial growth	5'-GCUACUGCCAUCCAAUCGAdTdT-3'

			factor	
NM_003376	Hs.73793	VEGF	Vascular endothelial growth factor	5'-GGAGUACCCUGAUGAGAUCdTdT-3'
NM_003376	Hs.73793	VEGF	Vascular endothelial growth factor	5'-CUGAGGAGUCCAACAUCACdTdT-3'
NM_003376	Hs.73793	VEGF	Vascular endothelial growth factor	5'-CCAAGGCCAGCACAUAGGAdTdT-3'
NM_005123	Hs.282735	NR1H4	Nuclear receptor subfamily 1, group H, member 4	5'-GTCGTGACTTGCGACAAG-3'
NM_004999	Hs.149387	MYO6	Myosin VI	5'-GCUGGCAGUUCAUAGGAAUdTdT-3'
NM_004999	Hs.149387	MYO6	Myosin VI	5'-CGUGCUCCAAAGUCUGUUAdTdT-3'
NM_014865	Hs.5719	CNAP1	Chromosome condensation-related SMC-associated protein 1	5'-UCAGUAUGUUGUGCAAGAGTT-3'
NM_014865	Hs.5719	CNAP1	Chromosome condensation-related SMC-associated protein 1	5'-GAAGAUACUCUGGAAUUCCTT-3'
NM_015261	Hs.438550	KIAA0056	KIAA0056 protein	5'-CUGGAUUUCACAGAGACUGTT-3'
NM_015261	Hs.438550	KIAA0056	KIAA0056 protein	5'-GCAGAGAUCAUAGAGACUGTT-3'
NM_015341	Hs.308045	BRRN1	Barren homolog (Drosophila)	5'-GACUUUCCUCAGAAUGACGTT-3'
NM_015341	Hs.308045	BRRN1	Barren homolog (Drosophila)	5'-CAUUACUCCACCUGUAUCATT-3'
NM_014551	Hs.180903	384D8-2	Hypothetical protein 384D8_6	5'-GGAUUUCAGGAUGAACACGTT-3'
NM_014551	Hs.180903	384D8-2	Hypothetical protein 384D8_6	5'-GCUGCAGGACUUCCACCAGTT-3'
NM_006031	Hs.474069	PCNT2	Pericentrin 2 (kendrin)	5'-AAUUGGAACAGCUGCAGCAGA-3'
NM_006031	Hs.474069	PCNT2	Pericentrin 2 (kendrin)	5'-AAGCUCUGAUUUAUCAAAAGA-3'
NM_012179.2	Hs.5912	FBXO7	F-box protein 7	5'-CCCACACCAUUCCAUUCUA-3'
NM_002467	Hs.202453	MYC	V-myc myelocytomatosis viral oncogene homolog (avian)	5'-AAGAUGAGGAAGAAAUCGAUGUU-3'
NM_002467	Hs.202453	MYC	V-myc myelocytomatosis viral oncogene homolog (avian)	5'-AAAAGGUCAGAGUCUGGAUCACC-3'
NM_002467	Hs.202453	MYC	V-myc myelocytomatosis viral	5'-CACGUCUCCACACAUCAGCACAA-3'

			oncogene homolog (avian)	
NM_002467	Hs.202453	MYC	V-myc myelocytomatosis viral oncogene homolog (avian)	5'-AAAUGAGAUAAAGGUGGCUAAUU-3'
NM_002392	Hs.369849	MDM2	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein	5'-UGGUUGCAUUGUCCAUGGC-3'
NM_003121	Hs.437905	SPIB	Spi-B transcription factor (Spi-1/PU.1 related)	5'-GATCGCTGTGTGTCTGTAA-3'
NM_003120.1	Hs.502511	SPI1	Spleen focus forming virus (SFFV) proviral integration oncogene spi1	5'-GTCCGTATGTAAATCAGAT-3'
NM_199002	Hs.278186	ARHGEF1	Rho guanine nucleotide exchange factor (GEF) 1	5'-CATACCATCTCTACCGACG-3'
NM_014784	Hs.516954	ARHGEF11	Rho guanine nucleotide exchange factor (GEF) 11	5'-ACTGAAGTCTCGGCCAGCT-3'
NM_015313	Hs.24598	ARHGEF12	Rho guanine nucleotide exchange factor (GEF) 12	5'-GAAACTCGTCGCATCTTCC-3'
NM_173842	Hs.81134	IL1RN	Interleukin 1 receptor antagonist	5'-AUCUGCAGAGGCCUCCGCAtt-3'
NM_032726	Hs.549218	PLCD4	Phospholipase C, delta 4	5'-GAGCAGAACCTTCAGAATAdTdT-3'
NM_032726	Hs.549218	PLCD4	Phospholipase C, delta 4	5'-GAGCAGGGCTTCACCATTGdTdT-3'
NM_032726	Hs.549218	PLCD4	Phospholipase C, delta 4	5'-GGAAGGAGAAGAATTCGTAdTdT-3'
NM_032726	Hs.549218	PLCD4	Phospholipase C, delta 4	5'-GATATCATCTTTCTCTGAAdTdT-3'
NM_004104	Hs.83190	FASN	Fatty acid synthase	5'-CAACTACGGCTTTGCCAAT-3'
NM_004104	Hs.83190	FASN	Fatty acid synthase	5'-GCAACTCACGCTCCGGAAA-3'
NM_004104	Hs.83190	FASN	Fatty acid synthase	5'-GCCCTGAGCTGGACTACTT-3'
NM_004104	Hs.83190	FASN	Fatty acid synthase	5'-GGTATGCGACGGGAAAGTA-3'
NM_002165.2	Hs.504609	ID1	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	5'-AACTCGGAATCCGAAGTTGGA-3'
NM_003200.1	Hs.371282	TCF3	Transcription factor 3	5'-AAAGACCTGAGGGACCGGGAG-3'

NM_015895	Hs.234896	GMNN	Geminin, DNA replication inhibitor	5'-GAGAAAATGAGCTGTCCGC-3'
NM_015895	Hs.234896	GMNN	Geminin, DNA replication inhibitor	5'-CTGGCAGAAGTAGCAGAAC-3'
NM_006704	Hs.281902	SUGT1	SGT1, suppressor of G2 allele of SKP1 (S. cerevisiae)	5'-AAGGCUUUGGAACAGAAACCA-3'
NM_002358	Hs.533185	MAD2L1	MAD2 mitotic arrest deficient-like 1	5'-AAGAGUCGGGACCACAGUUUA-3'
NM_006472	Hs.533977	TXNIP	Thioredoxin interacting protein	5'-ACAGACUUCGGAGUACCUGdTT-3'
NM_001379	Hs.202672	DNMT1	DNA (cytosine-5-)-methyltransferase 1	5'-CGGUGCUCAUGCUUACAACTT-3'
NM_001379	Hs.202672	DNMT1	DNA (cytosine-5-)-methyltransferase 1	5'-CGAGUUGCUAGACCGCUUCTT-3'
NM_006838	Hs.444986	METAP2	Methionyl aminopeptidase 2	5'-AAUGCCGGUGACACAACAUGA-3'
NM_007862.2	Mm.382	Dlgh1	Discs, large homolog 1 (Drosophila)	5'-TACGGGAGCAGATGATGAAA-3'
NM_007862.2	Mm.382	Dlgh1	Discs, large homolog 1 (Drosophila)	5'-AACCCAAATCCATGGAAAATA-3'
NM_008173.1	Mm.129481	Nr3c1	Nuclear receptor subfamily 3, group C, member 1	5'-GAGCAGTGGAAGGACAGCATTCAA GAGATGCTGTCCTTCCACTGCTCTTTTTT-3'
NM_008173.1	Mm.129481	Nr3c1	Nuclear receptor subfamily 3, group C, member 1	5'-GATCCCGAGCAGTGGAAGGACAGCATTCAA GAGATGCTGTCCTTCCACTGCTCTTTTTTGGAAA-3'
NM_007871.1	Mm.39292	Dnm2	Dynamin 2	5'-GGACCAGGCAGAGAATGAG-3'
NM_011155.1	Mm.3294	Ppp5c	Protein phosphatase 5, catalytic subunit	5'-AAG ACA CAG GCC AAC GAC UAC-3'
NM_011155.1	Mm.3294	Ppp5c	Protein phosphatase 5, catalytic subunit	5'-AAG AUU GUG AAG CAG AAG GCC-3'
NM_009263.1	Mm.288474	Spp1	Secreted phosphoprotein 1	5'-AATCTCCTTGCGCCACAGAAT-3'

NM_009263.1	Mm.288474	Spp1	Secreted phosphoprotein 1	5'-AAGTCAGCTGGATGAACCAAG-3'
NM_145978.1	Mm.283968	Pdlim2	PDZ and LIM domain 2	5'-AAGAUCCGACAGAGCGCCUCA-3'
NM_146386.1	Mm.32257	Myocd	Myocardin	5'-AATGCAACTGCAGAAGCAGAA-3'
NM_145541.3	Mm.333868	Rap1a	RAS-related protein-1a	5'-AAGCAAGTCGAGGTAGATTGC-3'
NM_010026.1	Mm.277236	Ddef1	RIKEN cDNA 1700010G06 gene	5'-CAGCUAACUGCACUCCGAG-3'
NM_010026.1	Mm.277236	Ddef1	RIKEN cDNA 1700010G06 gene	5'-UGAUAUUAUGGAAGCAAAU-3'
NM_007614.2	Mm.291928	Catnb	Catenin beta	5'-AAGGCTTTTCCCAGTCCTTCA-3'
NM_007614.2	Mm.291928	Catnb	Catenin beta	5'-AAGATGATGGTGTGCCAAGTG-3'
NM_011952.1	Mm.8385	Mapk3	Mitogen activated protein kinase 3	5'-AATGTTATAGGCATCCGAGAC-3'
NM_011952.1	Mm.8385	Mapk3	Mitogen activated protein kinase 3	5'-AACCCAAACAAGCGCATCACA-3'
NM_011949.2	Mm.196581	Mapk1	Mitogen activated protein kinase 1	5'-AAAGTTCGAGTTGCTATCAAG-3'
NM_010591.1	Mm.275071	Jun	Jun oncogene	5'-GCGCATGAGGAACCGCATT-3'
NM_008416.1	Mm.1167	Junb	Jun-B oncogene	5'-GACCAGGAGCGCATCAAAG-3'
NM_010592.3	Mm.1175	Jund1	Jun proto-oncogene related gene d1	5'-AAGCCAGAACACCGAGCTG-3'
NM_010234.2	Mm.246513	Fos	FBJ osteosarcoma related oncogene	5'-GCGGAGACAGATCAACTTG-3'
NM_010235.1	Mm.6215	Fosl1	Fos-like antigen 1	5'-ATTGGAGGATGAGAAATCG-3'
NM_008037.3	Mm.24684	Fosl2	Fos-like antigen 2	5'-TCAACGCCATCACCACCAG-3'
NM_009505.2	Mm.282184	Vegfa	Vascular endothelial growth factor A	5'-AACGAUGAAGCCCUGGAGUGC-3'
NM_015774.2	Mm.264435	Ero1l	ERO1-like (S. cerevisiae)	5'-ACTTCATCAGAATGGCAGGGTTT-3'
NM_021450.1	Mm.244705	Trpm7	Transient receptor potential cation channel, subfamily M, member 7	5'-AACCGGAGGTCAGGTCGAAAT-3'
NM_021450.1	Mm.244705	Trpm7	Transient receptor potential	5'-AAGCAGAGTGACCTGGTAGAT-3

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			cation channel, subfamily M, member 7	
NM_177407.2	Mm.131530	Camk2a	Calcium/calmodulin-dependent protein kinase II alpha	5'-CACCACCAUUGAGGACGAAdTdT-3'
NM_010118.1	Mm.290421	Egr2	Early growth response 2	5'- GUGACCACCUUACUACUCAdTdT -3'
NM_010118.1	Mm.290421	Egr2	Early growth response 2	5'- GUUUGCCAGGAGUGACGAAdTdT -3'
NM_015806.2	Mm.18856	Mapk6	Mitogen-activated protein kinase 6	5'-GGCUUUUCAUGUAUCAGCUTT-3'
NM_015806.2	Mm.18856	Mapk6	Mitogen-activated protein kinase 6	5'- GGCAAUGGCUUGGUUUUUUTT -3'
NM_015806.2	Mm.18856	Mapk6	Mitogen-activated protein kinase 6	5'- GGAGUACAUGGAGACAGACTT -3'
NM_009744.2	Mm.347398	Bcl6	B-cell leukemia/lymphoma 6	5'-GTCGAGACATCTTGACTGA-3'
NM_009744.2	Mm.347398	Bcl6	B-cell leukemia/lymphoma 6	5'-GACACGGATCTGAGAATCT-3'
NM_145533.1	Mm.136586	Smox	Spermine oxidase	5'-GGACGUGGUUGAGGAAUUC-3'
NM_008778.1	Mm.40035	Pak3	P21 (CDKN1A)-activated kinase 3	5'-TAGCAGCACATCAGTCGAATA-3'
NM_008778.1	Mm.40035	Pak3	P21 (CDKN1A)-activated kinase 3	5'-CCCAATATTGTCAATTATTTA-3'
NM_080428.2	Mm.196475	Fbxw7	F-box and WD-40 domain protein 7, archipelago homolog	5'-CACAAAGCTGGTGTGTGCA-3'
NM_021450.1	Mm.244705	Trpm7	Transient receptor potential cation channel, subfamily M, member 7	5'-AACCGGAGGTCAGGTCGAAAT-3'
NM_021450.1	Mm.244705	Trpm7	Transient receptor potential cation channel, subfamily M, member 7	5'-AAGCAGAGTGACCTGGTAGAT-3'
NM_009505.2	Mm.282184	Vegfa	Vascular endothelial growth factor A	5'-AAGCCGTCCTGTGTGCCGCTG-3'
NM_009505.2	Mm.282184	Vegfa	Vascular endothelial growth	5'-AACGATGAAGCCCTGGAGTGC-3'

			factor A	
NM_010228.2	Mm.3464	Flt1	FMS-like tyrosine kinase 1	5'-AAGTTAAAAGTGCCTGAACTG-3'
NM_010228.2	Mm.3464	Flt1	FMS-like tyrosine kinase 1	5'-AAGCAGGCCAGACTCTCTTTC-3'
NM_010612.2	Mm.285	Kdr	Kinase insert domain protein receptor	5'-AAGCTCAGCACACAGAAAGAC-3'
NM_010612.2	Mm.285	Kdr	Kinase insert domain protein receptor	5'-AATGCGGCGGTGGTGACAGTA-3'
AK019429.1	Mm.3049	Cks1b	CDC28 protein kinase 1b	5'-GGGACATAGCCAAGCTGGTCgagtactg GACCAGCTTGGCTATGTCC-3'
NM_008000.1	Mm.280819	Fert2	Fer (fms/fps related) protein kinase, testis specific 2	5'-AAC TAC GGT TGC TGG AGA CAG-3'
NM_009750.1	Mm.90787	Ngfrap1	Nerve growth factor receptor (TNFRSF16) associated protein 1	5'-CAACAACCACAACCATAAC-3'
NM_009750.1	Mm.90787	Ngfrap1	Nerve growth factor receptor (TNFRSF16) associated protein 1	5'-CATAACCACAACCACCACTdTdT-3'
NM_010431.1	Mm.3879	Hif1a	Hypoxia inducible factor 1, alpha subunit	5'-TGTGAGCTCACATCTTGAT-3'
NM_010838	Mm.1287	Mapt	Microtubule-associated protein tau	5'-CCAGGAGTTTGACACAATG-3'
NM_009045	Mm.249966	Rela	V-rel reticuloendotheliosis viral oncogene homolog A	5'-GATCAATGGCTACACAGGA-3'
NM_009689	Mm.8552	Birc5	Baculoviral IAP repeat-containing 5	5'-GAGCCAAGAACAAAATTGC-3'
NM_009689	Mm.8552	Birc5	Baculoviral IAP repeat-containing 5	5'-GAAAGTGCGCCGTGCCATC-3'
NM_007798.1	Mm.236553	CTSB	Cathepsin B	5'-CCACUGUGGCAUUGAAUCATT-3'
NM_011960	Mm.15962	Parg	Poly (ADP-ribose) glycohydrolase	5'-AACGCCACCTCGTTTGTTTTC-3'

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NM_010928.1	Mm.254017	Notch2	Notch gene homolog 2	5'-GAUGUGGACAGUGUCUGUTT-3'
NM_019984	Mm.41964	Tgm1	Transglutaminase 1, K polypeptide	5'-AUGCAGCUGGAGAUGGCACdTdT-3'
NM_009593	Mm.15691	Abcg1	ATP-binding cassette, sub-family G (WHITE), member 1	5'-CGTGGATGAGGTTGAGACA-3'
NM_009593	Mm.15691	Abcg1	ATP-binding cassette, sub-family G (WHITE), member 1	5'-GGTGGACAACAACTTCACA-3'
NM_138955	Mm.101876	Abcg4	ATP-binding cassette, sub-family G (WHITE), member 4	5'-GAAGGTGGAGAACCATATC-3'
NM_138955	Mm.101876	Abcg4	ATP-binding cassette, sub-family G (WHITE), member 4	5'-GCACTTGAACTACTGGTAT-3'
NM_011658	Mm.3280	Twist1	Twist gene homolog 1	5'-AAGCTGAGCAAGATTCAGACC-3'
NM_011658	Mm.3280	Twist1	Twist gene homolog 1	5'-AGGTACATCGACTTCCTGTAC-3'
NM_011658	Mm.3280	Twist1	Twist gene homolog 1	5'-AGCGGGTCATGGCTAACGTGC-3'
NM_009537	Mm.3868	Yy1	YY1 transcription factor	5'-GGGAGCAGAAGCAGGUGCAGAU-3'
NM_172689	Mm.86382	Ddx58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	5'-GCCCATTGAAACCAAGAAATT-3'
NM_008500.1	Mm.12881	Lhx6	LIM homeobox protein 6	5'-AGACGCAGAGGCCTTGGTTCAAGAGAC CAAGGCCTCTGCGTCTGACTTTTTC-3'
NM_009308	Mm.233846	Syt4	Synaptotagmin 4	5'-r(GAAGCACAGAGUGAAGACCA)d(TT)-3'
NM_009308	Mm.233846	Syt4	Synaptotagmin 4	5'-r(CGAGCAGGAGA ACAGCGAG)d(T T)-3'
NM_007615.1	Mm.35738	Catns	Expressed sequence AI225934	5'-GATGGTTATCCAGGTGGCA-3'
NM_007614.2	Mm.291928	Catnb	Catenin beta	5'-CUGUUGUGGUUAAACUCCUTT-3'
NM_011232	Mm.38376	Rad1	RAD1 homolog (S. pombe)	5'-GCCTTGACAACGTTAGGAATC-3'
NM_011232	Mm.38376	Rad1	RAD1 homolog (S. pombe)	5'-GCAGGAAGTTCCCACCTTGAC-3'
NM_011232	Mm.38376	Rad1	RAD1 homolog (S. pombe)	5'-GCCTGATGAAGAAGTTCC-3'
NM_010423	Mm.29581	Hey1	Hairy/enhancer-of-split related with YRPW motif 1	5'-GCTAGAAAAAGCTGAGATC-3'
NM_010133	Mm.2657	En1	Engrailed 1	5'-CAUCCUAAGGCCCGAUUUCTT-3'
NM_010133	Mm.2657	En1	Engrailed 1	5'-GUUCCCGGAACACAACCCUTT-3'

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NM_019390	Mm.243014	Lmna	Lamin A	5'-GCAGCUUCAGGAUGAGAUGTT-3'
NM_183355.1	Mm.43358	Pbx1	Pre B-cell leukemia transcription factor 1	5'-CAGUUUUGAGUAUUCGGGGTT-3'
NM_007430	Mm.5180	Nr0b1	Nuclear receptor subfamily 0, group B, member 1	5'-GAUCACCUGCACUUCGAGdTdT-3'
NM_007430	Mm.5180	Nr0b1	Nuclear receptor subfamily 0, group B, member 1	5'-CUGAACAGUGCCCUUUUCCdTdT-3'
NM_172203	Mm.233865	Nox1	NADPH oxidase 1	5'-TTATGAGAAGTCTGACAAG-3'
NM_172203	Mm.233865	Nox1	NADPH oxidase 1	5'-GATTCTTGGCTAAATCCCA-3'
NM_172203	Mm.233865	Nox1	NADPH oxidase 1	5'-GGACATTTGAACAACAGCA-3'
NM_080850	Mm.253287	Pask	PAS domain containing serine/threonine kinase	5'-AATTTATGGAGTCAACCACAGCTT-3'
NM_009330	Mm.7226	Tcf2	Transcription factor 2	5'-GCCGGUUUUCCAUACUCUCtt-3'
NM_009330	Mm.7226	Tcf2	Transcription factor 2	5'-CAAGAAGAUGCGCC GCAACtt-3'
NM_009330	Mm.7226	Tcf2	Transcription factor 2	5'-UGGUGGUCACAGA UACCAGtt-3'
NM_024148.1	Rn.5949	Apex1	Apurinic/apyrimidinic endonuclease 1	5'-GUCUGGUAAGACUGGAGUACC-3'
NM_017059.1	Rn.10668	Bax	Bcl2-associated X protein	5'-UUGGAGAUGAACUGGACAAUU-3'
NM_017059.1	Rn.10668	Bax	Bcl2-associated X protein	5'-CUG GAC AAU AAU AUG GAG CUU-3'
NM_023979.1	Rn.64522	Apaf1	Apoptotic protease activating factor 1	5'-AGA ACU UUG UGC UUU AAU GUU-3'
NM_023979.1	Rn.64522	Apaf1	Apoptotic protease activating factor 1	5'-UAU AGG CAU AUA CUG GAU GUU-3'
NM_021835.2	Rn.93714	Jun	V-jun sarcoma virus 17 oncogene homolog (avian)	5'-AGU GAA AAC CUU GAA AGC GUU-3'
NM_021835.2	Rn.93714	Jun	V-jun sarcoma virus 17 oncogene homolog (avian)	5'-AGU CAU GAA CCA CGU UAA CUU-3'
NM_012655.1	Rn.44609	Sp1	Sp1 transcription factor	AATGAGAACAGCAACAACTCC
XM_230974.2	Rn.102138	Sp3	Sp3 transcription factor	AAGTTCTCAGACAATGACTGC
NM_133551.1	Rn.10162	Pla2g4a	Phospholipase A2, group IVA (cytosolic, calcium-dependent)	5'-TCGAGACAGTAGTGGTTCTACGTGCCgagtactg

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				GGCACGTAGAACCACTACTGTTTTTT-3'
NM_001003959.1	Rn.117353	Dnmt3b	DNA methyltransferase 3B	5'-AGAUGACAGGUGCCCAGAGUU-3'
NM_019335.1	Rn.10022	Prkr	Protein kinase, interferon-inducible double stranded RNA dependent	5-GGUAGAUCAAAGCAGGAGGTT-3'
NM_053622.1	Rn.10474	Pom121	Nuclear pore membrane glycoprotein 121 kD	5'-AACGGAGUCCCUGCUGCAUUUdTdT-3'
NM_053622.1	Rn.10474	Pom121	Nuclear pore membrane glycoprotein 121 kD	5'-AACCAUGUCACCAGUCCAGUUdTdT-3'
NM_053622.1	Rn.10474	Pom121	Nuclear pore membrane glycoprotein 121 kD	5'-AAGCCUGUGUUUGGCUUUGGAdTdT-3'
NM_131907.2	Rn.5805	Atp2c1	ATPase, Ca++-sequestering	5'- AACCATTATGGAAGAAGTACATT-3'
NM_031337.1	Rn.22706	Siat9	Sialyltransferase 9	5'-GGGUUAUUCUGAACAUGUUtt-3'
NM_031010.2	Rn.11318	Alox15	Arachidonate 12-lipoxygenase	5'-GCAACTGGATTTCTGTGAAGG-3'
NM_031010.2	Rn.11318	Alox15	Arachidonate 12-lipoxygenase	5'-GAAGCGGATTTCTTCCTTCTG-3'
NM_031556.1	Rn.22518	Cav	Caveolin	5'-AAGGAGATCGACCTGGTCAAC-3'
NM_031556.1	Rn.22518	Cav	Caveolin	5'-AAGGGACACACAGTTTTGACG-3'
XM_232106.2	Rn.117974	Dok-1	Docking protein 1 (predicted)	5'-GAACTACACAAATTCAGCCAGGCGTA TCATCCGGTGTTTCGTCCTTTCCACAAG-3'
NM_133307.1	Rn.98279	Prkcd	Protein kinase C, delta	5'- AAAAGGCAAATTCACAAACAGCCTGTCTC -3'
NM_133307.1	Rn.98279	Prkcd	Protein kinase C, delta	5'- AAGTTCTCCGAAGTGTGAGAACCTGTCTC -3'
NM_012637.1	Rn.11317	Ptpn1	Protein tyrosine phosphatase, non-receptor type 1	5'-AAGCTGACACTGATCTCTGAA-3'
NM_017212	Rn.2455	Mapt	Microtubule-associated protein tau	5'-CCAGGAGTTTGACACAATG-3'
NM_013156	Rn.1294	CTSL	Cathepsin L	5'-GGACAGAUGUUCCUUAAGATT-3'
NM_012576.1	Rn.90070	Nr3c1	Nuclear receptor subfamily 3, group C, member 1	5'-GGCCAAGGGAGGGGGAGCGTA-3'

NM_013131.1	Rn.9678	Nr3c2	Nuclear receptor subfamily 3, group C, member 2	5'-GGCGCTGGAGTCAAGTGTCTC-3'
NM_031659	Rn.10039	Tgm1	Transglutaminase 1	5'-AUGCAGCUGGAGAUGGCACdTdT-3'
NM_019275	Rn.9774	Madh4	MAD homolog 4	5'-AAUACACCGACAAGCAAUGACdTdT-3'
NM_031132	Rn.9954	Tgfbr2	Transforming growth factor, beta receptor II	5'-AAAGUCGGUUAACAGCGAUCUdTdT-3'
NM_144741	Rn.16746	Retn	Resistin	5'-CCTTTCATTTCTCCTCCTT-3'
NM_144741	Rn.16746	Retn	Resistin	5'-AGCTGCTCCTGTGGCTCTG-3'
NM_144741	Rn.16746	Retn	Resistin	5'-GCCTCCTGCCCAGAAGGCA-3'
NM_012614	Rn.9714	Npy	Neuropeptide Y	5'-UGAGAGAAAGCACAGAAA-3'
NM_021597	Rn.35512	Eif2c2	GERp95	5'-UGGACAUCCCCAAAAUUGA-3'
NM_012547	Rn.87299	Drd2	Dopamine receptor 2	5'-CCCCAUCAUCUACACCACA-3'

TABLE 1

Oligonucleotide Stimulation of Mouse B Cells

	ODN Production	Sequence (5' to 3')*	Stimulation Index' ^{3}H Uridine	IgM
1	(SEQ ID NO:13)	GCTAGACGTTAGCGT	6.1 ± 0.8	17.9 ± 3.6
1a	(SEQ. ID NO:4)	......T.....	1.2 ± 0.2	1.7 ± 0.5
1b	(SEQ ID NO:14)	......Z.....	1.2 ± 0.1	1.8 ± 0.0
1c	(SEQ ID NO:15)	.....Z.....	10.3 ± 4.4	9.5 ± 1.8
1d	(SEQ ID NO:16)	..AT.....GAGC.	13.0 ± 2.3	18.3 ± 7.5
2	(SEQ ID NO:17)	ATGGAAGGTCCAGCGTTCTC	2.9 ± 0.2	13.6 ± 2.9
2a	(SEQ ID NO:18)	..C..CTC..G.....	7.7 ± 0.8	24.2 ± 3.2
2b	(SEQ ID NO:19)	..Z..CTC.ZG..Z.....	1.6 ± 0.8	3.8 ± 2.2
2c	(SEQ ID NO:20)	..Z..CTC..G.....	3.1 ± 0.6	7.3 ± 1.4
2d	(SEQ ID NO:21)	..C..CTC..G.....Z..	7.4 ± 1.4	27.7 ± 5.4
2e	(SEQ ID NO:22)	.....A.....	5.6 ± 2.8	ND
3D	(SEQ ID NO:23)	GAGAACGCTGGACCTTCCAT	4.9 ± 0.5	19.9 ± 3.6
3Da	(SEQ ID NO:24)	.....C.....	6.6 ± 1.5	33.9 ± 6.8
3Db	(SEQ ID NO:25)	.....C.....G..	10.1 ± 2.8	25.4 ± 0.8
3Dc	(SEQ ID NO:26)	...C.A.....	1.0 ± 0.1	1.2 ± 0.5
3Dd	(SEQ ID NO:27)	.....Z.....	1.2 ± 0.2	1.0 ± 0.4
3De	(SEQ ID NO:28)	.....Z.....	4.4 ± 1.2	18.8 ± 4.4
3Df	(SEQ ID NO:29)	.....A.....	1.6 ± 0.1	7.7 ± 0.4
3Dg	(SEQ ID NO:30)	.....CC.G.ACTG..	6.1 ± 1.5	18.6 ± 1.5
3M	(SEQ ID NO:31)	TCCATGTCGGTCCTGATGCT	4.1 ± 0.2	23.2 ± 4.9
3Ma	(SEQ ID NO:32)	......CT.....	0.9 ± 0.1	1.8 ± 0.5
3Mb	(SEQ ID NO:33)	.....Z.....	1.3 ± 0.3	1.5 ± 0.6
3Mc	(SEQ ID NO:34)	.....Z.....	5.4 ± 1.5	8.5 ± 2.6
3Md	(SEQ ID NO:35)	.....A..T.....	17.2 ± 9.4	ND
3Me	(SEQ ID NO:36)	.....C..A.	3.6 ± 0.2	14.2 ± 5.2
4		TCAACGTT	6.1 ± 1.4	19.2 ± 5.2
4a		....GC..	1.1 ± 0.2	1.5 ± 1.1
4b		...GCGC.	4.5 ± 0.2	9.6 ± 3.4
4c		...TCGA.	2.7 ± 1.0	ND

TABLE 1-continued

Oligonucleotide Stimulation of Mouse B Cells

ODN Production	Sequence (5' to 3')†	Stimulation Index' ^{3}H Uridine	IgM
4d	..TT$_{dd}$AA	1.3 ± 0.2	ND
4e	-..-..-	1.3 ± 0.2	1.1 ± 0.5
4f	C..-..-	3.9 ± 1.4	ND
4g	--..-..CT	1.4 ± 0.3	ND
4h	....-..C	1.2 ± 0.2	ND
LPS		7.8 ± 2.8	4.2 ± 1.0

'Stimulation indexes are the means and std. dev. derived from at least 3 separate experiments, and are compared to wells cultured with no added ODN.
ND = not done.
CpG dinucleotides are underlined.
Dots indicate identity; dashes indicate deletions.
$ indicates 5 methyl cytosine.

TABLE 2

Identification of the optimal CpG motif for Murine IL-6 production and B cell activation.

ODN (ng/ml)[a]	SEQUENCE (5'-3')	IL-6 (pg/ml)[a] CH12.LX	SPLENIC B CELL	SI[b]	IgM
512	(SEQ ID No:37) TCCATGTCGGTTCCTGATGCT	1300 ± 106	627 ± 43	5.8 ± 0.3	7315 ± 1324
1637	(SEQ ID No:38) .....C$_{dd}$.....	136 ± 27	46 ± 6	1.7 ± 0.2	770 ± 72
1615	(SEQ ID No:39) .....C$_{dd}$.....	1201 ± 155	850 ± 302	3.7 ±.0.3	3032 ± 617
1614	(SEQ ID No:40) .....A$_{dd}$.....	1533 ± 321	1812 ± 103	10.8 ± 0.6	7558 ± 414
1636	(SEQ ID No:41) .....$_{dd}$A.....	1181 ± 96	947 ± 132	5.4 ± 0.4	3983 ± 485
1634	(SEQ ID No:42) .....$_{dd}$C.....	1049 ± 223	1671 ± 175	9.2 ± 0.9	6256 ± 261
1619	(SEQ ID No:43) .....$_{dd}$T.....	1535 ± 304	2908 ± 129	12.5 ± 1.8	8243 ± 698
1618	(SEQ ID No:44) .....A$_{dd}$T.....	2109 ± 291	2596 ± 166	12.9 ± 0.7	10425 ± 674
1639	(SEQ ID No:45) .....AA$_{dd}$T.....	1827 ± 83	2012 ± 132	11.5 ± 0.4	9489 ± 103
1707	(SEQ ID No:46) .....A$_{dd}$TC.....	ND	1147 ± 175	4.0 ± 0.2	3534 ± 217
1708	(SEQ ID No:47) .....CA$_{dd}$TG.....	ND	59 ± 3	1.5 ± 0.1	466 ± 109

Dots indicate identity; CpG dinucleotides are underlined; ND = not done
[a]The experiment was done at least three times with similar results. The level of IL-6 of unstimulated control cultures of both CH12.LX and splenic B cells was ≤ 10 pg/ml. The IgM level of unstimulated culture was 547 ± 82 ng/ml. CpG dinucleotides are underlined and dots indicate identity.
[b]^{3}H] Uridine uptake was indicated as a fold increase (SI: stimulation index) from unstimulated control (2322.67 ± 213.68 cpm). Cells were stimulated with 20 μM of various CpG O-ODN. Data presents the mean ± SD of triplicates
[c]Measured by ELISA.

TABLE 3

Induction of Murine IL-6 secretion by CpG motifs in bacterial DNA or oligonucleotides.

Treatment		IL-6 (pg/ml)
calf thymus DNA		≤10
calf thymus DNA + DNase		≤10
E. coli DNA		1169.5 ± 94.1
E. coli DNA + DNase		≤10
CpG methylated E. coli DNA		≤10
LPS		280.1 ± 17.1
Media (no DNA)		≤10
ODN 5a SEQ. ID. No:1	ATGGACTCTCCAGCGTTCTC	1096.4 ± 372.0
5b SEQ. ID. No:2	.....AGG....A_....	1124.5 ± 126.2
5c SEQ. ID. No:3	..C_.....G_.._.....	1783.0 ± 189.5
5d SEQ. ID. No:4	.... AGG..C..T.....	≤10
5e SEQ. ID. No:5	..C_.....G..Z.....	851.1 ± 114.4
5f SEQ. ID. No:6	..Z.....ZG..Z.....	≤10
5g SEQ. ID. No:7	..C_.....G_...Z..	1862.3 ± 87.26

TABLE 5

Induction of human PBMC cytokine secretion by CpG oligos

ODN	Sequence (5'-3')	IL-6[1]	TNF-α[1]	IFN-γ[1]	GM-CSF	IL-12
512 SEQ ID NO:37	TCCATGTCGGTCCTGATGCT	500	140	15.6	70	250
1637 SEQ ID NO:38	......C..............	550	16	7.8	15.6	35
1615 SEQ ID NO:39	......G..............	600	145	7.8	45	250
1614	......A..............	550	31	0	50	250

TABLE 5-continued

Induction of human PBMC cytokine secretion by CpG oligos

ODN	Sequence (5'-3')	IL-6[1]	TNF-α[1]	IFN-γ[1]	GM-CSF	IL-12
SEQ ID NO:40						
1636 SEQ ID NO:41	.........A..........	325	250	35	40	0
1634 SEQ ID NO:42	.........C..........	300	400	40	85	200
1619 SEQ ID NO:43	.........T..........	275	450	200	80	>500
1618 SEQ ID NO:44	......A..T..........	300	60	15.6	15.6	62
1639 SEQ ID NO:45	.....AA..T..........	625	220	15.6	40	60
1707 SEQ ID NO:46	......A..TC.........	300	70	17	0	0
1708 SEQ ID NO:47	.....CA..TG.........	270	10	17	0	0

TABLE 9

Induction of NK Activity by DNA Containing CpG Motifs but not by Non-CpG DNA

	DNA or Cytokine Added		LU/10^6 Mouse Cells	LU/10^6 Human cells
Expt. 1	None		0.00	0.00
	IL-2		16.68	15.82
	E.Coli. DNA		7.23	5.05
	Calf thymus DNA		0.00	0.00
Expt. 2	None		0.00	3.28
	1585 ggGGGGTCAACGTTGAGGGGGGgggg	(SEQ ID No.12)	7.38	17.98
	1629 ----------gto----------	(SEQ ID No.59)	0.00	4.4
Expt. 3	None		0.00	
	1613 GCTAGACGTTAGTGT	(SEQ ID No.51)	5.22	
	1769 --------8------	(SEQ ID No.52)	0.02	ND
	1619 TCCATGTCGTTCCTGATGCT	(SEQ ID No.15)	3.35	
	1765 -------8---------	(SEQ ID No.53)	0.11	

TABLE 10

Different CpG motifs stimulate optimal murine B cell and NK activation

ODN	Sequence		B cell activation[1]	NK activation[2]
1668	TCCATGACGTTCCTGATGCT	(SEQ.ID.NO:54)	42,649	2.52
1758	TCTCCCAGCGTGCGCCAT	(SEQ.ID.NO.55)	1,747	6.66
NONE			367	0.00

CpG dinucleotides are underlined; oligonucleotides were synthesized with phosphorothioate modified backbones to improve their nuclease resistance.
[1]Measured by ^{3}H thymidine incorporation after 48 hr culture with oligodeoxynucleotides at a 200 nM concentration as described in Example 1.
[2]Measured in lytic units.

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TABLE 1

ODN Number	cells/ml x 10^4	PMN/ml x 10^5	% PMN
	expt 1, C3H/BFeJ mice		
1908	61.4 ± 35.9	59.2 ± 35.7	95.8 ± 0.95
1760	27.8 ± 3.5*	25.8 ± 3.0*	93.3 ± 2.3
1631	47.6 ± 31.1	46.1 ± 30.7	96.8 ± 1.11
1835	43.8 ± 7.1	44.4 ± 7.1	96.8 ± 0.75
1759	71.0 ± 19.8	67.7 ± 20.4	96.8 ± 2.6
1826	39.8 ± 7.8	38.3 ± 7.9	93.5 ± 1.4
None (saline)	71.0 ± 7.4	69.3 ± 6.9	97.8 ± 1.3
	expt 2, C57 Bl/6 mice		
1908	18.0 ± 2.6	16.6 ± 2.7	91.2 ± 3.7
1760	10.2 ± 2.3*	8.6 ± 2.1*	82.0 ± 3.0
1585	11.0 ± 2.2*	9.5 ± 2.2*	84.6 ± 2.9
2010	14.3 ± 2.1	11.8 ± 1.9	83.4 ± 2.1
None (saline)	17.9 ± 3.4	16.9 ± 2.1	
	expt 3		
1908	19.0 ± 2.5	16.9 ± 2.1	89.4 ± 1.4
1760	9.1 ± 0.8*	7.7 ± 0.7*	84.6 ± 0.5
1972	15.3 ± 1.6	13.5 ± 1.4	84.2 ± 1.2
2001	13.0 ± 1.6*	11.8 ± 1.6	90.4 ± 2.2

*P>0.05, Mann-Whitney U test

TABLE 2

Induction Of NK Activity By CpG Oligodeoxynucleotides (ODN)

	% YAC-1 Specific Lysis* Effector: Target		% 2C11 Specific Lysis Effector: Target	
ODN	50:1	100:1	50:1	100:1
None	−1.1	−1.4	15.3	16.6
1	16.1	24.5	38.7	47.2
3Dd	17.1	27.0	37.0	40.0
non-CpG ODN	−1.6	−1.7	14.8	15.4

TABLE 3

Induction of NK Activity by DNA Containing CpG Motifs but not by Non-CpG DNA

	DNA or Cytokine Added to Human Cells		LU/10^6 Cells: Mouse	LU/10^6 Cells: Human
Expt. 1	None		0.00	0.00
	IL-2		16.68	15.82
	E. Coli DNA		7.23	5.05
	Calf thymus DNA		0.00	0.00
Expt. 2	None		0.00	3.28
	1585 ggGGTCAACGTTGACgggg	(SEQ ID NO:21)	7.38	17.98
	1629 ---------gtc---------	(SEQ ID NO:22)	0.00	4.4
Expt. 3	None		0.00	
	1613 GCTAGACGTTAGTGT	(SEQ ID NO:23)	5.22	
	1769 --------X------	(SEQ ID NO:24)	0.02	ND
	1619 TCCATGTCGTTCCTGATGCT	(SEQ ID NO:5)	3.35	
	1765 ---------X----------	(SEQ ID NO:25)	0.11	

CpG dinucleotides in ODN sequences are indicated by underlining; X indicates methylcytosine. Lower case letters indicate nuclease resistant phosphorothioate modified internucleotide linkages which, in titration experiments, were more than 20 times as potent as non-modified ODN, depending on the flanking bases. Poly G ends (g) were used in some ODN, because they significantly increase the level of ODN uptake. Dashes indicate some bases are identical to those in the directly preceding sequence, with the exception of changes noted.

TABLE 4

ODN induction of NK Lytic Activity (LU)

ODN	Sequence (5'–3')		LU
None			0.01
1754	ACCATGGACGATCTGTTTCCCCTC	(SEQ ID NO:26)	0.02
1759	TCTCCCAGCGTGCGCCAT	(SEQ ID NO:27)	0.05
1761	TACCGCGTGCGACCCTCT	(SEQ ID NO:28)	0.05
1776	ACCATGGACGAACTGTTTCCCCTC	(SEQ ID NO:29)	0.03
1777	ACCATGGACGAGCTGTTTCCCCTC	(SEQ ID NO:30)	0.05
1778	ACCATGCACGACCTGTTTCCCCTC	(SEQ ID NO:31)	0.01
1779	ACCATGGACGTACTGTTTCCCCTC	(SEQ ID NO:32)	0.02
1780	ACCATGGACGGTCTGTTTCCCCTC	(SEQ ID NO:33)	0.29
1781	ACCATGGACGTTCTGTTTCCCCTC	(SEQ ID NO:34)	0.38
1823	GCATGACGTTGAGCT	(SEQ ID NO:35)	0.08
1824	CACGTTGAGGGGCAT	(SEQ ID NO:36)	0.01
1825	CTGCTGAGACTGGAG	(SEQ ID NO:37)	0.01
1828	TCAGCGTGCGCC	(SEQ ID NO:38)	0.01
1829	ATGACGTTCCTGACGTT	(SEQ ID NO:39)	0.42
1830[2]	RANDOM SEQUENCE		0.25
1834	TCTCCCAGCGGGCGCAT	(SEQ ID NO:40)	0.00
1835	TCTCCCAGCGCGCGCCAT	(SEQ ID NO:41)	0.46
1840	TCCATGTCGTTCCTGTCGTT	(SEQ ID NO:42)	2.70
1841	TCCATAGCGTTCCTAGCGTT	(SEQ ID NO:43)	1.45
1842	TCGTCGCTGTCTCCGCTTCTT	(SEQ ID NO:44)	0.06
1851	TCCTGACGTTCCTGACGTT	(SEQ ID NO:45)	2.32

[1]Lytic units (LU) were measured as described (8). Briefly, PBMC were collected from normal donors and spun over Ficoll, then cultured with or without the indicated ODN (which were added to cultures at 6 μg/ml) for 24 hr. Then their ability to lyse ^{51}Cr-labeled K562 cells was determined. The results shown are typical of those obtained with several different normal human donors.

[2]This oligo mixture contained a random selection of all 4 bases at each position.

TABLE 5

Induction of NK LU by Phosphorothioate CpG ODN with Good Motifs

ODN[1]	sequence (5'–3')		expt. 1	expt. 2	expt. 3
None			0.00	1.26	0.46
1840	TCCATGTCGTTCCTGTCGTT	(SEQ ID NO:42)	2.33	ND	ND
1960	TCCTGTCGTTCCTGTCGTT	(SEQ ID NO:46)	ND	0.48	8.99
1961	TCCATGTCGTTTTTTGTCGTT	(SEQ ID NO:47)	4.03	1.23	5.08
1962	TCCTGTCGTTCCTTGTCGTT	(SEQ ID NO:13)	ND	1.60	5.74
1963	TCCTTGTCGTTCCTGTCGTT	(SEQ ID NO:48)	3.42	ND	ND
1965	TCCTGTCGTTTTTTGTCGTT	(SEQ ID NO:14)	0.46	0.42	3.48
1966	TCGTCGCTGTCTCCGCTTCTT	(SEQ ID NO:44)	2.62	ND	ND
1967	TCGTCGCTGTCTGCCCTTCTT	(SEQ ID NO:15)	5.82	1.64	8.32
1968	TCGTCGCTGTTGTCGTTTCTT	(SEQ ID NO:16)	3.77	5.26	6.12
1979[2]	TCCATGT&GTTCCTGT%GTT	(SEQ ID NO:42)	1.32	ND	ND
1982	TCCAGGACTTCTCTCAGGTT	(SEQ ID NO:50)	0.05	ND	0.90
1990	TCCATGCGTGCGTGCGTTTT	(SEQ ID NO:51)	2.10	ND	ND
1991	TCCATGCGTTGCGTTGCGTT	(SEQ ID NO:52)	0.89	ND	ND
2002	TCCACGACGTTTTCGACGTT	(SEQ ID NO:53)	4.02	1.31	9.79
2005	TCGTCGTTGTCGTTGTCGTT	(SEQ ID NO:7)	ND	4.22	12.75
2006	TCGTCGTTTTGTCGTTTTGTCGTT	(SEQ ID NO:6)	ND	6.17	12.82
2007	TCGTCGTTGTCGTTTTGTCGTT	(SEQ ID NO:8)	ND	2.68	9.66
2008	GCGTGCGTTGTCGTTGTCGTT	(SEQ ID NO:9)	ND	1.37	8.15
2010	GCGGCGGGCGGGGCGCGCCC	(SEQ ID NO:54)	ND	0.01	0.05
2012	TGTCGTTTGTCGTTTGTCGTT	(SEQ ID NO:10)	ND	2.02	11.61
2013	TGTCGTTGTCGTTGTCGTTGTCGTT	(SEQ ID NO:55)	ND	0.56	5.22
2014	TGTCGTTGTCGTTGTCGTT	(SEQ ID NO:11)	ND	5.74	10.89
2018	TCGTCGTCGTTCGTT	(SEQ ID NO:12)	ND	4.53	10.13
2016	TGTCGTTGTCGTT	(SEQ ID NO:56)	ND	6.54	8.06

[1]PBMC essentially as described herein. Results are representative of 6 separate experiments; each experiment represents a different donor.
[2]This is the methylated version of ODN 1840; % = 5-methyl cytosine LU is lytic units; ND = not done; CpG dinucleotides are underlined for clarity.

TABLE 6

Induction of human B cell proliferation by Phosphorothioate CpG ODN

DR	sequence (5'-3')		Stimulation Index[1] expt. 1	expt. 2	expt. 3	expt. 4	expt. 5	expt. 6
1840	TCCATGTCGTTCCTGTCGTT	(SEQ ID NO:42)	4	ND	ND	ND	ND	34
1841	TCCATAGCGTTCCTAGCGTT	(SEQ ID NO:43)	3	ND	ND	ND	ND	ND
1960	TCCTGTCGTTCCTGTCGTT	(SEQ ID NO:46)	ND	2.0	2.8	3.6	ND	ND
1961	TCCATGTCGTTTTTGTCGTT	(SEQ ID NO:47)	2	3.9	1.9	3.7	ND	37
1962	TCCTGTCGTTCCTTGTCGTT	(SEQ ID NO:13)	ND	3.8	1.9	3.9	5.4	35
1963	TCCTGTCGTTCCTGTCGTT	(SEQ ID NO:48)	3	ND	ND	ND	ND	ND
1965	TCCTGTCGTTTTTTGTCGTT	(SEQ ID NO:14)	4	3.7	2.4	4.7	6.0	43
1967	TCGTCGCTGTCTGCCCTTCTT	(SEQ ID NO:15)	ND	4.4	2.0	4.5	5.0	36
1968	TCGTCGCTGTTGTCGTTTCTT	(SEQ ID NO:16)	ND	4.0	2.6	4.9	6.7	38
1982	TCCAGGACTTCTCTCAGGTT	(SEQ ID NO:50)	3	1.8	1.3	3.1	3.2	12
2052	TCCAGGACGTTTTCGAGGTT	(SEQ ID NO:53)	ND	2.7	1.4	4.4	ND	14
2005	TCGTCGTTGTCGTTGTCGTT	(SEQ ID NO:7)	5	3.2	1.2	3.0	7.9	37
2006	TCGTCGTTTTGTCGTTTTGTCGTT	(SEQ ID NO:6)	4	4.5	2.2	5.8	8.3	43
2007	TCGTCGTTGTCGTTTTGTCGTT	(SEQ ID NO:8)	3	4.8	4.3	4.1	ND	22
2008	GCGTGCGTTGTCGTTGTCGTT	(SEQ ID NO:9)	ND	3.0	2.4	1.6	ND	12
2010	GCGGCGGGCGGCGCCGCCC	(SEQ ID NO:54)	ND	1.6	1.9	3.2	ND	ND
2012	TGTCGTTTGTCGTTTGTCGTT	(SEQ ID NO:10)	2	2.8	0	3.2	ND	33
2013	TGTCGTTGTCGTTGTCGTTGTCGTT	(SEQ ID NO:55)	3	2.3	3.1	2.8	ND	7
2014	TGTCGTTGTCGTTGTCGTT	(SEQ ID NO:11)	3	2.5	4.0	3.2	6.7	14
2015	TCGTCGTCGTCGTT	(SEQ ID NO:12)	5	1.8	2.6	4.5	9.4	1
2016	TGTCGTTGTCGTT	(SEQ ID NO:56)	ND	1.1	1.7	2.7	7.3	1

[1]Cells = human spleen cells stored at -70° C. after surgical harvest or PBMC collected from normal donors and spun over Ficoll. Cells were cultured in 96 well U-bottom microtiter plates with or without the indicated ODN (which were added to cultures at 6 μg/ml). N = 12 experiments. Cells were cultured for 4-7 days, pulsed with 1 μCi of ^{3}H thymidine for 16 hr before harvest and scintillation counting. Stimulation index = the ratio of cpm in wells without ODN to that in wells that had been stimulated throughout the culture period with the indicated ODN (there were no further additions of ODN after the cultures were set up). ND = not done

TABLE 7

Induction of human IL-12 secretion by Phosphorothioate CpG ODN

ODN	sequence (5'-3')		IL-12 (pg/ml) expt. 1	expt. 2
None			0	0
1962	TCCTGTCGTTCCTTGTCGTT	(SEQ ID NO:13)	19	0
1965	TCCTGTCGTTTTTTGTCGTT	(SEQ ID NO:14)	36	0
1967	TCGTCGCTGTCTGCCCTTCTT	(SEQ ID NO:15)	41	0
1968	TCGTCGCTGTTGTCGTTTCTT	(SEQ ID NO:16)	24	0
2006	TCGTCGTTGTCGTTGTCGTT	(SEQ ID NO:7)	25	0
2006	TCGTCGTTTTGTCGTTTTGTCGTT	(SEQ ID NO:6)	29	15
2014	TGTCGTTGTCGTTGTCGTT	(SEQ ID NO:11)	28	0
2015	TCGTCGTCGTCGTT	(SEQ ID NO:12)	14	0
2016	TGTCGTTGTCGTT	(SEQ ID NO:56)	3	0

[1]PBMC were collected from normal donors and spun over Ficoll, then cultured at 10^6 cells/well in 96 well microtiter plates with or without the indicated ODN which were added to cultures at 6 μg/ml. Supernatants were collected at 24 hr and tested for IL-12 levels by ELISA as described in methods. A standard curve was run in each experiment, which represents a different donor.

TABLE 8

Different CpG motifs stimulate optimal murine B cell and NK activation

ODN	Sequence		B cell activation[1]	NK activation[2]
1668	TCCATGACGTTCCTGATGCT	(SEQ ID NO:56)	42,849	2.52
1758	TCTCCCAGCGTGCGCCAT	(SEQ ID NO:27)	1,747	6.66
NONE			367	0.00

CpG dinucleotides are underlined; oligonucleotides were synthesized with phosphorothioate modified backbones to improve their nuclease resistance.
[1]Measured by ^{3}H thymidine incorporation after 48 hr culture with oligodeoxynucleotides at a 200 nM concentration as described in Example 1.
[2]Measured in lytic units.

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TABLE 1

sequences	
GCTAGACGTTAGCGT	(SEQ ID NO: 1)
GCTAGATGTTAGCGT	(SEQ ID NO: 2)
GCTAGAZGTTAGCGT	(SEQ ID NO: 3)
GCTAGACGTTAGZGT	(SEQ ID NO: 4)
GCATGACGTTGAGCT	(SEQ ID NO: 5)
ATGGAAGGTCCAGCGTTCTC	(SEQ ID NO: 6)
ATCGACTCTCGAGCGTTCTC	(SEQ ID NO: 7)
ATZGACTCTZGAGZGTTCTC	(SEQ ID NO: 8)
ATZGACTCTCGAGCGTTCTC	(SEQ ID NO: 9)
ATCGACTCTCGAGCGTTZTC	(SEQ ID NO: 10)
ATCGACTCTCGAACGTTCTC	(SEQ ID NO: 11)
GAGAACGCTGGACCTTCCAT	(SEQ ID NO: 12)
GAGAACGCTCGACCTTCCAT	(SEQ ID NO: 13)
GAGAACGCTCGACCTTCGAT	(SEQ ID NO: 14)
GAGCAAGCTGGACCTTCCAT	(SEQ ID NO: 15)
GAGCAZGCTGGACCTTCCAT	(SEQ ID NO: 16)
GAGAACGCTGGACZTTCCAT	(SEQ ID NO: 17)
GAGAACGATGGACCTTCCAT	(SEQ ID NO: 18)

TABLE 1-continued

sequences	
GAGAACGCTCCAGCACTGAT	(SEQ ID NO: 19)
CCATGTCGGTCCTGATGCT	(SEQ ID NO: 20)
TCCATGCTCGTCCTGATGCT	(SEQ ID NO: 21)
TCCATGT2GGTCCTGATGCT	(SEQ ID NO: 22)
TCCATGTCGGTZCTGATGCT	(SEQ ID NO: 23)
TCCATGACGTTCCTGATGCT	(SEQ ID NO: 24)
TCCATGTCGGTCCTGACGCA	(SEQ ID NO: 25)
TCAACGTT	(SEQ ID NO: 26)
TCAAGCTT	(SEQ ID NO: 27)
TCAGCGCT	(SEQ ID NO: 28)
TCTTCGAT	(SEQ ID NO: 29)
TCTTCGAA	(SEQ ID NO: 30)
CAACGTT	(SEQ ID NO: 31)
CCAACGTT	(SEQ ID NO: 32)
CAACGTTCT	(SEQ ID NO: 33)
TCAACGTC	(SEQ ID NO: 34)
ATGGACTCTCCAGCGTTCTC	(SEQ ID NO: 35)
ATAGGAGGTCCAACGTTCTC	(SEQ ID NO: 36)
ATCGACTCTCGAGCGTTCTC	(SEQ ID NO: 37)
ATGGAGGCTCCATCGTTCTC	(SEQ ID NO: 38)
AT2GGACTCTCGAG2GTTCTC	(SEQ ID NO: 39)
ATNNGACTCTCGAGNGTTCTC	(SEQ ID NO: 40)
GCATGACGTTGAGCT3'	(SEQ ID NO: 41)
TCCATGTCGGTCCTGATGCT	SEQ ID NO: 42
TCCATGCCGGTCCTGATGCT	SEQ ID NO: 43
TCCATGGCGGTCCTGATGCT	SEQ ID NO: 44
TCCATGACGGTCCTGATGCT	SEQ ID NO: 45
TCCATGTCGATCCTGATGCT	SEQ ID NO: 46
TCCATGTCGCTCCTGATGCT	SEQ ID NO: 47
TCCATGTCGTTCCTGATGCT	SEQ ID NO: 48
TCCATAACGTTCCTGATGCT	SEQ ID NO: 49
TCCATGACGTCGCTGATGCT	SEQ ID NO: 50
TCCATCACNTGCCTGATGCT	SEQ ID NO: 51
GGGGTCAACGTTGACGGGG	(SEQ ID NO: 52)
GGGGTCAGTCGTGACGGGG	(SEQ ID NO: 53)
GCTAGAACGTTAGTGT	(SEQ ID NO: 54)
GCTAGAAGTTAGTGT	(SEQ ID NO: 55)
TCCATGTCGTTCCTGATGCT	(SEQ ID NO: 56)

TABLE 1-continued

sequences	
TCCATGTZGTTCCTGATGCT	(SEQ ID NO: 57)
ACCATGGACGATCTGTTTCCCCTC	(SEQ ID NO: 58)
TCTCCCAGCGTGCGCCAT	(SEQ ID NO: 59)
TACCGCGTGCGACCCTCT	(SEQ ID NO: 60)
ACCATGGACGAACTGTTTCCCCTC	(SEQ ID NO: 61)
ACCATGGACGAGCTGTTTCCCCTC	(SEQ ID NO: 62)
ACCATGGACGACCTGTTTCCCCTC	(SEQ ID NO: 63)
ACCATGGACGTACTGTTTCCCCTC	(SEQ ID NO: 64)
ACCATGGACGGTCTGTTTCCCCTC	(SEQ ID NO: 65)
ACCATGGACGTTCTGTTTCCCCTC	(SEQ ID NO: 66)
CACGTTGAGGGGCAT	(SEQ ID NO: 67)
CTGCTGAGACTGGAG	(SEQ ID NO: 68)
TCAGCGTGCGCC	(SEQ ID NO: 69)
ATGACGTTCCTGACGTT	(SEQ ID NO: 70)
TCTCCCAGCGGGCGCAT	(SEQ ID NO: 71)
TCTCCCAGCGCGCGCCAT	(SEQ ID NO: 72)
TCCATGTCGTTCCTGTCGTT	(SEQ ID NO: 73)
TCCATAGCGTTCCTAGCGTT	(SEQ ID NO: 74)
TCGTCGCTGTCTCCGCTTCTT	(SEQ ID NO: 75)
TCCTGACGTTCCTGACGTT	(SEQ ID NO: 76)
TCCTGTCGTTCCTGTCGTT	(SEQ ID NO: 77)
TCCATGTCGTTTTTGTCGTT	(SEQ ID NO: 78)
TCCTGTCGTTTCCTTGTCGTT	(SEQ ID NO: 79)
TCGTTGTCGTTCCTGTCGTT	(SEQ ID NO: 80)
TCCTGTCGTTTTTTGTCGTT	(SEQ ID NO: 81)
TCGTCGCTGTCTGCCCTTCTT	(SEQ ID NO: 82)
TCGTCGCTGTTGTCGTTTCTT	(SEQ ID NO: 83)
TCCATGTZGTTCCTGTZGTT	(SEQ ID NO: 84)
TCCAGGACTTCTCTCAGGTT	(SEQ ID NO: 85)
TCCATGCGTGCGTGCGTTTT	(SEQ ID NO: 86)

TABLE 1-continued

sequences	
TCCATGCGTTGCGTTGCGTT	(SEQ ID NO: 87)
TCCACGACGTTTTCGACGTT	(SEQ ID NO: 88)
TCGTCGTTGTCGTTGTCGTT	(SEQ ID NO: 89)
TCGTCGTTTTGTCGTTTTGTCGTT	(SEQ ID NO: 90)
TCGTCGTTGTCGTTTTGTCGTT	(SEQ ID NO: 91)
GCGTGCGTTGTCGTTGTCGTT	(SEQ ID NO: 92)
GCGGCGGGCGGCGCGCGCCC	(SEQ ID NO: 93)
TGTCGTTTGTCGTTTGTCGTT	(SEQ ID NO: 94)
TGTCGTTGTCGTTGTCGTTGTCGTT	(SEQ ID NO: 95)
TGTCGTTGTCGTTGTCGTT	(SEQ ID NO: 96)
TCGTCGTCGTCGTT	(SEQ ID NO: 97)
TGTCGTTGTCGTT	(SEQ ID NO: 98)
TCCATAGCGTTCCTAGCGTT	(SEQ ID NO: 99)
TCCATGACGTTCCTGACGTT	(SEQ ID NO: 100)
GTCG(T/C)T	(SEQ ID NO: 101)
TGTCG(T/C)T	(SEQ ID NO: 102)
TCCATGAGCTTCCTGAGTCT	(SEQ ID NO: 103)
TCTCCCAGCGTGCGCCAT	(SEQ ID NO: 104)
TCCATGACGTTCCTGACGTT	(SEQ ID NO: 105)

TABLE 2

Induction of human IL-12 secretion by Phosphorothioate CpG oligonucleotide

		IL-12 (pg/ml)	
ODN[1]	sequence (5'-3')	expt. 1	expt. 2
None		0	0
1962	TCCTGTCGTTCCTTGTCGTT (SEQ. ID NO:79)	19	0
1965	TCCTGTCGTTTTTTGTCGTT (SEQ. ID NO:81)	36	0
1967	TCGTCGCTGTCTGCCCTTCTT (SEQ. ID NO:82)	41	0
1968	TCGTCGCTGTTGTCGTTTCTT (SEQ. ID NO:83)	24	0
2005	TCGTCGTTGTCGTTGTCGTT (SEQ. ID NO:89)	25	0
2006	TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO: 90)	29	15
2014	TGTCGTTGTCGTTGTCGTT (SEQ. ID NO:96)	28	0
2015	TCGTCGTCGTCGTT (SEQ ID NO:97)	14	0
2016	TGTCGTTGTCGTT (SEQ. ID NO:98)	3	0

[1]PBMC were collected from normal donors and spun over Ficoll, then cultured at 10^6 cells/well in 96 well microtiter plates with or without the indicated oligonucleotide which were added to cultures at μg/ml. Supernatants were collected at 24 hr and tested for IL-12 levels by ELISA as described in methods. A standard curve was run in each experiment, which represents a different donor.

TABLE 3

Compound	CD86 (5 Exp)	CD40 (4 Exp.)	T cell proliferation
GM-CSF	1.9	2.5	13.3
CpG	3.9	3.5	19.7
CpG + GM-CSF	7.0	8.5	25.6

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TABLE 1

Oligonucleotide Stimulation of Mouse B Cells

ODN		Sequence (5'to 3')†	Stimulation Index' ^{3}H Uridine	IgM Production
1	(SEQ ID NO:91)	GCTAGACGTTAGCGT	6.1 ± 0.8	17.9 ± 3.6
1a	(SEQ. ID NO:4)	.....T.....	1.2 ± 0.2	1.7 ± 0.5
1b	(SEQ ID NO:13)	.....Z.....	1.2 ± 0.1	1.8 ± 0.0
1c	(SEQ ID NO:14)	.....Z..	10.3 ± 4.4	9.5 ± 1.8

TABLE 1-continued

Oligonucleotide Stimulation of Mouse B Cells

ODN	Sequence (5'to 3')†	Stimulation Index* ^{3}H Uridine	Stimulation Index* IgM Production
1d (SEQ ID NO:92)	..AT.....GAGC.	13.0 ± 2.3	18.3 ± 7.5
2 (SEQ ID NO:1)	ATGGAAGGTCCAGCGTTCTC	2.9 ± 0.2	13.6 ± 2.0
2a (SEQ ID NO:35)	..C..CTC..G.....	7.7 ± 0.8	24.2 ± 3.2
2b (SEQ ID NO:16)	..Z..CTC.ZG..Z.....	1.6 ± 0.5	2.8 ± 2.2
2c (SEQ ID NO:17)	..Z..CTC..G.....	3.1 ± 0.6	7.3 ± 1.4
2d (SEQ ID NO:18)	..C..CTC..G.....Z..	7.4 ± 1.4	27.7 ± 5.4
2e (SEQ ID NO:19)	.....A.....	5.6 ± 2.0	ND
3D (SEQ ID NO:20)	GAGAACGCGGACGTTCCAT	4.9 ± 0.5	19.9 ± 3.6
3Da (SEQ ID NO:21)	.....C.....	6.6 ± 1.5	33.9 ± 6.8
3Db (SEQ ID NO:22)	.....C.....G..	10.1 ± 2.8	25.4 ± 0.8
3Dc (SEQ ID NO:23)	...C.A.....	1.0 ± 0.1	1.2 ± 0.5
3Dd (SEQ ID NO:24)	.....Z.....	1.2 ± 0.2	1.8 ± 0.4
3De (SEQ ID NO:25)	.....Z.....	4.4 ± 1.2	18.8 ± 4.4
3Df (SEQ ID NO:26)	.....A.....	1.6 ± 0.1	7.7 ± 0.4
3Dg (SEQ ID NO:27)	.....CC.G.ACTG..	6.1 ± 1.5	18.6 ± 1.5
3M (SEQ ID NO:28)	TCCATGTCGGTCCTGATGCT	4.1 ± 0.2	23.2 ± 4.9
3Ma (SEQ ID NO:29)	.....CT.....	0.9 ± 0.1	1.8 ± 0.5
3Mb (SEQ ID NO:30)	.....Z.....	1.3 ± 0.3	1.5 ± 0.6
3Mc (SEQ ID NO:31)	.....Z.....	5.4 ± 1.5	8.5 ± 2.6
3Md (SEQ ID NO:7)	.....A..T.....	17.2 ± 9.4	ND
3Me (SEQ ID NO:93)	.....C..A.	3.6 ± 0.2	14.2 ± 5.2
4 (SEQ ID NO:94)	TCAACGTT	6.1 ± 1.4	19.2 ± 5.2
4a (SEQ ID NO:95)	....GC..	1.1 ± 0.2	1.5 ± 1.1
4b (SEQ ID NO:96)	...GCGC.	4.5 ± 0.2	9.6 ± 3.4
4c (SEQ ID NO:97)	...TCGA.	2.7 ± 1.0	ND
4d (SEQ ID NO:98)	..TT..AA	1.3 ± 0.2	ND
4e (Residue 2-8 of SEQ ID NO:94)	-......	1.3 ± 0.2	1.1 ± 0.5
4f (SEQ ID NO:99)	C.....	3.9 ± 1.4	ND
4g (Residue 11-18 of SEQ ID NO:19)	--.....CT	1.4 ± 0.3	ND
4h (SEQ ID NO:100)	C	1.2 ± 0.2	ND
LPS		7.8 ± 2.5	4.8 ± 1.0

*Stimulation indexes are the means and std. dev. derived from at least 3 separate experiments, and are compared to wells cultured with no added ODN.
ND = not done.
CpG dinucleotides are underlined.
Dots indicate identity; dashes indicate deletions.
Z indicates 5 methyl cytosine.

TABLE 2

Identification of the optimal CpG motif for Murine IL-6 production and B cell activation

ODN	SEQUENCE (5'→3')	IL-6 (pg/ml)[a] CH12.LX	SPLENIC B CELL SI[b]	IgM (ng/ml)[c]
512 (SEQ ID No:28)	TCCATGTCGGTCCTGATGCT	1300 ± 106	627 ± 43	5.8 ± 0.3	7315 ± 1324
1637 (SEQ ID No:29)	.....C.....	136 ± 27	46 ± 6	1.7 ± 0.2	770 ± 72
1615 (SEQ ID No:101)	.....G.....	1201 ± 155	850 ± 202	3.7 ± 0.3	3212 ± 617
1614 (SEQ ID No:102)	.....A.....	1533 ± 321	1812 ± 103	10.8 ± 0.6	7558 ± 414
1636 (SEQ ID No:103)	.....A.....	1181 ± 76	947 ± 132	5.4 ± 0.4	3983 ± 485
1634 (SEQ ID NO:104)	.....C.....	1049 ± 223	1671 ± 178	9.2 ± 0.9	6256 ± 261
1619 (SEQ ID No:105)	.....T.....	1555 ± 304	2908 ± 129	12.5 ± 1.0	8243 ± 698
1618 (SEQ ID No:7)	.....A..T.....	2109 ± 291	2596 ± 166	12.9 ± 0.7	10425 ± 674
1639 (SEQ ID No:3)	.....AA..T.....	1827 ± 83	2012 ± 132	11.5 ± 0.4	9489 ± 103
1707 (SEQ ID No:88)	.....A..TC.....	ND	1147 ± 175	4.0 ± 0.2	3534 ± 217
1708 (SEQ ID No:106)	.....CA..TG.....	ND	59 ± 3	1.5 ± 0.1	466 ± 109

Dots indicate identity; CpG dinucleotides are underlined; ND = not done
[a]The experiment was done at least three times with similar results. The level of IL-6 of unstimulated control cultures of both CH12.LX and splenic B cells was ≤ 10 pg/ml. The IgM level of unstimulated culture was 547 ± 82 ng/ml. CpG dinucleotides are underlined and dots indicate identity.
[b][^{3}H] Uridine uptake was indicated as a fold increase (SI: stimulation index) from unstimulated control (2322.67 ± 213.68 cpm). Cells were stimulated with 20 μM of various CpG O-ODN. Data present the mean ± SD of triplicates
[c]Measured by ELISA.

TABLE 3

Induction of Murine IL-6 secretion by CpG motifs in bacterial DNA or oligonucleotides.

Treatment	IL-6 (pg/ml)
calf thymus DNA	≤10
calf thymus DNA + DNase	≤10
E. coli DNA	1169.5 ± 94.1
E. coli DNA + DNase	≤10
CpG methylated *E. coli* DNA	≤10
LPS	280.1 ± 17.1
Media (no DNA)	≤10

ODN			
5a	SEQ. ID. No:1	ATGGACTCTCCAGCGTTCTC	1096.4 ± 372.0
5b	SEQ. ID. No:2	.....AGG....A.....	1124.5 ± 126.2
5c	SEQ. ID. No:3	..C.....G.....	1783.0 ± 189.5
5d	SEQ. ID. No:4	.... AGG..C..T.....	≤10
5e	SEQ. ID. No:5	..C.....G..Z.....	851.1 ± 114.4

TABLE 3-continued

Induction of Murine IL-6 secretion by CpG motifs in bacterial DNA or oligonucleotides.

5f	SEQ. ID. No:6	..Z.....ZG..Z.....	≦10
5g	SEQ. ID. No:7	..C.....G.....Z..	1862.3 ± 87.26

T cell depleted spleen cells from DBA/2 mice were stimulated with phosphodiester modified oligonucleotides (O-ODN) (20 μM), calf thymus DNA (50 μg/ml) or *E. Coli* DNA (50 μg/ml) with or without enzyme treatment, or LPS (10 μg/ml) for 24 hr. Data represent the mean (pg/ml) ± SD of triplicates. CpG dinucleotides are underlined and dots indicate identity. Z indicates 5-methylcytosine.

TABLE 4

Secretion of Murine IL-6 induced by CpG DNA stimulation in vivo.

Stimulant	IL-6 (pg/ml)
PBS	<50
E. coli DNA	13858 ± 3143
Calf Thymus DNA	<50
CpG S-ODN	20715 ± 606
non-CpG S-ODN	<50

Mice (2 mice/group) were i.v. injected with 100 μl of PBS, 200 μg of *E. coli* DNA or calf thymus DNA, or 800 μg of CpG S-ODN or non-CpG control S-ODN. Mice were bled 2 hr after injection and 1:10 dilution of each serum was analyzed by IL-6 ELISA. Sensitivity limit of IL-6 ELISA was 5 pg/ml. Sequences of the CpG S-ODN is 5'GCATGACGTTGAGCT3' (SEQ. ID. No: 6) and of the non-stimulatory S-ODN is 5'GCTAGATGTTAGCGT3' (SEQ. ID. No: 49). Note that although there is a CpG in sequence 48, it is too close to the 3' end to effect stimulation, as explained herein. Data represent mean ± SD of duplicates. The experiment was done at least twice with similar results.

TABLE 5

Induction of human PBMC cytokine secretion by CpG oligos

ODN	Sequence (5'–3')	IL-6[1]	TNF-α[1]	IFN-γ[1]	GM-CSF	IL-12
512 SEQ ID NO:28	TCCATGTCGGTCCTGATGCT	500	140	15.6	70	250
1637 SEQ ID NO:29	.....C.....	550	16	7.8	15.6	16
1615 SEQ ID NO:101	.....G.....	600	145	7.8	45	145

TABLE 5-continued

Induction of human PBMC cytokine secretion by CpG oligos

ODN	Sequence (5'–3')	IL-6[1]	TNF-α[1]	IFN-γ[1]	GM-CSF	IL-12
1614 SEQ ID NO:102	.....A.....	550	31	0	80	31
1636 SEQ ID NO:103	.....AA.....	325	250	35	40	250
1634 SEQ ID NO:104	.....CC.....	300	400	40	85	400
1619 SEQ ID NO:105	.....TT.....	275	450	200	80	450
1618 SEQ ID NO:7	.....AT.....	300	60	15.6	15.6	62
1639 SEQ ID NO:3	....AAT.....	625	220	15.6	40	220
1707 SEQ ID NO:88	.....ATC.....	300	70	17	0	70
1708 SEQ ID NO:106	....CATG.....	270	10	17	ND	10

dots indicate identity; CpG dinucleotides are underlined

[1]measured by ELISA using Quantikine kits from R&D Systems (pg/ml) Cells were cultured in 10% autologous serum with the indicated oligodeoxynucleotides (12 μg/ml) for 4 hr in the case of TNF-α or 24 hr for the other cytokines before supernatant harvest and assay. Data are presented as the level of cytokine above that in wells with no added oligodeoxynucleotide.

TABLE 6

CpG DNA induces cytokine secretion by human PBMC

DNA	TNF-α(pg/ml)[1]	IL-6 (pg/ml)	IFN-γ (pg/ml)	RANTES (pg/ml)
EC DNA (50 μg/ml)	980	12,000	700	1560
EC DNA (5 μg/ml)	850	11,000	400	750
EC DNA (0.5 μg/ml)	500	ND	200	0
EC DNA (0.05 μg/ml)	62.5	10,000	15.6	0
EC DNA (50 μg/ml) + L-LME[2]	0	ND	ND	ND
EC DNA (10 μg/ml) Methyl.[3]	0	5	ND	ND
CT DNA (50 μg/ml)	0	600	0	0

[1]Levels of all cytokines were determined by ELISA using Quantikine kits from R&D Systems as described in the previous table. Results are representative using PBMC from different donors.
[2]Cells were pretreated for 15 min. with L-leucyl-L-leucine methyl ester (M-LME) to determine whether the cytokine production under these conditions was from monocytes (or other L-LME-sensitive cells).
[3]EC DNA was methylated using 2U/μg DNA of CpG methylase (New England Biolabs) according to the manufacturer's directions, and methylation confirmed by digestion with Hpa-II and Msp-I. As a negative control, samples were included containing twice the maximal amount of LPS contained in the highest concentration of EC DNA which failed to induce detectable cytokine production under these experimental conditions.
ND = not done

TABLE 7

CpG DNA induces cytokine expression in purified human macrophages

	IL-6 (pg/ml)	GM-CSF (pg/ml)	TNF-α(pg/ml)
Cells alone	0	0	0
CT DNA (50 μg/ml)	0	0	0
EC DNA (50 μg/ml)	2000	15.6	1800

TABLE 8

Induction Of NK Activity By CpG Oligodeoxynucleotides (ODN)

	% YAC-1 Specific Lysis* Effector: Target		% 2C11 Specific Lysis Effector: Target	
ODN	50:1	100:1	50:1	100:1
None	−1.1	−1.4	15.3	16.6
1	16.1	24.5	38.7	47.2
3Dd	17.1	27.0	37.0	40.0
non-CpG ODN	−1.6	−1.7	14.8	15.4

TABLE 9

Induction of NK Activity by DNA Containing CpG Motifs but not by Non-CpG DNA

	DNA or Cytokine Added		LU/10^6 Mouse Cells	LU/10^6 Human Cells
Expt. 1	None		0.00	0.00
	IL-2		16.68	15.82
	E.Coli. DNA		7.23	5.05
	Calf thymus DNA		0.00	0.00
Expt. 2	None		0.00	3.28
	1585 ggGGTCAACGTTGACgggg	(SEQ ID No.12)	7.38	17.98
	1629 -------gtc------	(SEQ ID No.50)	0.00	4.4
Expt. 3	None		0.00	
	1613 GCTAGACGTTAGTGT	(SEQ ID No.42)	5.22	
	1769 -------Z------	(SEQ ID No.52)	0.02	ND
	1619 TCCATGTCGTTCCTGATGCT	(SEQ ID No:38)	3.35	
	1765 -------Z---------	(SEQ ID No.53)	0.11	

CpG dinucleotides in ODN sequences are indicated by underlying; Z indicates methylcytosine. Lower case letters indicate nuclease resistant phosphorothioate modified internucleotide linkages which, in titration experiments, were more than 20 times as potent as non-modified ODN, depending on the flanking bases. Poly G ends (g) were used in some ODN, because they significantly increase the level of ODN uptake.

TABLE 10

ODN Induction of NK Lytic Activity (LU)

ODN cells alone	Sequence (5'→3')	LU 0.01	
1754	ACCATGGACGATCTGTTTCCCCTC	0.02	SEQ ID NO: 59
1758	TCTCCCAGCGTGCGCCAT	0.05	SEQ ID NO: 45
1761	TACCGCGTGCGACCCTCT	0.05	SEQ ID NO: 60
1776	ACCATGGACGAACPGTTTCCCCTC	0.03	SEQ ID NO: 61
1777	ACCATGGACGAGCTGTTTCCCCTC	0.05	SEQ ID NO: 62
1778	ACCATGGACGACCTGTTTCCCCTC	0.01	SEQ ID NO: 63
1779	ACCATGGACGTACTGTTTCCCCTC	0.02	SEQ ID NO: 64
1780	ACCATGGACGGTCTGTTTCCCCTC	0.29	SEQ ID NO: 65
1781	ACCATGGACGTTCTGTTTCCCCTC	0.38	SEQ ID NO: 66
1823	GCATGACGTTGAGCT	0.08	SEQ ID NO: 6
1824	CACGTTGAGGGGCAT	0.01	SEQ ID NO: 67
1825	CTGCTGAGACTGGAG	0.01	SEQ ID NO: 68
1828	TCAGCGTGCGCC	0.01	SEQ ID NO: 69
1829	ATGACGTTCCTGACGTT	0.42	SEQ ID NO: 70
1830[2]	RANDOM SEQUENCE	0.25	
1834	TCTCCCAGCGGGCGCAT	0.00	SEQ ID NO: 71
1836	TCTCCCAGCGCGCGCCAT	0.46	SEQ ID NO: 72
1840	TCCATGTCGTTCCTGTCGTT	2.70	SEQ ID NO: 73
1841	TCCATAGCGTTCCTAGCGTT	1.48	SEQ ID NO: 74
1842	TCGTCGCTGTCTCCGCTTCTT	0.06	SEQ ID NO: 75
1851	TCCTGACGTTCCTGACGTT	2.32	SEQ ID NO: 76

[1]Lytic units (LU) were measured as described (8). Briefly, PBNC were collected from normal donors and spun over Ficoll, then cultured with or without the indicated ODN (which were added to cultures at 6 μg/ml) for 24 hr. Then their ability to lyse ^{51}Cr-labeled K562 cells was determined. The results shown are typical of those obtained with several different normal human donors. [2]This oligo mixture contained a random selection of all 4 bases at each position.

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TABLE 11

Induction of NK LU by Phosphorothioate CpG ODN with Good Motifs

ODN[1] cells alone	sequence (5'→3')	SEQ ID NO:	expt. 1	expt. 2	expt. 3
			0.00	1.26	0.46
1840	TCCATGTCGTTCCTGTCGTT	73	2.33	ND	ND
1960	TCCTGTCGTTCCTGTCGTT	77	ND	0.48	8.99
1961	TCCATGTCGTTTTTGTCGTT	78	4.03	1.23	5.08
1962	TCCTGTCGTTCCTTGTCGTT	52	ND	1.60	5.74
1963	TCCTTGTCGTTCCTGTCGTT	79	3.42	ND	ND
1965	TCCTGTCGTTTTTTGTCGTT	53	0.46	0.42	3.48
1966	TCGTCGCTGTCTCCGCTTCTT	75	2.62	ND	ND
1967	TCGTCGCTGTCTGCCCTTCTT	54	5.82	1.64	8.32
1968	TCGTCGCTGTTGTCGTTTCTT	55	3.77	5.26	6.12
1979[2]	TCCATGTZGTTCCTGTZGTT		1.32	ND	ND
1982	TCCAGGACTTCTCTCAAGTT	79	0.05	ND	0.98
1990	TCCATGCGTGCGTGCGTTTT	80	2.10	ND	ND
1991	TCCATGCGTTGCGTTGCGTT	81	0.89	ND	ND
2002	TCCACGACGTTTTCGACGTT	82	4.02	1.31	9.79
2005	TCGTCGTTGTCGTTGTCGTT	47	ND	4.22	12.75
2006	TCGTCGTTTTGTCGTTTTGTCGT	56	ND	6.17	12.82
2007	TCGTCGTTGTCGTTTTGTCGTT	49	ND	2.68	9.66
2008	GCGTGCGTTGTCGTTGTCGTT	56	ND	1.37	8.15
2010	GCGGCGGGCGGCGCGCGCCC	83	ND	0.01	0.05
2012	TGTCGTTTGTCGTTTGTCGTT	48	ND	2.02	11.61
2013	TGTCGTTGTCGTTGTCGTTGTCGTT	84	ND	0.56	5.22
2014	TGTCGTTGTCGTTGTCGTT	60	ND	5.74	10.89
2015	TCGTCGTCGTCGTT	51	ND	4.53	10.13
2016	TGTCGTTGTCGTT	85	ND	6.54	8.06

[1]PBMC essentially as described herein. Results are representative of 6 separate experiments; each experiment represents a different donor.
[2]This is the methylated version of ODN 1840 (SEQ ID NO:83); Z = 5-methyl cytosine at residues 8 and 17; LU is lytic units; ND = not done; CpG dinucleotides are underlined for clarity

TABLE 12

Induction of human B cell proliferation by Phosphorothioate CpG ODN

		Stimulation Index[1,]					
ODN sequence (5' 3')	SEQ ID NO:	expt. 1	expt. 2	expt. 3	expt. 4	expt. 5	expt. 6
1840 TCCATGTCGTTCCTGTCGTT	73	4	ND	ND	ND	ND	34
1841 TCCATAGCGTTCCTAGCGTT	74	3	ND	ND	ND	ND	ND

TABLE 12-continued

Induction of human B cell proliferation by Phosphorothioate CpG ODN

		Stimulation Index[1,]					
ODN sequence (5' 3')	SEQ ID NO:	expt. 1	expt. 2	expt. 3	expt. 4	expt. 5	expt. 6
1960 TCCTGTCGTTCCTTGTCGTT	77	ND	2.0	2.0	3.6	ND	ND
1961 TCCATGTCGTTTTTGTCGTT	78	2	3.9	1.9	3.7	ND	37
1962 TCCTGTCGTTCCTTGTCGTT	52	ND	3.8	1.9	3.9	9.4	35
1963 TCCTTGTCGTTCCTGTCGTT	79	3	ND	ND	ND	ND	ND
1965 TCCTGTCGTTTTTTGTCGTT	53	4	3.7	2.4	4.7	6.0	43
1967 TCGTCGCTGTCTGCCCTTCTT	54	ND	4.4	2.0	4.5	5.6	36
1968 TCGTCGCTGTTGTCGTTTCTT	55	ND	4.0	2.0	4.9	8.7	38
1982 TCCAGGACTTCTCTCAGGTT	79	3	1.8	1.3	3.1	3.2	12
2002 TCCACGACGTTTTCGACGTT	86	ND	2.7	1.4	4.4	ND	14
2005 TCGTCGTTGTCGTTGTCGTT	47	5	3.2	1.2	3.0	7.9	37
2006 TCGTCGTTTTGTCGTTTTGTCGTT	46	4	4.5	2.2	5.8	8.3	40
2007 TCGTCGTTGTCGTTTTGTCGTT	49	3	4.0	4.2	4.1	ND	22
2008 GCGTGCGTTGTCGTTGTCGTT	56	ND	3.0	2.4	1.6	ND	12
2010 GCGGCGGGCGGCGCGCCC	83	ND	1.6	1.9	3.2	ND	ND
2012 TGTCGTTTGTCGTTTGTCGTT	48	2	2.8	0	3.2	ND	33
2013 TGTCGTTGTCGTTGTCGTTGTCGTT	84	3	2.3	3.1	2.8	ND	7
2014 TGTCGTTGTCGTTGTCGTT	50	3	2.5	4.0	3.2	6.7	14
2015 TCGTCGTCGTCGTT	51	5	1.8	2.6	4.5	9.4	1
2016 TGTCGTTGTCGTT	85	ND	1.1	1.7	2.7	7.3	1

[1]Cells = human spleen cells stored at -70° C. after surgical harvest or PBMC collected from normal donors and spun over Ficoll. Cells were cultured in 96 well U-bottom microtiter plates with or without the indicated ODN (which were added to cultures at 6 μml). N = 12 experiments. Cells were cultured for 4–7 days, pulsed with 1 μCi of ^{3}H thymidine for 18 hr before harvest and scintillation counting. Stimulation index = the ratio of cpm in wells without ODN to that in wells that had been stimulated throughout the culture period with the indicated ODN (there were no further additions of ODN after the cultures were set up). ND = not done

TABLE 13

Induction of human IL-12 secretion by Phosphorothioate CpG ODN

ODN[1]	sequence (5'-3')	SEQ ID NO	IL-12 (pg/ml) expt. 1	IL-12 (pg/ml) expt. 2
cells alone			0	0
1962	TCCTGTCGTTCCTTGTCGTT	52	19	0
1965	TCCTGTCGTTTTTTGTCGTT	53	36	0
1967	TCGTCGCTGTCTGCCCTTCTT	54	41	0
1968	TCGTCGCTGTTGTCGTTTCTT	55	24	0
2005	TCGTCGTTGTCGTTGTCGTT	47	25	0
2006	TCGTCGTTTTGTCGTTTTGTCGTT	46	29	15

TABLE 13-continued

Induction of human IL-12 secretion by Phosphorothioate CpG ODN

ODN[1]	sequence (5'-3')	SEQ ID NO	IL-12 (pg/ml) expt. 1	expt. 2
2014	TGTCGTTGTCGTTGTCGTT	50	28	0
2015	TCGTCGTCGTCGTT	51	14	0
2016	TGTCGTTGTCGTT	85	3	0

[1]PBMC were collected from normal donors and spun over Ficoll, then cultured at 10^6 cells/well in 96 well microtiter plates with or without the indicated ODN which were added to cultures at 6 μg/ml. Supernatants were collected at 24 hr and tested for IL-12 levels by ELISA as described in methods. A standard curve was run in each experiment, which represents a different donor.

TABLE 14

Different CpG motifs stimulate optimal murine B cell and NK activation

ODN	Sequence		B cell activation[1]	NK activation[2]
1668	TCCATGACGTTCCTGATGCT	(SEQ.ID.NO:7)	42,849	2.52
1758	TCTCCCAGCGTGCGCCAT	(SEQ.ID.NO.45)	1,747	5.66
NONE			367	0.00

CpG dinucleotides are underlined; oligonucleotides were synthesized with phosphorothioate modified backbones to improve their nuclease resistance. [1]Measured by ^{3}H thymidine incorporation after 48 hr culture with oligodeoxynucleotides at a 200 nM concentration as described in Example 1. [2]Measured in lytic units.

TABLE 15

Specific blockade of CpG-induced TNF-α and IL-12 expression by inhibitors of endosomal acidification or NFκB activation

	Medium		Inhibitors: Bafilomycin (250 nM)		Chloroquine (2.5 μg/ml)		Monensin (10 μM)		NAC (50 mM)	TPCK (50 μM)	Gliotoxin (0.1 μg/ml)	Bisgliotoxin (0.1 μg/ml)
activators	TNF-α	IL-12	TNF-α	IL-12	TNF-α	IL-12	TNF-α	IL-12	TNF-α	TNF-α	TNF-α	TNF-α
Medium	37	147	46	102	27	20	22	73	30	24	17	41
CpG ODN	455	17,314	71	316	28	6	49	777	54	23	31	441
LPS	903	22,485	1370	4051	3025	12418	493	4796	437	46	178	3120

TABLE 15 legend IL-12 and TNF-α assays: The murine monocyte cell line 3774 (3×10^5 cells/ml for IL-12 or 1×10^6 cells/ml for TNF-α), were cultured with or without the indicated inhibitors at the concentrations shown for 2 hr and then stimulated with the CpG oligodeoxynucleotide (ODN) 1826 (TCCATGACGTTCCTGACGTT SEQ ID NO:16) at 2 μM or LPS (10 μg/ml) for 4 hr (TNF-α or 24 hr (IL-12) at which time the supernatant was harvested. ELISA for IL-12 or TNF-α (pg/ml) was performed on the supernatants essentially as described (A. K. Krieg, A.-K. Yi, S. Matson, T. J. Waldschmidt, G. A. Bishop, R. Teasdale, G. Koretzky and D. Klinman, Nature 374, 546 (1995); Yi A.-K., D. M. Klinman, T. L. Martin, S. Matson and A. M. Krieg, J Immunol., 157, 5394-5402 (1996); Krieg, A. M, J Lab. Clin. Med., 128, 128-133 (1996). Cells cultured with ODN that lacked CpG motifs did not induce cytokine secretion. Similar specific inhibition of CpG responses was seen with IL-6 assays, and in experiments using primary spleen cells or the B cell lines CH12.LX and WEHI-231. 2.5 μg/ml of chloroquine is equivalent to <5 μM. Other inhibitors of NF-κB activation including PDTC and calpain inhibitors I and II gave similar results to the inhibitors shown. The results shown are representative of those obtained in ten different experiments.

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TABLE 3

Plasmids containing immunostimulatory CpG motifs

Plasmid	Backbone	No. CpG, Motifs	Species Specificity and ODN Equivalence of CpG-S Insert
pMCG-16	pMAS	16	mouse-specific CpG motif #1826[1]
pMCG-50	pMAS	50	
pMCG-100	pMAS	100	
pMCG-200	pMAS	200	
pHCG-30	pMAS	30	human-specific CpG motif - no ODN equivalent[2]
pHCG-50	pMAS	50	
pHCG-100	pMAS	100	
pHCG-200	pMAS	200	
pHIS-40	pMAS	40	human-specific CpG motif

TABLE 3-continued

Plasmids containing immunostimulatory CpG motifs

Plasmid	Backbone	No. CpG, Motifs	Species Specificity and ODN Equivalence of CpG-S Insert
pHIS-64	pMAS	64	#2006[3]
pHIS-128	pMAS	128	
pHIS-192	pMAS	192	

[1]sequence of 1826 is TCCATGACGTTCCTGACGTT

[2]sequence used as a source of CpG motifs is GACTT CGTGTCGTTCTTCTGTCGTCTTTAGCGCTTCTCCTGCGTG CGTCCTTG(SEQ ID NO:14)

[3]sequence of 2006 is TCGTCGTTTTGTCGTTTTGTCGTT(SEQ ID NO:3)

TABLE 4

Plasmids encoding hepatitis B surface antigen (derived from ayw or adw subtypes of HBV)

Plasmid	Backbone	Insert
pUK-S	pUK21-A2	HBV-S (ayw)
pUKAX-S	pUK21-AX*	HBV-S (ayw)
pMAS-S	pMAS	HBV-S (ayw)
pMCG16-S	pMCG-16	HBV-S (ayw)
pMCG50-S	pMCG-50	HBV-S (ayw)
pMCG100-S	pMCG-100	HBV-S (ayw)
pMCG200-S	pMCG-200	HBV-S (ayw)

TABLE 4-continued

Plasmids encoding hepatitis B surface antigen (derived from ayw or adw subtypes of HBV)

Plasmid	Backbone	Insert
pHCG30-S	pHCG-30	HBV-S (ayw)
pHCG50-S	pHCG-50	HBV-S (ayw)
pHCG100-S	pHCG-100	HBV-S (ayw)
pHCG200-S	pHCG-200	HBV-S (ayw)
pHIS40-S(ad)	pHIS-40	HBV-S (adw2)
pHIS64-S(ad)	pHIS-64	HBV-S (adw2)
pHIS128-S(ad)	pHIS-128	HBV-S (adw2)
pHIS192-S(ad)	pHIS-192	HBV-S (adw2)

*pUK21-AX was created by deleting f1 origin from pUK21-A

TABLE 6

CpG used with plasmid DNA

Backbone	CpG code number	Sequence
		(SEQ ID NO:51)
S-CpG	1826	TCCATGACGTTCCTGACGTT
		(SEQ ID NO:52)
	1628	GGGGTCAACGTTGAGGGGGG
		(SEQ ID NO:53)
	1911	TCCAGGACTTTCCTCAGGTT
		(SEQ ID NO:54)
	1982	TCCAGGACTTCTCTCAGGTT
		(SEQ ID NO:55)
	2017	CCCCCCCCCCCCCCCCCCCC
		(SEQ ID NO:56)
O-CpG	2061	TCCATGACGTTCCTGACGTT
		(SEQ ID NO:57)
	2001	GGCGGCGGCGGCGGCGGCGG
		(SEQ ID NO:58)
SOS-CpG	1980	TCCATGACGTTCCTGACGTT
		(SEQ ID NO:59)
	1585	GGGTCAACGTTGAGGGGGG
		(SEQ ID NO:60)
	1844	TCTCCCAGCGTGCGCCATAT
		(SEQ ID NO:61)
	1972	GGGGTCTGTGCTTTTGGGGGG
		(SEQ ID NO:62)
	2042	TCAGGGGTGGGGGGAACCTT
		(SEQ ID NO:63)
	1981	GGGGTTGACGTTTTGGGGGG
		(SEQ ID NO:64)
	2019	TCTAGCGTTTTTAGCGTTCC
		(SEQ ID NO:65)
	2021	TCGTCGTTGTCGTTGTCGTT
		(SEQ ID NO:66)
	2022	TCGTCGTTTTGTCGTTTTGTCGTT
		(SEQ ID NO:67)
	2023	NCGTCGTTGTCGTTTTGTCGTT

Note: (SEQ ID NO: 51-67, respectively)

TABLE 10

Inhibitory CpG motifs can block B cell proliferation induced by a stimulatory CpG motif

Oligonucleotide added	cpm
medium	194
1668 (TCCATGACGTTCCTGATGCT)(SEQ ID NO:68)	34,669
1668 + 1735 (GCGTTTTTTTTTGCG)(SEQ ID NO:69)	24,452
1720 (TCCATGAGCTTCCTGATGCT)(SEQ ID NO:70)	601
1720 + 1735	1109

TABLE 11

Inhibitory effects of "bad" CpG motifs on the "good" CpG Oligo 1619

Oligonucleotide added	IL-12 in pg/ml
medium	0
1619 alone	6
1619 + 1949 (TCCATGTCGTTCCTGATGCG(SEQ ID NO:72))	16
1619 + 1952 (TCCATGTCGTTCCGCGCGCG(SEQ ID NO:73))	0
1619 + 1953 (TCCATGTCGTTCCTGCCGCT(SEQ ID NO:74))	0
1619 + 1955 (GCGGCGGGCGGCGCGCCC(SEQ ID NO:75))	0

Notes:
The sequence of oligo 1619 is TCCATGTCGTTCCTGATGCT (SEQ ID NO:71)
1949 has only 1 GCG at the 3' end, which has essentially no inhibitory activity

TABLE 13

Identification of neutralizing CpG motifs which reduce the induction of cytokine secretion by a CpG-S motif in the same ODN (cis-neutralization)

ODN	sequence 5'-3'[1]		ODN-induced cytokine expression[2] IL-6[2]	IL-12	IFN-γ
None			<5	206	898
1619	TCCATGTCGTTCCTGATGCT	(SEQ ID NO:71)	1405	3130	4628
1952	.....GCGCGCG	(SEQ ID NO:73)	559	1615	2135
1953	.....CC...	(SEQ ID NO:74)	557	1854	2000

[1]Dots in the sequence of ODN 1952 and 1953 indicate identity to ODN 1619; CpG dinucleotides are underlined for clarity. ODN without CpG-N or CpG-S motifs had little or no effect on cytokine production. The data shown are representative of 4 experiments.

[2]All cytokines are given in pg/ml; measured by ELISA on supernatants from DBA/2 spleen cells cultured in 96 well plates at 2 x 10^7 cells/ml for 24 hr with the indicated ODN at 30 μg/ml. Std. dev. of the triplicate wells was <7%. None of the ODN induced significant amounts of IL-5.

TABLE 14

Inhibition of CpG-induced cytokine secretion by ODN containing CpG-N motifs

ODN	sequence 5'-3'	IL-12 secretion[1]	CpG-S-induced IL-12 secretion[2]
none		268	5453
1895	GCGCGCGCGCGCGCGCGCGC(SEQ ID NO:76)	123	2719
1896	CGGCCGGCCGGCCGGCCGG(SEQ ID NO:77)	292	2740
1955	GCGCGGGCGGCGCGCGCCC(SEQ ID NO:78)	270	2539
2037	TCCATGCGTTCCTGCGTT(SEQ ID NO:78)	423	2847

[1]BALB/c spleen cells were cultured in 96 well plates at 2 x 10^7 cells/ml with the indicated ODN for 24 hr and then the supernatants were assayed for IL-12 by ELISA (pg/ml).
[2]Cells were set up the same as in [1] except that IL-12 secretion was induced by the addition of the CpG ODN 1619 (TCCATGTCGTTCCTGATGCT) at 30 μg/ml. The data shown are representative of 5 experiments.

SUBSTITUTE SHEET (RULE 26)

APPENDIX (C)

Exemplary Human miRNA Sequences

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SUBSTITUTE SHEET (RULE 26)

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SUBSTITUTE SHEET (RULE 26)

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Claims

1. A chirally controlled oligonucleotide composition comprising a plurality of oligonucleotides of at least one type, wherein each type is defined by:
1) base sequence;
2) pattern of backbone linkages;
3) pattern of backbone chiral centers; and
4) pattern of backbone X-moieties.

2. The composition of claim 1, wherein the oligonucleotide of at least one type is a unimer.

3. The composition of claim 2, wherein the oligonucleotide of at least one type is a stereounimer, P-modification unimer, or linkage unimer.

4. The composition of claim 1, wherein the oligonucleotide of at least one type is an altmer.

5. The composition of claim 4, wherein the oligonucleotide of at least one type is a stereoaltmer, P-modification altmer, or linkage altmer.

6. The composition of claim 1, wherein the oligonucleotide of at least one type is a blockmer.

7. The composition of claim 6, wherein the oligonucleotide of at least one type is a stereoblockmer, P-modification blockmer, or linkage blockmer.

8. The composition of claim 1, wherein the oligonucleotide of at least one type is a gapmer.

9. The composition of claim 1, wherein the oligonucleotide of at least one type is a skipmer

10. The composition of claim 1, wherein the oligonucleotide of at least one type is an antisense oligonucleotide, an antagomir, a microRNA, a pre-microRNA, an antimir, a supermir,

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a ribozyme, a Ul adaptor, RNA activator, RNAi agent, a decoy oligonucleotide, a triplex forming oligonucleotide, or an aptamer.

11. The composition of claim 1, wherein the oligonucleotide of at least one type is useful in regulating the level of a protein, or an RNA.

12. The composition of claim 11, wherein the oligonucleotide of at least one type is useful in regulating the level of an mRNA.

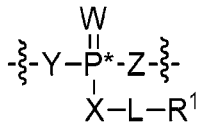
13. The composition of claim 11, wherein the oligonucleotide of at least one type is useful in regulating the level of a microRNA.

14. The composition of claim 1, wherein the at least one oligonucleotide type comprises one or more phosphorothioate triester internucleotide linkages.

15. The composition of claim 1, wherein the at least one oligonucleotide type comprises at least 2, at least 3, at least 4, at least 5, or at least 10 phosphorothioate triester internucleotide linkages.

16. The composition of claim 1, wherein the at least one oligonucleotide type comprises at least 10, at least 12, or at least 15 phosphorothioate triester internucleotide linkages.

17. The composition of claim 1, wherein the at least one oligonucleotide type comprises one or more modified internucleotide linkages independently having the structure of formula **I**:



(I)

wherein:

P* is an asymmetric phosphorus atom and is either *R*p or *S*p;

W is O, S or Se;

each of X, Y and Z is independently –O–, –S–, –N(–L–R^1)–, or L;

L is a covalent bond or an optionally substituted, linear or branched C_1–C_{50} alkylene, wherein one or more methylene units of L are optionally and independently replaced by an optionally substituted C_1–C_6 alkylene, C_1–C_6 alkenylene, —C≡C—, –C(R′)$_2$–, –Cy–, –O–, –S–, –S–S–, –N(R′)–, –C(O)–, –C(S)–, –C(NR′)–, –C(O)N(R′)–, –N(R′)C(O)N(R′)-, –N(R′)C(O)–, –N(R′)C(O)O–, –OC(O)N(R′)-, –S(O)–, –S(O)$_2$–, –S(O)$_2$N(R′)–, –N(R′)S(O)$_2$–, –SC(O)–, –C(O)S–, –OC(O)–, or –C(O)O–;

R^1 is halogen, R, or an optionally substituted C_1–C_{10} aliphatic wherein one or more methylene units are optionally and independently replaced by an optionally substituted C_1–C_6 alkylene, C_1–C_6 alkenylene, —C≡C—, –C(R′)$_2$–, –Cy–, –O–, –S–, –S–S–, –N(R′)–, –C(O)–, –C(S)–, –C(NR′)–, –C(O)N(R′)–, –N(R′)C(O)N(R′)-, –N(R′)C(O)–, –N(R′)C(O)O–, –OC(O)N(R′)-, –S(O)–, –S(O)$_2$–, –S(O)$_2$N(R′)–, –N(R′)S(O)$_2$–, –SC(O)–, –C(O)S–, –OC(O)–, or –C(O)O–;

each R′ is independently –R, -C(O)R, -CO_2R, or –SO_2R, or:

two R′ on the same nitrogen are taken together with their intervening atoms to form an optionally substituted heterocyclic or heteroaryl ring, or

two R′ on the same carbon are taken together with their intervening atoms to form an optionally substituted aryl, carbocyclic, heterocyclic, or heteroaryl ring;

–Cy– is an optionally substituted bivalent ring selected from phenylene, carbocyclylene, arylene, heteroarylene, or heterocyclylene;

each R is independently hydrogen, or an optionally substituted group selected from C_1–C_6 aliphatic, phenyl, carbocyclyl, aryl, heteroaryl, or heterocyclyl; and

each $-\!\!\lesssim$ independently represents a connection to a nucleoside.

18. The composition of claim 1, wherein the at least one oligonucleotide type comprises one or more modified internucleotide linkages independently having the structure of formula **I-a**, **I-b**, or **I-c**.

19. The composition of claim 18, wherein the at least one oligonucleotide type comprises one or more modified internucleotide linkages independently having the structure of formula **I-c**.

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20. The composition of any of claims 1-19, wherein the at least one oligonucleotide type further comprises one or more phosphate diester linkage.

21. An oligonucleotide composition that is chirally controlled in that the composition contains predetermined levels of one or more individual oligonucleotide types, wherein an oligonucleotide type is defined by:
1) base sequence;
2) pattern of backbone linkages;
3) pattern of backbone chiral centers; and
4) pattern of backbone X-moieties.

22. The oligonucleotide composition of claim 21, wherein the composition is chirally pure.

23. The oligonucleotide composition of claim 22, wherein the composition is chirally uniform.

24. The oligonucleotide composition of claim 22, wherein the composition contains predetermined levels of at least two oligonucleotide types.

25. The oligonucleotide composition of claim 22, wherein the composition contains predetermined levels of at least three or at least four oligonucleotide types.

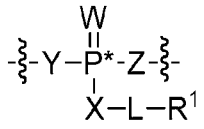
26. The oligonucleotide composition of claim 1 or claim 21, wherein one or more oligonucleotide types is selected from Table 2

27. A chirally controlled oligonucleotide comprising one or more phosphorothioate triester internucleotide linkages.

28. The composition of claim 27, comprising at least 2, at least 3, at least 4, at least 5, or at least 10 phosphorothioate triester internucleotide linkages.

29. A chirally controlled oligonucleotide comprising at least 10, at least 12, or at least 15 phosphorothioate triester internucleotide linkages.

30. A chirally controlled oligonucleotide comprising one or more modified internucleotide linkages independently having the structure of formula **I**:



(I)

wherein:

P* is an asymmetric phosphorus atom and is either *R*p or *S*p;

W is O, S or Se;

each of X, Y and Z is independently –O–, –S–, –N(–L–R^1)–, or L;

L is a covalent bond or an optionally substituted, linear or branched C_1–C_{50} alkylene, wherein one or more methylene units of L are optionally and independently replaced by an optionally substituted C_1–C_6 alkylene, C_1–C_6 alkenylene, —C≡C—, –C(R′)$_2$–, –Cy–, –O–, –S–, –S–S–, –N(R′)–, –C(O)–, –C(S)–, –C(NR′)–, –C(O)N(R′)–, –N(R′)C(O)N(R′)-, –N(R′)C(O)–, –N(R′)C(O)O–, –OC(O)N(R′)-, –S(O)–, –S(O)$_2$–, –S(O)$_2$N(R′)–, –N(R′)S(O)$_2$–, –SC(O)–, –C(O)S–, –OC(O)–, or –C(O)O–;

R^1 is halogen, R, or an optionally substituted C_1–C_{10} aliphatic wherein one or more methylene units are optionally and independently replaced by an optionally substituted C_1–C_6 alkylene, C_1–C_6 alkenylene, —C≡C—, –C(R′)$_2$–, –Cy–, –O–, –S–, –S–S–, –N(R′)–, –C(O)–, –C(S)–, –C(NR′)–, –C(O)N(R′)–, –N(R′)C(O)N(R′)-, –N(R′)C(O)–, –N(R′)C(O)O–, –OC(O)N(R′)-, –S(O)–, –S(O)$_2$–, –S(O)$_2$N(R′)–, –N(R′)S(O)$_2$–, –SC(O)–, –C(O)S–, –OC(O)–, or –C(O)O–;

each R′ is independently –R, -C(O)R, -CO_2R, or –SO_2R, or:

two R′ on the same nitrogen are taken together with their intervening atoms to form an optionally substituted heterocyclic or heteroaryl ring, or

two R′ on the same carbon are taken together with their intervening atoms to form an optionally substituted aryl, carbocyclic, heterocyclic, or heteroaryl ring;

–Cy– is an optionally substituted bivalent ring selected from phenylene, carbocyclylene, arylene, heteroarylene, or heterocyclylene;

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each R is independently hydrogen, or an optionally substituted group selected from C_1–C_6 aliphatic, phenyl, carbocyclyl, aryl, heteroaryl, or heterocyclyl; and

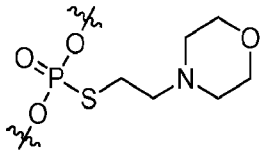
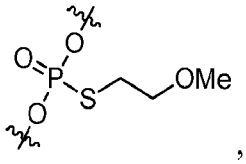
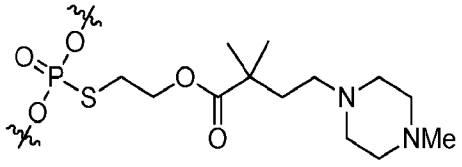
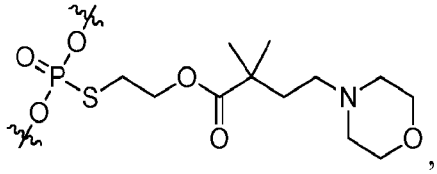
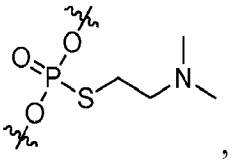
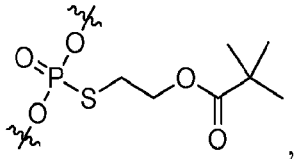
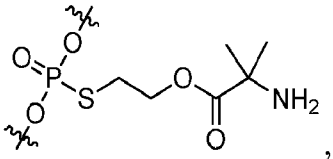
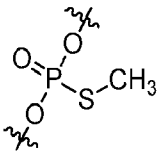
each $-\!\!\lesssim\!\!-$ independently represents a connection to a nucleoside.

31. A chirally controlled oligonucleotide, comprising one or more modified internucleotide linkages independently having the structure of formula **I-a**, **I-b**, or **I-c**.

32. A chirally controlled oligonucleotide, comprising one or more modified internucleotide linkages independently having the structure of formula **I-c**.

33. The chirally controlled oligonucleotide of any of claims 27-32, wherein the at least one oligonucleotide type further comprises one or more phosphate diester linkage.

34. The chirally controlled oligonucleotide of any of claims 27-33 comprising at least 5, at least 6, or at least 7 consecutive modified internucleotidic linkages, and at least one of the consecutive modified internucleotidic linkages is not a phosphorothioate diester linkage.

35. The chirally controlled oligonucleotide of any of claims 27-34, comprising at least one internucleotidic linkage having the structure of , , , , , , , or .

36. A chirally controlled oligonucleotide selected from Table 2.

37. A method for making a chirally controlled oligonucleotide comprising steps of:

(1) coupling;

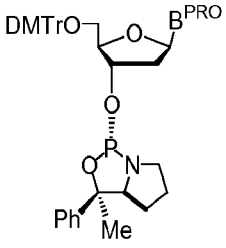
(2) capping;

(3) modifying;

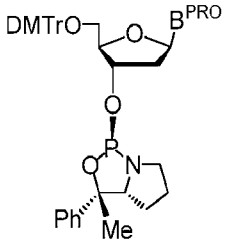
(4) deblocking; and

(5) repeating steps (1) – (4) until a desired length is achieved.

38. The method of claims 37, wherein the step of coupling comprises the use of CMPT and



or



, wherein B^{PRO} is a protected nucleobase.

39. The method of claims 37 or 38, wherein the step of capping comprises steps of capping of the amino group in the chiral auxiliary and the capping of unreacted 5'–OH.

40. The method of claims 39, wherein the step of capping comprises using of $(Pac)_2O$.

41. The method of claims 37, wherein the modifying step comprises sulfurization.

42. The method of claim 41, wherein the sulfurization step comprises using a sulfurization reagent is of formula S-I or S-II.

43, The method of claim 41, wherein the cycle comprising the said sulfurization step forms an internucleotidic linkage having the structure of formula I.

44. The method of claim 37, wherein the step of deblocking comprises steps of using an acid.

45. The method of claim 37, wherein all oligonucleotides that are one nucleotide shorter than the desired length, when combined, are less than 10% of the crude product.

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46. The method of claim 45, wherein all oligonucleotides that are one nucleotide shorter than the desired length, when combined, are less than 5% of the crude product.

47. The method of claim 45, wherein all oligonucleotides that are one nucleotide shorter than the desired length, when combined, are less than 4% of the crude product.

48. The method of claim 45, wherein all oligonucleotides that are one nucleotide shorter than the desired length, when combined, are less than 3% of the crude product.

49. The method of claim 45, wherein all oligonucleotides that are one nucleotide shorter than the desired length, when combined, are less than 2% of the crude product.

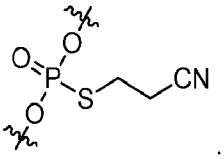
50. The method of claim 45, wherein all oligonucleotides that are one nucleotide shorter than the desired length, when combined, are less than 1% of the crude product.

51. The method of claim 45, wherein all oligonucleotides that are one nucleotide shorter than the desired length, when combined, are less than 0.5% of the crude product.

52. A method for making a chirally controlled oligonucleotide composition, comprising providing one or more oligonucleotide types in a pre-determined amount.

53. The method of claim 37, wherein a phosphorothioate diester precursor is used for each phosphorothioate diester linkage.

54. The method of claim 53, wherein the phosphorothioate diester precursor is



55. The method of claim 53 or 54, wherein each phosphorothioate diester precursor is

converted to a phosphorothioate diester linkage after the desired oligonucleotide length is achieved.

56. The method of any of claims 37-55, wherein at least one modifying step is replaced by an oxidation step.

57. A method for modifying an oligonucleotide comprising steps of:

(1) providing a chirally controlled oligonucleotide;

(2) providing a modification reagent; and

(3) reacting the chirally controlled oligonucleotide with the modification reagent under conditions suitable to effect modification of the linkage phosphorus of the chirally controlled oligonucleotide.

58. The method of claim 57, wherein the modification reagent is of formula S-I or S-II.

59. The method of claim 57, wherein at least one phosphorothioate diester precursor is converted to a phosphorothioate diester linkage.

60. The method of claim 59, wherein each phosphorothioate diester precursor is converted to a phosphorothioate diester linkage.

61. A sulfurization reagents having the formula of S-I.

62. A sulfurization reagents having the formula of S-II.

63. A sulfurization reagent selected from Table 5.

64. A pharmaceutical composition for treating a disease comprising a therapeutically effective amount of the chirally controlled oligonucleotide of any of claims 27-36, and a pharmaceutically acceptable excipient.

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65. A method comprising steps of administering to a subject suffering from or susceptible to cancer an amount of an oligonucleotide composition of any of claims 1-26 and 64, sufficient to partially or completely alleviate, ameliorate, relieve, inhibit, prevent, delay onset of, reduce severity of, and/or reduce incidence of one or more symptoms or features of cancer.

66. The chirally controlled oligonucleotide of any of claims 27-35, wherein the chirally controlled oligonucleotide comprises a sequence found in GCCTCAGTCTGCTTCGCACC.

67. The chirally controlled oligonucleotide of claim 66, selected from Table 4.

68. The chirally controlled oligonucleotide of any of the claims 27-36, 66 and 67, wherein the chirally controlled oligonucleotide has a purity of >50%.

69. The chirally controlled oligonucleotide of claim 68, wherein the chirally controlled oligonucleotide has a purity of >60%.

70. The chirally controlled oligonucleotide of claim 68, wherein the chirally controlled oligonucleotide has a purity of >70%.

71. The chirally controlled oligonucleotide of claim 68, wherein the chirally controlled oligonucleotide has a purity of >80%.

72. The chirally controlled oligonucleotide of claim 68, wherein the chirally controlled oligonucleotide has a purity of >85%.

73. The chirally controlled oligonucleotide of claim 68, wherein the chirally controlled oligonucleotide has a purity of >90%.

74. The chirally controlled oligonucleotide of claim 68, wherein the chirally controlled oligonucleotide has a purity of >95%.

75. The chirally controlled oligonucleotide of claim 68, wherein the chirally controlled oligonucleotide has a purity of >98%.

76. The chirally controlled oligonucleotide of claim 68, wherein the chirally controlled oligonucleotide has a purity of >99%.

77. A chirally controlled oligocucleotide of one of the structures depicted in Figure 26.

78. A chirally controlled oligocucleotide of one of the structures depicted in Figure 27.

79. A chirally controlled oligocucleotide of one of the structures depicted in Figure 28.

80. A chirally controlled oligocucleotide of one of the structures depicted in Figure 29.

81. A chirally controlled oligocucleotide of one of the structures depicted in Figure 30.

82. A chirally controlled oligocucleotide composition comprising one or more of the oligonucleotides depicted in Figure 26.

83. A chirally controlled oligocucleotide composition comprising one or more of the oligonucleotides depicted in Figure 27.

84. A chirally controlled oligocucleotide composition comprising one or more of the oligonucleotides depicted in Figure 28.

85. A chirally controlled oligocucleotide composition comprising one or more of the oligonucleotides depicted in Figure 29.

86. A chirally controlled oligocucleotide composition comprising one or more of the oligonucleotides depicted in Figure 30.

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87. A chirally controlled oligonucleotide of any of the structures depicted in WO 2012/030683.

88. A chirally controlled oligonucleotide composition comprising an oligonucleotide of any of the structures depicted in WO 2012/030683.

89. A chirally controlled oligonucleotide having any of the base sequences described or depicted in WO 2012/030683.

90. A chirally controlled oligonucleotide prepared by a method of any one of claims 37-60.

91. A chirally controlled oligonucleotide prepared by a method comprising one or more cycles depicted in Scheme I.

92. A chirally controlled oligonucleotide prepared by a method comprising one or more cycles depicted in Scheme I-b.

93. A chirally controlled oligonucleotide prepared by a method comprising one or more cycles depicted in Scheme I-c.

94. A chirally controlled oligonucleotide prepared by a method comprising one or more cycles depicted in Scheme I-d.

95. The chirally controlled oligonucleotide of any one of claims 27-36 and 66-94, wherein the chirally controlled oligonucleotide is not linked to a solid support.

96. A chirally controlled oligonucleotide composition comprising an oligonucleotide having any of the base sequences described or depicted in WO 2012/030683.

97. A chirally controlled oligonucleotide composition comprising a chirally controlled oligonucleotide of any one of claims 27-36 and 66-95.

98. A method comprising steps of:

a) performing a first analysis of a first composition, which first composition comprises a plurality of different types of an oligonucleotide; and

b) comparing the performed first analysis with a second analysis, under comparable conditions as the first analysis, of a second composition, which second composition is a chirally controlled composition of the oligonucleotide, where differences between the first and second analyses reflect differences in presence or level of at least one type of the oligonucleotide in the first as compared with the second composition.

99. The method of claim 98, wherein the second composition contains only a single type of the oligonucleotide.

100. The method of claim 98, wherein the second composition is a composition of any one of claim 1-26 and 95-96.