

ACID-FREE THERMAL DEPROTECTION OF TRITYL PROTECTED OLIGONUCLEOTIDES**CROSS REFERENCE TO RELATED APPLICATIONS**

[0001] The present application claims priority of US Provisional Application No. 63/534,076, filed August 22, 2023, which is hereby incorporated by reference in its entirety.

FIELD

[0002] The present disclosure relates to processes for facile removal of trityl-based protecting groups from oligonucleotides, including 5'-amino-modified oligonucleotides, by heating the protected oligonucleotide in an acid-free aqueous solution at a temperature of at least 60°C, optionally with removal of an insoluble trityl-hydroxyl byproduct to further drive the deprotection reaction.

REFERENCE TO SEQUENCE LISTING

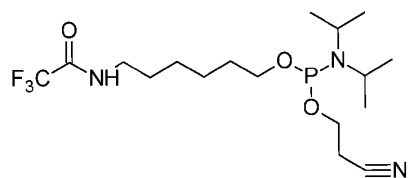
[0003] The official copy of the Sequence Listing is submitted concurrently with the specification via USPTO Patent Center as an WIPO Standard ST.26 formatted XML file with file name "17033-510WO1.xml", a creation date of August 17, 2024, and a size of 9,813 bytes. This Sequence Listing filed via USPTO Patent Center is part of the specification and is incorporated in its entirety by reference herein.

BACKGROUND

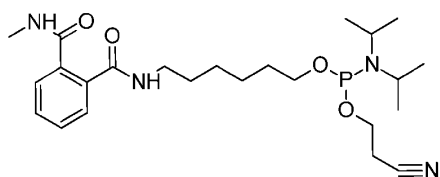
[0004] Nucleic acid conjugates have revolutionized disparate fields ranging from therapeutic intervention to early diagnostic detection and DNA based data storage. The impact of nucleic acid conjugates has been accelerated by the constant evolution of synthetic methods for the incorporation of chemically reactive handles for downstream derivatization. One clear example being the field of nucleic acid therapeutics where the incorporation of ligands has greatly improved both the drug-like properties and cell specific delivery of antisense oligonucleotides (ASOs) and small interfering RNAs (siRNAs) (see e.g., references 1-4). Installation of reactive chemical handles during synthesis that enable efficient post-synthesis conjugation is critical for the generation of these conjugates. Although several handles exist for which robust conjugation reactions are widely employed (see e.g., references 5-6), the amide bond forming reaction between an amine and activated ester is universal due to the cost-effectiveness and relatively simple preparation of starting materials (see e.g., references 7-9).

[0005] There exist two major classes of phosphoramidite reagents capable of labeling oligonucleotides with 5'-amines that differ in their amino protecting group and therefore their method of deprotection. The first group contains base labile protection including both

trifluoroacetyl (TFA) (compound 1) and phthalic acid diamide (PDA) (compound 2), the structures of which are shown below.



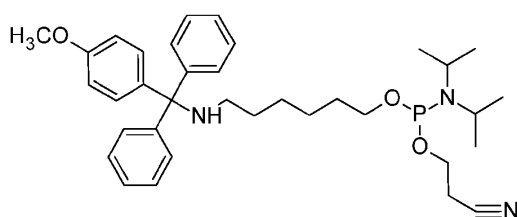
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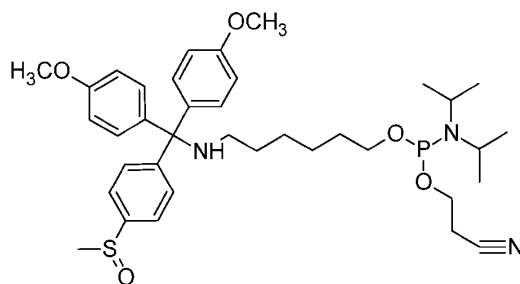
(2)

[0006] In the former case, the TFA group is readily deprotected during standard cleavage and deprotection in concentrated aqueous ammonia, while in the latter case, the PDA group requires a 1:1 mixture of concentrated aqueous ammonia and methylamine (AMA). This approach requires conjugation to occur in the crude synthesis mixture and hinges on the subsequent conjugate being sufficiently distinct chemically, to allow isolation from the truncated oligonucleotide sequences generated during synthesis. This requires the use of HPLC purification, which is expensive, time consuming, and not accessible in every lab.

[0007] The second group contains the reagents with trityl-based protecting groups. MMTr (compound 3) and 5'-DMS(O)MT (compound 4), the structures of which are shown below, are two of the most widely used reagents for labeling oligonucleotides with 5'-amines which due to the convenient hydrophobic handle provided by the trityl-based protecting group enables routine cartridge purification.



(3)



(4)

[0008] The compatibility of these reagents with cartridge purification greatly accelerates the ability to generate many high-quality conjugates without the need for laborious purifications. A significant challenge in using these reagents with trityl-based protecting groups (which is noted on supplier websites), however, is the propensity of the trityl group to remain attached during deprotection or to unexpectedly detach during routine handling (see e.g., reference 10). In addition to these challenges, the acidic treatment required to deprotect these reagents can cause undesired depurination of the oligonucleotide resulting in abasic sites and cleavage products (see e.g., references 11-13). Previous studies have explored alternative approaches to minimizing depurination, such as using less acidic deprotection cocktails for DMTr and MMTr protected oligonucleotides (see e.g., references 14-15). Alternative protecting groups for amino amidites have received much attention, however, none have made significant improvements over the MMTr-protected amine linker phosphoramidite reagent of compound (3), which to date remains the most practical and utilized option (see e.g., reference 16).

SUMMARY

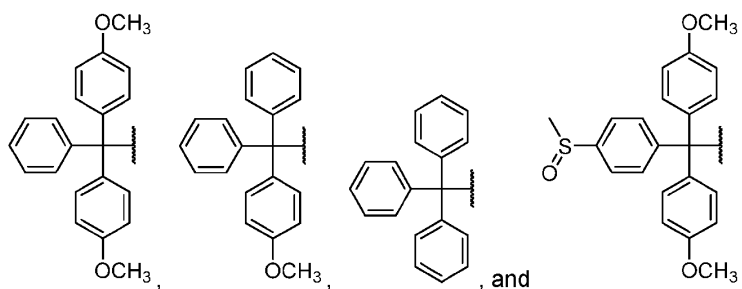
[0009] A practical method for synthesizing 5'-amine labeled oligonucleotides using phosphoramidite reagents with trityl-based protecting groups, such as compound (3), that solves the challenge of incomplete deprotection and/or oligonucleotide depurination, would greatly facilitate the routine synthesis of oligonucleotide conjugates, and open avenues for oligonucleotide modifications that are not amenable to conventional deprotection strategies. Accordingly, there remains a need for improved processes of deprotecting the trityl protecting groups that are commonly used in the synthesis of oligonucleotides, such as 5'-amino-modified oligonucleotides.

[0010] The present disclosure relates to methods of deprotecting a trityl-based protecting group from an amine group or a hydroxyl group during synthesis of oligonucleotide, such as an oligonucleotide modified with a 5'-amino group. The methods generally comprise heating in an acid-free aqueous solution at a temperature of at least 60°C the protected oligonucleotide comprising a trityl-based protecting group on an amine or hydroxyl group, thereby resulting in an oligonucleotide with a deprotected amine group or hydroxyl group. The method optionally comprises concomitant removal of a trityl-hydroxyl byproduct of deprotection to further drive the

deprotection reaction to rapid completion. The methods allow facile deprotection of trityl-based protecting groups with little or no depurination of the product oligonucleotide. This summary is intended to introduce the subject matter of the present disclosure, but does not cover each and every embodiment, combination, or variation that is contemplated and described within the present disclosure. Further embodiments are contemplated and described by the disclosure of the detailed description, drawings, and claims.

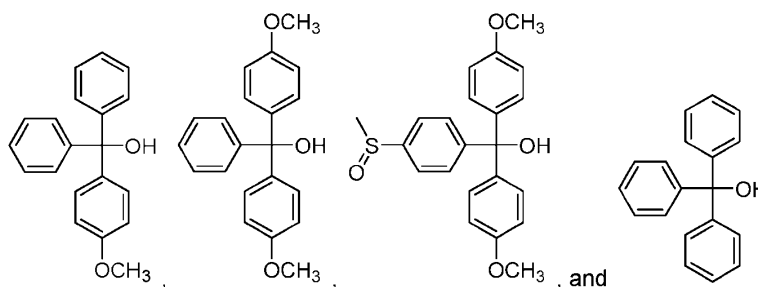
[0011] In at least one embodiment, the present disclosure provides a method of deprotecting an oligonucleotide comprising an amine group or a hydroxyl group protected by a trityl-based protecting group, the method comprising heating the oligonucleotide in an acid-free aqueous solution at a temperature of at least 60°C, thereby cleaving the trityl based protecting group to provide the oligonucleotide comprising the deprotected amine group or hydroxyl group.

[0012] In at least one embodiment of the method, the trityl-based protecting group is selected from: trityl, monomethoxytrityl (MMTr), dimethoxytrityl (DMTr), and dimethoxymethylsulfonyltrityl (DMS(O)MTr). In at least one embodiment, the trityl-based protecting group has a structure selected from:



wherein  represents a covalent bond to the nitrogen atom of the amine group, or the oxygen atom of the hydroxyl group.

[0013] In at least one embodiment of the method, the deprotection of the amine group or hydroxyl group produces an insoluble trityl byproduct, and the method further comprises separating the insoluble trityl byproduct from the deprotected oligonucleotide. In at least one embodiment, the insoluble trityl byproduct is separated by extraction, desalting, or precipitation and filtration. In at least one embodiment, the insoluble trityl byproduct is selected from: MMTr-OH, DMTr-OH, Tr-OH, and DMS(O)Tr-OH. In at least one embodiment, the insoluble trityl byproduct has a structure selected from:



[0014] In at least one embodiment of the method, the amine group or hydroxyl group is attached to the 5' phosphate group of the oligonucleotide.

[0015] In at least one embodiment of the method, the amine group or hydroxyl group is attached to the 5' phosphate group of the oligonucleotide through a linker group. In at least one embodiment, the linker group comprises a 5-carbon to 12-carbon unbranched alkylene chain. In at least one embodiment, the linker group comprises a 4-carbon to 12-carbon unbranched alkylene chain with at least one ether linkage.

[0016] In at least one embodiment, the linker is selected from: a succinate linker, a PEG3 linker, a PEG4 linker, a sarcosine-glutarate linker, a hydrazone linker, a disulfide linker, a valine-citrulline linker, a valine-alanine linker, a tris-hexylamino linker, a hydroquinone-O,O'-diacetate ("Q") linker, a hexylamine linker, a hexyloxy linker, pentethyleneglycol linker, and a derivative thereof. In one embodiment, the linker is selected from a PEG3 linker $-(\text{CH}_2\text{CH}_2\text{O})_3-$ and a PEG4 linker $-(\text{CH}_2\text{CH}_2\text{O})_4-$.

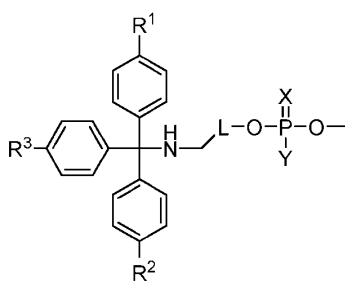
[0017] In at least one embodiment of the method, the amine group of the oligonucleotide protected by a trityl-based protecting group is a primary amine group.

[0018] In at least one embodiment of the method, the method further comprises conjugating a ligand to the oligonucleotide by reacting the deprotected primary amine group of the oligonucleotide with a ligand molecule comprising an amine-reactive group, thereby forming a ligand-oligonucleotide conjugate. In at least one embodiment, the amine-reactive group is selected from an ester, an NHS ester, and a carboxylic acid. In at least one embodiment, the ligand is selected from N-acetylgalactosamine (GalNAc), a triantennary cluster of N-acetylgalactosamine moieties, a lipid molecule, a small molecule peptide (e.g., RGD, a cell penetrating peptide, an integrin), a protein, and an antibody.

[0019] In at least one embodiment of the method, the amine group of the oligonucleotide protected by a trityl-based protecting group is a secondary amine of a phosphorodiamidate morpholino oligomer (PMO) linkage.

[0020] In at least one embodiment of the method, the percentage conversion of the oligonucleotide with trityl-based protecting group to the oligonucleotide with deprotected amine group or hydroxyl group is at least 95%, at least 97%, at least 99%, at least 99.5%, or at least 100%.

[0021] In at least one embodiment, the present disclosure provides a method of deprotecting a 5'-amine modified oligonucleotide compound of Formula (I), the method comprising heating the 5'-amino modified oligonucleotide compound of Formula (I) in an acid-free aqueous solution at a temperature of at least 60°C to form an oligonucleotide compound of Formula (II) and a byproduct compound of Formula (III):



(I)

or a salt thereof or a pharmaceutically acceptable salt thereof,

wherein:

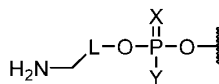
each of R^1 , R^2 , and R^3 is independently hydrogen, $-OCH_3$, or $-S(O)CH_3$;

L is an optional linker;

X is O or S;

Y is OH or SH; and

— represents a covalent bond to the 5'-end of the oligonucleotide;



(II)

or a salt thereof, or a pharmaceutically acceptable salt thereof,

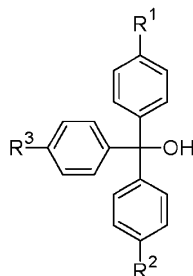
wherein:

X is O or S;

Y is -OH or -SH;

L is an optional linker; and

— represents a covalent bond to the 5'-end of the oligonucleotide;



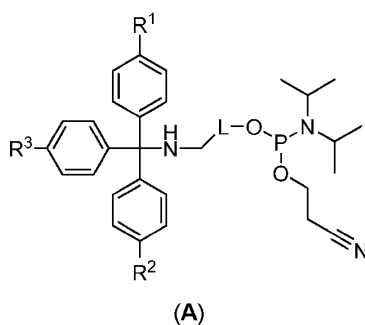
(III)

wherein:

each of R^1 , R^2 , and R^3 is independently hydrogen, $-OCH_3$, or $-S(O)CH_3$.

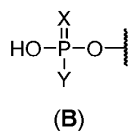
[0022] In at least one embodiment of the method of deprotecting an oligonucleotide compound of Formula (I), the method further comprises separating the oligonucleotide compound of Formula (II) from the byproduct compound of Formula (III) by extraction, desalting, or precipitation and filtration.

[0023] In at least one embodiment of the method of deprotecting an oligonucleotide compound of Formula (I), the oligonucleotide compound of Formula (I) is prepared by reacting a phosphoramidite compound of Formula (A) with an oligonucleotide compound of Formula (B):



wherein:

each of R^1 , R^2 , and R^3 is independently hydrogen, $-OCH_3$, or $-S(O)CH_3$; and
L is an optional linker;



or a salt thereof, or a pharmaceutically acceptable salt thereof,

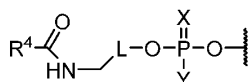
wherein:

X is O or S;

Y is $-OH$ or $-SH$; and

represents a covalent bond to the 5'-end of the oligonucleotide.

[0024] In at least one embodiment of the method of deprotecting an oligonucleotide compound of Formula (I), the method further comprises reacting the oligonucleotide compound of Formula (II) with a ligand molecule comprising an amine-reactive group to form a ligand-oligonucleotide conjugate compound of Formula (IV):



(IV)

or a salt thereof, or a pharmaceutically acceptable salt thereof,

wherein:

L is a divalent linker;

X is S or O;

Y is -SH or -OH;

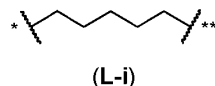
R⁴ is a ligand; and

 represents a covalent bond to the 5'-end of the oligonucleotide.

[0025] In at least one embodiment of the method of deprotecting an oligonucleotide compound of Formula (I), the amine-reactive group is selected from an ester, an NHS ester, and a carboxylic acid.

[0026] In at least one embodiment of the method of deprotecting an oligonucleotide of Formula (I), the ligand R⁴ is selected from N-acetylgalactosamine (GalNAc), a triantennary cluster of N-acetylgalactosamine moieties, a lipid molecule, a small molecule peptide (e.g., RGD, a cell penetrating peptide, an integrin), a protein, and an antibody.

[0027] In at least one embodiment of the method of deprotecting an oligonucleotide compound of Formula (I), the linker moiety L is a divalent linker of Formula (L-i):



wherein:

 represents a covalent bond to the nitrogen atom of the primary amine group; and

 represents a covalent bond to the oxygen atom of the phosphate group.

[0028] In at least one embodiment of the method of deprotecting an oligonucleotide compound of Formula (I), the linker L comprises: (i) a 5-carbon to 20-carbon branched or unbranched alkylene chain; or (ii) a 4-carbon to 20-carbon branched or unbranched alkylene chain with at least one ether linkage (or wherein at least one carbon atom of the chain is replaced with an oxygen atom). In another embodiment of the method of deprotecting an oligonucleotide compound of Formula (I), the linker L comprises a 4-carbon to 12-carbon unbranched alkylene chain, such as a 4-carbon, 5-carbon, 6-carbon, 7-carbon, 8-carbon, 9-carbon, 10-carbon, 11-carbon, or 12-carbon unbranched alkylene chain. In another embodiment of the method of deprotecting an oligonucleotide compound of Formula (I), the linker L comprises a 4-carbon to 12-carbon unbranched alkylene chain, such as a 4-carbon, 5-carbon, 6-carbon, 7-carbon, 8-carbon, 9-carbon, 10-carbon, 11-carbon, or 12-carbon unbranched alkylene chain, with at least one ether linkage (or wherein at least one carbon atom of the chain is replaced with an oxygen atom).

[0029] In at least one embodiment of the method of deprotecting an oligonucleotide compound of Formula (I), the linker L comprises: a succinate linker, a PEG3 linker, a PEG4 linker, a sarcosine-glutarate linker, a hydrazone linker, a disulfide linker, a valine-citrulline linker, a valine-alanine linker, a tris-hexylamino linker, a hydroquinone-O,O'-diacetate ("Q") linker, a hexylamine linker, a hexyloxy linker, pentethyleneglycol, or a derivative thereof. In one embodiment, the linker L comprises a PEG3 linker $-(\text{CH}_2\text{CH}_2\text{O})_3-$. In another embodiment, the linker L comprises a PEG4 linker $-(\text{CH}_2\text{CH}_2\text{O})_4-$.

[0030] In at least one embodiment of the methods of deprotecting an oligonucleotide of the present disclosure, the acid-free aqueous solution is heated to a temperature of at least about 65°C, at least about 70°C, at least about 75°C, at least about 80°C, at least about 85°C, at least about 90°C, or at least about 95°C. In at least one embodiment, the acid-free aqueous solution is heated to a temperature of about 60°C to about 100°C, about 60°C to about 80°C, about 60°C to about 75°C, or about 60°C to about 70°C.

[0031] In at least one embodiment of the methods of deprotecting of the present disclosure, the acid-free aqueous solution is heated for at least about one hour, or at least about two hours. In at least one embodiment, the acid-free aqueous solution is heated for about one hour to about two hours.

[0032] In at least one embodiment of the methods of deprotecting an oligonucleotide of the present disclosure, the acid-free aqueous solution has a pH of about pH 6.5 to about pH 8.0, about pH 6.8 to about pH 7.8, about pH 6.8 to about pH 7.6, about pH 7.0, about pH 7.2, or about pH 7.4.

[0033] In at least one embodiment of the methods of deprotecting an oligonucleotide compound of Formula (I), the percentage of the oligonucleotide compound of Formula (I) converted to oligonucleotide compound of Formula (II) is at least 95%, at least 97%, at least 99%, at least 99.5%, or at least 100%.

[0034] In at least one embodiment of the methods of deprotecting an oligonucleotide of the present disclosure, the percentage of depurinated oligonucleotide relative to the total oligonucleotide detected by MS analysis after the deprotection is less than 5%, less than 2%, less than 1%, less than 0.5%, less than 0.1%, or less than 0.01%.

[0035] In at least one embodiment of the methods of deprotecting an oligonucleotide of the present disclosure, the oligonucleotide length is from 2-mer to 100-mer; optionally, wherein the oligonucleotide length is from 3-mer to 75-mer, from 5-mer to 50-mer, 15-mer to 50-mer, 15-mer to 40-mer, 15-mer to 30-mer, or 2-mer to 10-mer.

BRIEF DESCRIPTION OF THE DRAWINGS

[0036] A better understanding of the novel features and advantages of the present disclosure will be obtained by reference to the following detailed description that sets forth illustrative

embodiments, in which the principles of the disclosure are utilized, and the accompanying drawings (also "Figure" and "FIG." herein), of which:

[0037] FIG. 1A depicts liquid chromatography (LC) plots over time showing the incomplete deprotection of oligonucleotide MMTr-ON #5 under the standard acid-catalyzed deprotection protocol as described in Example 2.

[0038] FIG. 1B and **FIG. 1C** depict plots of MS peaks detected for the acid-catalyzed deprotection of oligonucleotide MMTr-ON #5 (**FIG. 1B**) versus the MS peaks detected for the acid-free thermal deprotection (**FIG. 1C**) as described in Example 2. The MS peaks in **FIG. 1B** indicate detection of >5% depurinated species due to the acid-catalyzed deprotection protocol.

[0039] FIG. 1D depicts LC plots showing how varying conditions of % acetic acid, temperature, and incubation time affects the acid-catalyzed deprotection of oligonucleotide MMTr-ON #5 as described in Example 2.

[0040] FIG. 1E depicts a plot of MS peaks observed for an acid-catalyzed deprotection of oligonucleotide MMTr-ON #5 reaction carried out at 45°C as described in Example 2. The MS peaks indicate increased depurination of the deprotected oligonucleotide MMTr-ON #5 product.

[0041] FIG. 1F depicts LC plots over time showing that the use of the acid-free thermal deprotection conditions resulted in full deprotection of the oligonucleotide MMTr-ON #5 in 60 minutes as described in Example 2.

[0042] FIG. 2A depicts NMR spectra confirming that the white precipitate formed during the acid-free thermal deprotection protocol is the reaction byproduct, MMTr-OH. **FIG. 2A**, top spectrum is from a commercial sample of MMTr-OH; **FIG. 2A**, bottom spectrum is from the isolated white precipitate reaction byproduct.

[0043] FIG. 2B depicts a schematic comparison of the acid-catalyzed deprotection reaction and the acid-free thermal deprotection of an MMTr protected oligonucleotide (indicated by "A") to form the deprotected 5'-amine linker modified oligonucleotide product (indicated by "B"). The reactions illustrated how the insolubility of the byproduct MMTr-OH in water helps to drive the acid-free thermal deprotection reaction to completion, whereas the solubility of the positive ion byproduct, MMTr⁺ formed in the acid-catalyzed reaction can allow the reverse reaction in equilibrium reform the undesired MMTr protected oligonucleotide starting material.

[0044] FIG. 3A, FIG. 3B, FIG. 3C, FIG. 3D, FIG. 3E, and FIG. 3F depict exemplary LC plots comparing the acid-catalyzed and acid-free thermal deprotection of six MMTr protected oligonucleotides with different sequences as described in Example 4. As shown in **FIG. 3A**, "A" indicates the peak due to the protected MMTr-ON starting material and "B" indicates the peak due to the deprotected 5'-amine linker modified oligonucleotide product. **FIG. 3A**: MMTr-ON #6; **FIG. 3B**: MMTr-ON #7; **FIG. 3C**: MMTr-ON #9; **FIG. 3D**: MMTr-ON #11; **FIG. 3E**: MMTr-ON #12; and **FIG. 3F**: MMTr-ON #13.

[0045] FIG. 4A depicts a schematic of the comparative reaction and conditions used for deprotection of a 5'-DMTr-O-protected oligonucleotide (indicated by "A") to yield the deprotected oligonucleotide with 5'-OH group as described in Example 5.

[0046] FIG. 4B depicts LC plots showing that full deprotection of 5'-DMTr-ON starting material (peak indicated by "A") to form deprotected oligonucleotide with 5'-OH group (peak indicated by "B") was achieved using acid-free thermal deprotection conditions of 1.5 hours at 95°C as described in Example 5.

DETAILED DESCRIPTION

[0047] For the descriptions herein and the appended claims, the singular forms "a", and "an" include plural referents unless the context clearly indicates otherwise. Thus, for example, reference to "an oligonucleotide" includes more than one oligonucleotide, and reference to "a compound" refers to more than one compound. It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely," "only" and the like in connection with the recitation of claim elements, or use of a "negative" limitation. The use of "comprise," "comprises," "comprising" "include," "includes," and "including" are interchangeable and not intended to be limiting. It is to be further understood that where descriptions of various embodiments use the term "comprising," those skilled in the art would understand that in some specific instances, an embodiment can be alternatively described using language "consisting essentially of" or "consisting of."

[0048] Where a range of values is provided, unless the context clearly dictates otherwise, it is understood that each intervening integer of the value, and each tenth of each intervening integer of the value, unless the context clearly dictates otherwise, between the upper and lower limit of that range, and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of these limits, ranges excluding (i) either or (ii) both of those included limits are also included in the invention. For example, "1 to 50," includes "2 to 25," "5 to 20," "25 to 50," "1 to 10," etc.

[0049] Ranges can be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, another aspect includes from the one particular value of the range and/or to the other particular value of the range. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint. Similarly, when values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another aspect.

[0050] Generally, the nomenclature used herein and the techniques and procedures described herein include those that are well understood and commonly employed by those of ordinary skill in the art. Singleton, et al., *DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY*, 2D ED., John Wiley and Sons, New York (1994), and Hale & Marham, *THE HARPER COLLINS DICTIONARY OF BIOLOGY*, Harper Perennial, NY (1991) provide one of skill with a general dictionary of many of the terms used in this invention. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred techniques and methodologies are described in e.g., Green and Sambrook, *Molecular Cloning: A Laboratory Manual* (Fourth Edition), Vols. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 2012 (hereinafter "Sambrook"); and *Current Protocols in Molecular Biology*, F. M. Ausubel et al., eds., originally published in 1987 in book form by Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., and regularly supplemented through 2011, and now available in journal format online as *Current Protocols in Molecular Biology*, Vols. 00 - 130, (1987-2020), published by Wiley & Sons, Inc. in the Wiley Online Library (hereinafter "Ausubel"). It is to be understood that this invention is not limited to the particular methodology, protocols, and reagents described, as these may vary.

[0051] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the present invention pertains. It is to be understood that the terminology used herein is for describing particular embodiments only and is not intended to be limiting.

[0052] "Oligonucleotide" as used herein is defined as it is generally understood by the skilled person as a molecule including two or more covalently linked nucleotides. Such covalently bound nucleotides may also be referred to as nucleic acid molecules or oligomers. Oligonucleotides are commonly made in the laboratory by solid-phase chemical synthesis followed by purification, but can also be produced enzymatically using purified enzymes such as ligases and/or polymerases. When referring to a sequence of the oligonucleotide, reference is made to the sequence or order of nucleobase moieties, or modifications thereof, of the covalently linked nucleotides or nucleosides. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively. The present disclosure is generally directed to oligonucleotides that are chemically synthesized (i.e., man-made) and then, typically purified or isolated. However, oligonucleotides used in the methods of the present disclosure can also be produced enzymatically and then optionally purified and/or isolated. Oligonucleotides as used herein are intended to include modified oligonucleotides, such as those having modified sugars, modified internucleotide linkages, and/or attached conjugate groups, such as a 5' attached amino group protected by a trityl-based protecting group. Oligonucleotides as used herein can also be therapeutic oligonucleotides, such as antisense oligonucleotides and small interfering RNAs (siRNAs).

[0053] “Nucleotide” as used herein refers to a glycoside including a sugar moiety, a nucleobase moiety, and a phosphate or modified phosphate group (e.g., phosphorothioate), and covers both the naturally occurring nucleotides found in DNA (dA, dC, dG, and dT) and RNA (A, C, G, U), , and non-naturally occurring nucleotides with modified sugar and/or base moieties that are well known in the art of therapeutic antisense oligonucleotides (ASOs) and small interfering RNAs (siRNAs), such locked nucleic acid (LNA) nucleotides, and phosphorodiamidate morpholino oligomer (PMO) nucleotides.

[0054] “Phosphorothioate linkage” or “PS linkage” as used herein refers to an internucleotide phosphate linkage in an oligonucleotide where one of the non-bridging oxygens has been substituted with a sulfur. The substitution of one of the non-bridging oxygens with a sulfur introduces a chiral center, and as such within a single phosphorothioate oligonucleotide, the phosphorothioate internucleotide linkage will be either in the *S* (Sp) or *R* (Rp) stereoisomers. Such internucleotide linkages are referred to as “chiral internucleotide linkages.” By comparison, phosphodiester internucleotide linkages are non-chiral as they have two non-terminal oxygen atoms. As those skilled in the art will appreciate, a given phosphorothioate oligonucleotide can have a mixture of different *S* (Sp) or *R* (Rp) linkages.

[0055] As used herein, a “nucleobase” includes the purine (e.g. adenine and guanine) and pyrimidine (e.g. uracil, thymine and cytosine) moiety present in nucleosides and nucleotides which form hydrogen bonds in nucleic acid hybridization. In the context of the present disclosure, the term nucleobase also includes modified (or non-naturally occurring) nucleobases which may differ from naturally occurring nucleobases, but are functional during nucleic acid hybridization and/or suitable for incorporation into a therapeutic or diagnostic oligonucleotide.

[0056] The nucleobase moieties may be indicated by the letter code for each corresponding nucleobase, e.g. A, T, G, C or U, wherein each letter may optionally include modified nucleobases of equivalent function. For example, in the exemplified oligonucleotides, the nucleobase moieties are selected from A, T, G, C, and 5-methyl cytosine. Optionally, for LNA gapmers, 5-methyl cytosine LNA nucleosides may be used.

[0057] A “modified nucleotide” refers to a nucleotide having a modified sugar moiety, a modified linkage, or a modified base.

[0058] A “modified oligonucleotide” refers to an oligonucleotide including one or more nucleobase modifications, one or more sugar-modified nucleotides, one or more modified internucleotide linkages, and/or one or more 5'-end or 3'-end modifications, such as 5'-amino-linker moiety that can act as a reactive handle to attach ligands or conjugate moieties to the oligonucleotide.

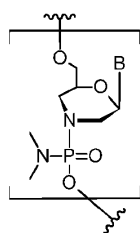
[0059] “Trityl-based protecting group” as used herein refers to a protecting group that comprises a triphenylmethyl radical.

[0060] “Ligands” or “conjugate moieties” refers to molecular groups or moieties that can be attached to oligonucleotides, directly or indirectly through a linker.

[0061] “Linker,” as used herein, refers to any divalent molecular moiety that covalently attaches two or more molecules, molecular groups, and/or molecular moieties, such as an oligonucleotide and a ligand. Linkers useful in the methods of the present disclosure include but are not limited to saturated divalent aliphatic radical chains of carbon atoms (also referred to herein as “alkylene” chains). For example, exemplary linkers, as described elsewhere herein, can comprise a 5-carbon to 12-carbon branched or unbranched alkylene chain, and optionally, can further comprise one or more ether linkages (e.g., PEG).

[0062] “Locked nucleic acid” or “LNA” refers to a bicyclic nucleoside analogue that includes a bridge between the 2' and 4' position in the ribose ring (2' to 4' bicyclic nucleotide analogue). For example, the LNA contains one or more nucleotides with an extra methylene bridge that fixes the ribose moiety either in the C3'-endo (beta-D-LNA) or C2'-endo (alpha-L-LNA) conformation. As those skilled in the art will appreciate, LNAs are also referred to as BNAs (bridged nucleic acid or bicyclic nucleic acid) and the two terms may be used interchangeably. The term LNA may also refer to an LNA monomer, or, when used in the context of an “LNA oligonucleotide,” LNA refers to an oligonucleotide containing one or more such bicyclic nucleotide analogues. In some aspects, bicyclic nucleoside analogues are LNA nucleotides, and these terms may therefore be used interchangeably, and in such embodiments, both are characterized by the presence of a linker (such as a bridge) between C2' and C4' of the ribose sugar ring. LNAs, for example, provide stability against enzymatic degradation and provide improved specificity and affinity in base-pairing as a monomer or a constituent of an oligonucleotide.

[0063] “Phosphorodiamidate morpholino oligomer” or “PMO” as used herein refers to an oligomeric molecule that includes a nucleobase attached to a backbone of 6-membered methylenemorpholine rings linked via phosphorodiamidate linkages, as illustrated by the backbone structure depicted below



PMO backbone structure

[0064] “Antisense oligonucleotide,” abbreviated “ASO,” refers to a single-stranded oligomer of polymer of nucleosides having a nucleobase sequence that permits hybridization to a corresponding region (target region) or segment of a target nucleic acid. As those skilled in the

art will appreciate, antisense oligonucleotides are capable of modulating expression of a target gene by hybridizing to a target nucleic acid, in particular to a contiguous sequence (a sub-sequence) on a target nucleic acid.

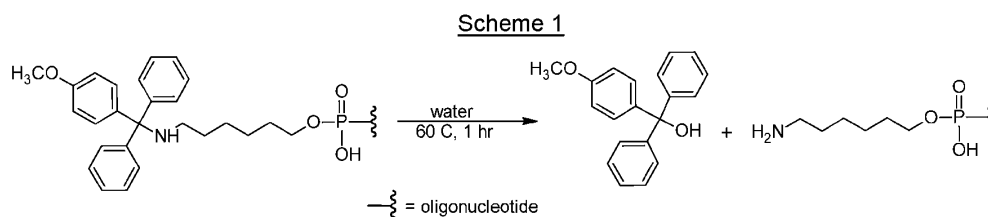
[0065] Acid-free thermal deprotection of trityl-based protecting groups

[0066] The present disclosure provides methods useful for deprotecting trityl-based protecting groups from oligonucleotides comprising a trityl-protected amine group or hydroxyl group, such as an oligonucleotide modified with a 5'-amino group. The methods of the present disclosure provide facile deprotection of trityl-based protecting groups with the advantage of providing complete deprotection with little or no depurination of the oligonucleotide product.

[0067] The deprotection method comprises heating the oligonucleotide comprising a trityl-based protecting group protecting an amine or hydroxyl group in an acid-free aqueous solution (e.g., about pH 6.5 to about pH 8.0) at a temperature of at least 60°C for a period of time, typically at least about 1 hour. As described in greater detail below and in the Examples, the present disclosure contemplates that the method can be carried out with a range of trityl-based protecting groups, and under a range of conditions of temperature and pH.

[0068] Further, as is illustrated in the Examples, the deprotection method can work on any protected oligonucleotide independent of its sequence or length. Accordingly, the deprotection method can be carried out where the oligonucleotide length is from 2-mer to 100-mer; optionally, wherein the oligonucleotide length is from 3-mer to 75-mer, from 5-mer to 50-mer, 15-mer to 50-mer, 15-mer to 40-mer, 15-mer to 30-mer, or 2-mer to 10-mer. Also, as illustrated in the Examples, the deprotection method can work on oligonucleotides having modified groups commonly used in therapeutic oligonucleotides (such as ASOs or siRNAs), including but not limited to phosphorothioate linkages, phosphorodiamidate linkages, 2'-sugar modifications, and/or LNA nucleotides.

[0069] The combination of heating in an acid-free aqueous solution as used in the method of the present disclosure results in complete or nearly complete deprotection of the trityl-based protecting group protecting an amine group or hydroxyl group, such as a 5'-amino or 5'-hydroxyl. The acid-free thermal reaction that results in the deprotection of trityl-based protecting group on a 5'-amine modified oligonucleotide according to an embodiment of the methods described herein is illustrated in Scheme 1 below.



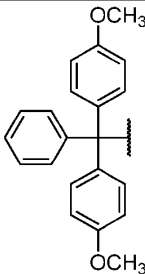
[0070] As illustrated by the reaction of Scheme 1, the acid-free conditions used in the deprotection method of the present disclosure results in the formation of a free primary amine

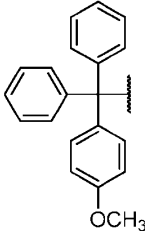
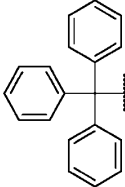
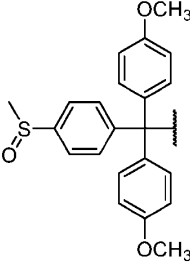
on a C6 linker attached to the 5' end of an oligonucleotide and a trityl hydroxide byproduct. Under the acid-free conditions of the reaction, this byproduct is insoluble and precipitates. Without intending to be bound by any particular mechanism, the formation of this insoluble trityl-hydroxide byproduct helps to drive the reaction to completion. The concomitant removal or sequestration of the insoluble byproduct from the reaction can act to further accelerate the deprotection reaction to rapid completion. Accordingly, in at least one embodiment, the method of acid-free thermal deprotection is carried out wherein deprotection of the amine group or hydroxyl group produces an insoluble trityl byproduct, and the method further comprises separating the insoluble trityl byproduct from the deprotected oligonucleotide. Separation can be carried out using standard methods and techniques for separating out insoluble byproducts from a reaction in aqueous solution. In at least one embodiment, the method can further comprise separating the insoluble trityl byproduct by extraction, desalting, or precipitation and filtration.

[0071] The formation of this insoluble byproduct during trityl-based deprotection contrasts to the byproduct formed under the standard acidic conditions used in the art for such a deprotection (e.g., 20% acetic acid, 25°C, 1 hour). The standard acidic deprotection conditions result in formation of a soluble positively charged triphenyl ion byproduct that undergoes the reverse "protection" reaction in equilibrium with the deprotection reaction. Thus, the presence of the soluble positively charged byproduct slows the progress of the deprotection reaction and can reduce overall yield of the desired deprotected product.

[0072] Trityl-based protecting groups share a common triphenylmethyl (or trityl) moiety structure that has been found to be particularly useful as a protecting group. A wide range of trityl groups with different groups substituted on the phenyl rings are known in the art of protecting groups. Any of these known trityl-based protecting groups are contemplated for use in the methods of the present disclosure. Exemplary trityl-based protecting groups useful in the compositions and methods of the present disclosure include but are not limited to the chemical moieties shown in Table 1 below.

[0073] TABLE 1: Exemplary trityl-based protecting groups

Name	Structure
Dimethoxytrityl- (DMTr)	

Monomethoxytrityl- (MMTr)	
Trityl- (Tr)	
6-(4,4'-Dimethoxy-4''-methylsulfonyl-trityl) OR Dimethoxymethylsulfonyltrityl- (DMS(O)MTr)	
In the above structures,  represents a covalent bond to a nitrogen atom or an oxygen atom	

[0074] Table 1 (above) provides structures for a few exemplary trityl-based protecting groups that are well-known and that can be used with the acid-free thermal deprotection method: Trityl- (Tr), Dimethoxytrityl- (DMTr), Monomethoxytrityl- (MMTr), Dimethoxymethylsulfonyltrityl- (DMS(O)MTr).

[0075] Trityl-based protecting groups, such as DMTr, are widely used as the protecting group of the 5'-hydroxyl group during standard automated solid-phase oligonucleotide synthesis (SPOS). During the cycles of SPOS an acidic solution at room temperature is added to remove DMTr and provide a free 5'-OH that can couple to the next phosphoramidite reagent. Oligonucleotides prepared in SPOS also commonly include a protected 5' "handle" in the final step that can be deprotected to provide a reactive group for subsequent 5'-conjugation to the oligonucleotide. This reactive 5' handle is usually a primary amine group, or a hydroxyl group. Either of these groups can be easily conjugated to a ligand using a variety of well-known chemical reactions. Standard SPOS conditions for deprotection of the trityl-based protecting groups are acidic conditions. But as noted in the Background and elsewhere herein, deprotection of trityl-based protecting groups under acidic conditions, particularly in the case of a 5'-protected amine group or hydroxyl group can be incomplete or result in deleterious effects such as depurination of the oligonucleotide. As described elsewhere herein and shown in the

Examples, the acid-free thermal deprotection method of the present disclosure results in little or no depurination of the deprotected oligonucleotide. Accordingly, in at least one embodiment of the methods disclosed herein, the percentage of depurinated oligonucleotide relative to the total oligonucleotide detected by mass spectral (MS) analysis after the deprotection is less than 5%, less than 2%, less than 1%, less than 0.5%, less than 0.1%, or less than 0.01%.

[0076] As noted above, oligonucleotide modified with a trityl-based protecting group protecting a reactive amine or hydroxyl group can be prepared using standard automated SPOS systems and commercially available phosphoramidite reagents from well-known SPOS reagent vendors such as Glen Research Inc. (Sterling, VA, USA) and Hongene Biotech Corporation (Shanghai, China). For example, the phosphoramidite reagent, "MMTr-Amine Linker Phosphoramidite," can be used to add a MMTr-protected amine group attached to a C6 alkyl linker to the 5' end of an oligonucleotide during the final step of SPOS. After this MMTr-protected oligonucleotide is cleaved from the solid support and purified it can be stored. Then when desired, the MMTr-protected oligonucleotide can be subjected to the acid-free thermal deprotection method in aqueous solution as disclosed herein to produce the deprotected oligonucleotide with a reactive 5'-amine group that is available for further reaction, such as conjugation to a ligand, as described below.

[0077] The deprotection method of the present disclosure is carried out in an acid-free aqueous solution. The term "acid-free" as used in this context means any aqueous solution having a pH of about pH 6.5 to about pH 8.0. Buffer salts can be included in the aqueous solutions. Exemplary acid-free aqueous solutions useful in the methods of the present disclosure include a neutral unbuffered water solution (e.g., pH 7.0 water), or buffered solutions (e.g., pH 7.4, PBS or pH 8.0, TES buffer). Generally, it is preferred that the acid-free aqueous solution is not strongly basic (e.g., 28% NH_4OH). A strongly basic solution (e.g., > pH 9) can have deleterious effects on the deprotection reaction as is illustrated in the Examples.

[0078] The presence of organic solvents (e.g., CH_3CN) in the acid-free aqueous solution can result in decreased efficiency of the deprotection reaction, as is shown in the Examples. This decrease in efficiency is likely due to the presence of the organic solvent acting to solubilize the trityl-hydroxide byproduct formed during the deprotection reaction. Accordingly, in at least one embodiment, the acid-free aqueous solution comprises less than 25%, less than 10%, less than 5%, or less than 1% of an organic solvent, optionally, wherein the organic solvent is CH_3CN .

[0079] The acid-free thermal deprotection method of the present disclosure generally uses a temperature of at least 60°C, and higher temperatures are contemplated depending on the particular trityl-based protecting group and the particular amine or hydroxyl group it is protecting. As illustrated in the Examples disclosed herein, a temperature of 60 °C, or 80 °C for 1 hour resulted in 100% deprotection of an MMTr-protected 5'-amine group of an oligonucleotide. Lower temperatures of 35 °C or 45 °C resulted in only 45% or 94% deprotection, respectively. The acid-free thermal deprotection of a DMTr 5'-hydroxyl group of

an oligonucleotide could also be carried out, but as shown in the Examples, using a higher temperature of 95 °C (for 1.5 hours) to achieve full deprotection to provide the 5'-hydroxyl oligonucleotide. Thus, the deprotection of hydroxyl groups protected by a trityl-based protecting groups preferably is carried out at a temperature higher than 60 °C, such as 95 °C.

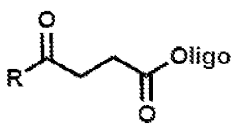
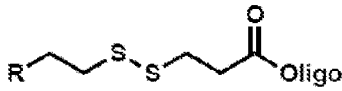
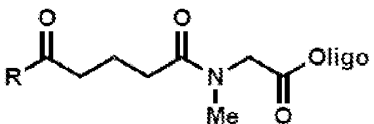
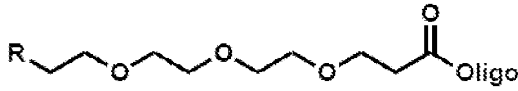
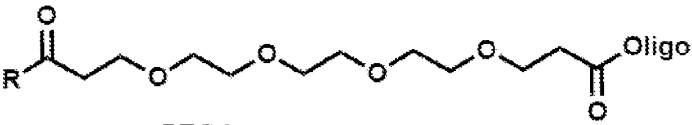
[0080] It is further contemplated that the acid-free thermal deprotection method can be used in other deprotection reactions, for example, the deprotection of a trityl-based group protecting a secondary amine group, such as in a phosphorodiamidate morpholino oligomer (PMO) linkage that occurs in a PMO oligonucleotide synthesis. In such a deprotection reaction, it is contemplated that a temperature of up to 100 °C is used.

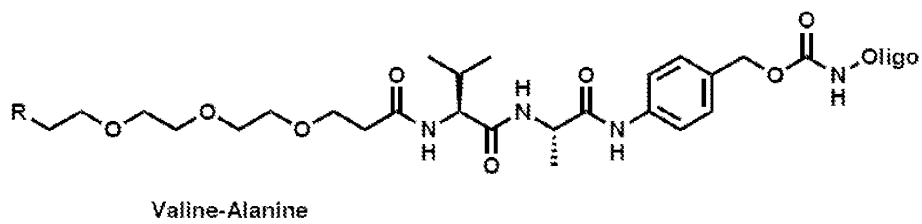
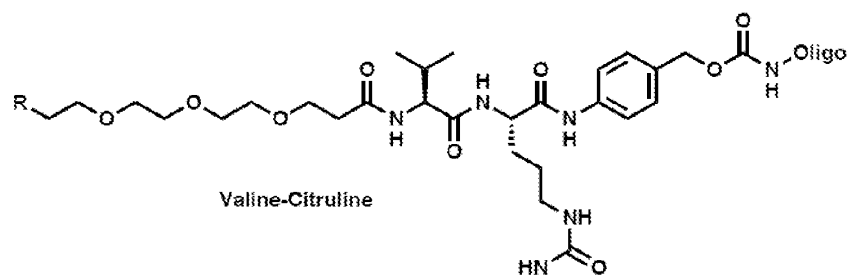
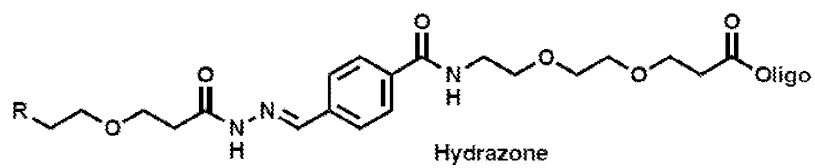
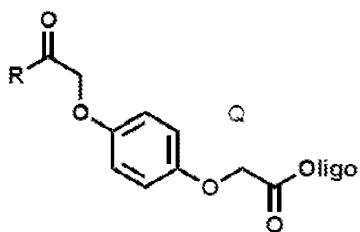
[0081] Accordingly, in at least one embodiment, the method of acid-free thermal deprotection is carried out wherein the temperature of the aqueous solution is at least 60 °C, at least 70 °C, at least 80 °C, at least 90 °C, or at least 95 °C. In some embodiments, the temperature of the aqueous solution is about 60 °C to about 100 °C.

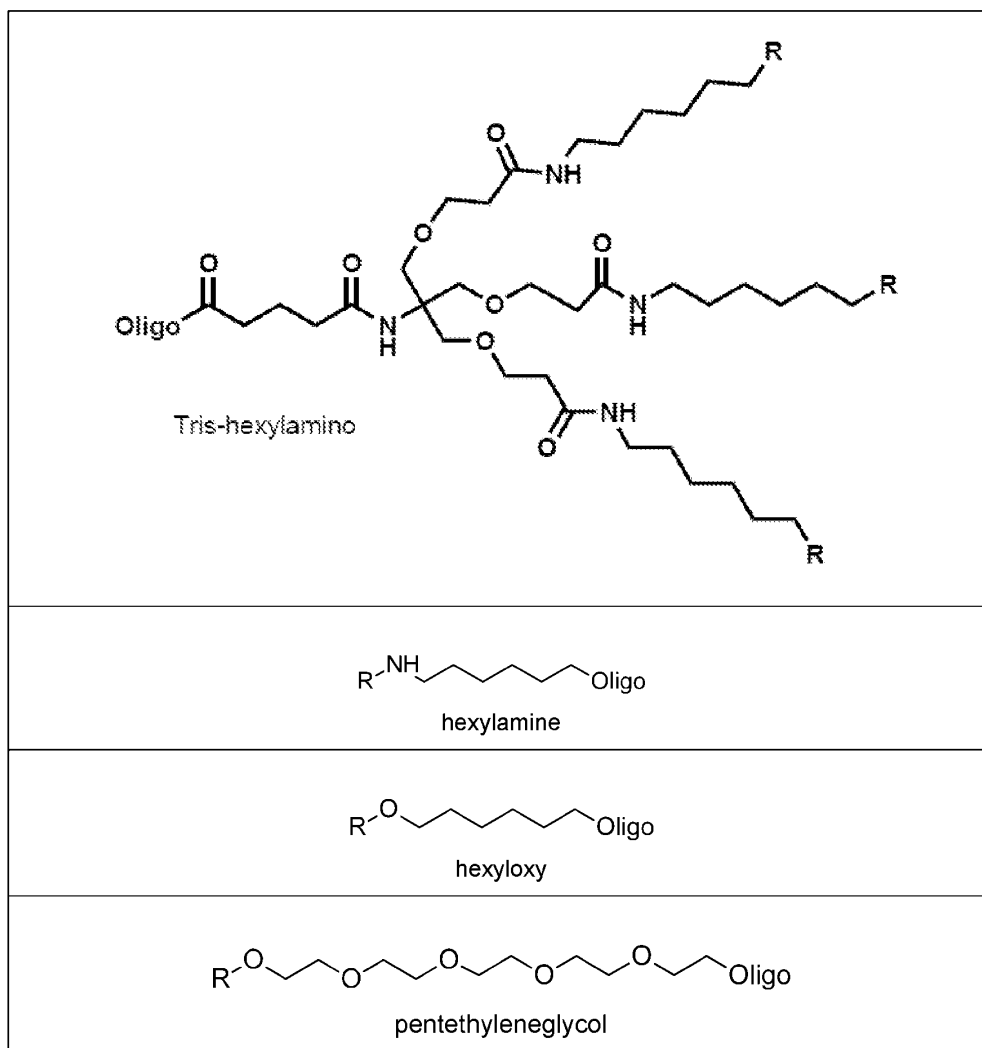
[0082] As noted elsewhere herein, it is a surprising advantage of the deprotection methods of the present disclosure that acid-free conditions can be used to provide fully deprotect trityl-based protecting groups and produce free amine or hydroxyl groups on a modified oligonucleotide. For example, in at least one embodiment, the acid-free thermal deprotection method of the present disclosure yields a percentage conversion of the oligonucleotide with trityl-based protecting group to the oligonucleotide with deprotected amine group or hydroxyl group that is at least 95%, at least 97%, at least 99%, at least 99.5%, or at least 100%. These fully deprotected reactive groups can then be used as handles for further modification of the oligonucleotide, such as by conjugation at the 5'-end to provide a ligand-oligonucleotide conjugate. Accordingly, in at least one embodiment of the deprotection method of the present disclosure, the reactive amine group or hydroxyl group yielded by deprotection of the trityl-based group is attached to the 5' phosphate group of the oligonucleotide. The amine group or hydroxyl group can be attached to the 5' phosphate group of the oligonucleotide directly or indirectly through a linker group. Suitable linker groups include, but are not limited to, those known and used for attachment of 5' reactive groups to oligonucleotides during SPOS.

[0083] Exemplary linkers useful in the compositions and methods of the present disclosure can include: (i) a branched or unbranched alkylene chain of 5-carbons to 20-carbons; (ii) a branched or unbranched alkylene chain of at least 4-carbons to 20-carbons and at least one ether linkage; (iii) a polymeric chain of from 2 to 100 polyethylene glycol (PEG) moieties, which polymeric chains can further include alkyl, alkene, alkyne, ester, ether, amide, imide, and/or phosphodiester groups; or (iv) a succinate linker, a PEG3 linker, a PEG4 linker, a sarcosine-glutarate linker, a hydrazone linker, a disulfide linker, a valine-citrulline linker, a valine-alanine linker, a tris-hexylamino linker, a hydroquinone-O,O'-diacetate ("Q") linker, a hexylamine linker, a hexyloxy linker, pentethyleneglycol, or a derivative thereof. Other exemplary linkers include those shown in Table 2 below.

[0084] TABLE 2: Exemplary linkers (depicted with attached oligonucleotide and wherein R is a ligand)

 Succinate
 Disulfide
 Sarcosine-Glutarate
 PEG3
 PEG4





[0085] Other exemplary linkers can be found, for example, in Doronina et al., *Bioconjug Chem.* 2006 Jan-Feb;17(1):114-24; Sheyi et al., *Pharmaceutics* 2022, 14(2), 396; Acchione et al., *MAbs.* 2012 May 1; 4(3): 362–372; Lu et al., *Int J Mol Sci.* 2016 Apr; 17(4): 561; and, Giese et al., *Bioconjugate Chem.* 2021, 32, 10, 2257–2267, each of which are incorporated herein in their entirety.

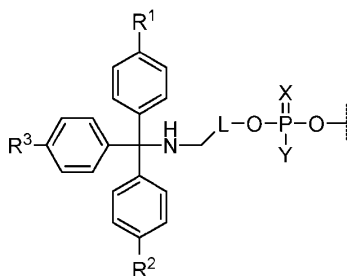
[0086] As noted elsewhere herein, the deprotection reaction of the present disclosure yields a deprotected reactive group, such as a 5'-amine or 5'-hydroxyl, that that can be used as a handle for further conjugation to the oligonucleotide. Accordingly, it is contemplated that the method of the present disclosure can further include conjugating the deprotected reactive group of the oligonucleotide to a ligand thereby forming a ligand-oligonucleotide conjugate. For example, in at least one embodiment, the deprotected reactive group is a primary amine, and

the further conjugation reaction involves contacting the deprotected oligonucleotide with an amine-reactive group such as an ester, an N-hydroxysuccinimide (NHS) ester, or a carboxylic acid.

[0087] In such an embodiment of the method with further conjugation to the deprotected reactive group, the present disclosure contemplates that a wide range of ligands can be used including but not limited to: a carbohydrate complex (e.g., N-acetylgalactosamine (GalNAc)); peptide molecule (e.g., RGD, a cell penetrating peptide, an integrin); a lipophilic molecule (aromatic and non-aromatic) including steroid molecules; a protein molecule (e.g., antibodies, enzymes, serum proteins); a vitamin (water-soluble or lipid-soluble); a polymer (water-soluble or lipid-soluble); a small molecule, such as a drug, toxin, reporter molecule, or receptor ligand; a nucleic acid cleaving complex; a metal chelator (e.g., porphyrins, texaphyrins, crown ethers, etc.); an intercalator, including a hybrid photonuclease/intercalator; a crosslinking agent (e.g., photoactive, redox active); or combination or derivative thereof.

[0088] In one exemplary embodiment, the method comprises conjugating the deprotected oligonucleotide to a ligand that is a multi-valent carbohydrate. Such multi-valent carbohydrates can include 2, 3, or 4 identical or non-identical carbohydrate moieties, and are conjugated to the oligonucleotide (such as an ASO compound or siRNA compound) directly or indirectly via a linker. For example, the ligand can be N-acetylgalactosamine (GalNAc), in a monovalent, divalent, trivalent, or tetravalent conjugate moiety form, such as those described in WO 2014/076196, WO 2014/207232 and WO 2014/179620, each of which is hereby incorporated by reference herein. GalNAc has a strong binding affinity for an asialoglycoprotein (ASGP) receptors, and a trivalent GalNAc conjugated to an oligonucleotide (such as an ASO or siRNA) can be used to target the compound to the liver. In another exemplary embodiment, the ligand or conjugate moiety can be a galactose or galactose derivative, *i.e.*, any galactose-based molecule having affinity for the ASGP receptor equal to or greater than that of galactose to the ASGP receptor.

[0089] As described elsewhere herein, the deprotection method of the present disclosure can be carried out wherein the trityl-based protecting group is protecting a reactive amine group that is attached to the 5'-end of an oligonucleotide, optionally via a linker. Accordingly, in at least one embodiment the method can be used to deprotect the 5'-amine modified oligonucleotide of Formula (I) (or a salt thereof or a pharmaceutically acceptable salt thereof)



(I)

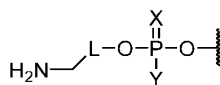
wherein:

each of R^1 , R^2 , and R^3 is independently hydrogen, $-OCH_3$, or $-S(O)CH_3$; L is an optional linker; X is O or S; Y is OH or SH; and

the oligonucleotide attached at its 5'-end of the phosphate oxygen is indicated by:



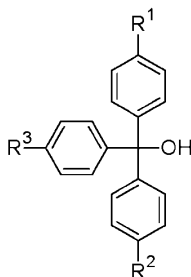
[0090] The deprotection method comprises heating the 5'-amine modified oligonucleotide of Formula (I) in an acid-free aqueous solution at a temperature of at least 60°C to form a compound of Formula (II) (or a salt thereof, or a pharmaceutically acceptable salt thereof)



(II)

wherein: X is O or S; Y is -OH or -SH; L is the optional linker; and the oligonucleotide attached at its 5'-end of the phosphate oxygen is indicated as above for the compound of Formula (I).

[0091] The method of deprotection of compound of Formula (I) also produces the insoluble trityl-hydroxide byproduct compound of Formula (III):

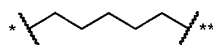


(III)

wherein: each of R^1 , R^2 , and R^3 is independently hydrogen, $-OCH_3$, or $-S(O)CH_3$.

[0092] The method of deprotecting a compound of Formula (I) can further comprise separating the insoluble byproduct compound of Formula (II) from the compound of Formula (III) by extraction, desalting, or precipitation and filtration.

[0093] As with the general deprotection method of the present disclosure, it is contemplated that when a linker, L, is present in the compound of Formula (I), a wide-range of linker structures can be used. In at least one embodiment, the linker L in the compound of Formula (I) is a divalent linker of Formula (L-i):



(L-i)

wherein:

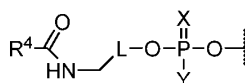


represents a covalent bond to the nitrogen atom of the primary amine group, and



represents a covalent bond to the oxygen atom of the phosphate group.

[0094] The compound of Formula (II), as described above, represents the 5'-amine modified oligonucleotide that is the desired product of the acid-free thermal deprotection reaction of the compound of Formula (I). As noted elsewhere herein, the compound of Formula (II) can be further reacted with a ligand molecule comprising an amine-reactive group to provide a ligand-oligonucleotide conjugate compound of Formula (IV):



(IV)

or a salt thereof, or a pharmaceutically acceptable salt thereof,

wherein:

L is a divalent linker;

X is S or O;

Y is -SH or -OH;

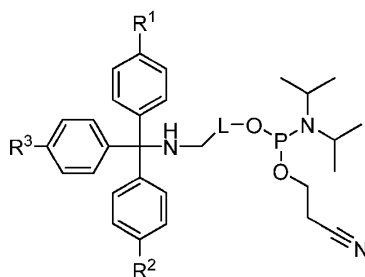
R⁴ is a ligand; and



represents a covalent bond to the 5'-end of the oligonucleotide.

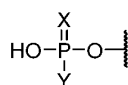
[0095] The ligand R⁴ can be any of those useful ligands described elsewhere herein, including but not limited to, N-acetylgalactosamine (GalNAc), a triantennary cluster of N-acetylgalactosamine moieties, a lipid molecule, a small molecule peptide (e.g., RGD, a cell penetrating peptide, an integrin), a protein, and an antibody. Ligand molecules, such as these, can be modified if necessary with an amine-reactive group, such as an ester, NHS ester, or carboxylic acid, using standard techniques or in some cases are commercially available with an amine-reactive group for conjugation.

[0096] As described elsewhere herein, the oligonucleotide with trityl-based protecting group protecting a reactive primary amine at the 5'-end of the oligonucleotide can be prepared using the appropriate protected phosphoramidite reagent, such as at the final step of an automated SPOS. Accordingly, a compound of Formula (I) useful in the methods of the present disclosure can be prepared by reacting a compound of Formula (A) with a compound of Formula (B):



(A)

wherein: each of R^1 , R^2 , and R^3 is independently hydrogen, $-OCH_3$, or $-S(O)CH_3$; and L is an optional linker;



(B)

or a salt thereof, or a pharmaceutically acceptable salt thereof,
wherein: X is O or S; Y is $-OH$ or $-SH$; and



represents a covalent bond to the 5'-end of the oligonucleotide.

[0097] As described elsewhere herein, and shown in the Examples, the acid-free thermal deprotection method results in complete or nearly complete conversion of the protected oligonucleotide compound of Formula (I) to the desired deprotected oligonucleotide compound of Formula (II) with little or no depurination detectable by MS analysis. Thus, in at least one embodiment of the method, the percentage of oligonucleotide compound of Formula (I) converted to oligonucleotide compound of Formula (II) is at least 95%, at least 97%, at least 99%, at least 99.5%, or at least 100%. Additionally, in at least one embodiment of the method, the percentage of depurinated oligonucleotide species detected by MS analysis relative to the total deprotected oligonucleotide compound of Formula (II) after the deprotection treatment is less than 5%, less than 2%, less than 1%, less than 0.5%, less than 0.1%, or less than 0.01%.

EXAMPLES

[0098] Various features and embodiments of the disclosure are illustrated in the following representative examples, which are intended to be illustrative, and not limiting. Those skilled in the art will readily appreciate that the specific examples are only illustrative of the invention as described more fully in the claims which follow thereafter. Every embodiment and feature described in the application should be understood to be interchangeable and combinable with every embodiment contained within.

Example 1: Synthesis of 5'-Amino Modified Oligonucleotides with MMTr-Protected Amine

[0099] This example illustrates the exemplary synthesis of oligonucleotides with MMTr-protected 5' amino groups used in the Examples of the present disclosure.

[0100] *Materials and Methods*

[0101] *Synthesis of 5'-MMTr protected oligonucleotides*

[0102] Synthesis of 5'-MMTr protected oligonucleotides ("MMTr-ON") used in the examples was carried out using the standard phosphoramidite method on an LGC MerMade-192x synthesizer on a 1 μmol scale using phosphoramidites from Glen Research Inc. (Sterling, VA, USA) and Hongene Biotech Corporation (Shanghai, China). Exemplary phosphoramidite reagents used in the oligonucleotide syntheses are provided in Table 3 below.

[0103] TABLE 3

Name	Catalog Number
MMTr-Amine Linker Phosphoramidite	OP-002
DMTr-2'-O-LNA-A(Bz)-CE-Phosphoramidite	PR1-002
DMTr-2'-O-LNA-G(dmf)-CE-Phosphoramidite	PR2-005
5-Me-DMTr-2'-O-LNA-C(Bz)-CE-Phosphoramidite	PR3-009
DMTr-2'-O-LNA-T-CE-Phosphoramidite	PR4-001
DMTr-dA(Bz)-CE-Phosphoramidite	PD1-004
DMTr-dG(iBu)-CE-Phosphoramidite	PD2-004
DMTr-dG(dmf)-CE-Phosphoramidite	PD2-006
DMTr-dC(Bz)-CE-Phosphoramidite	PD3-003

[0104] Glen UnySupport™ CPG 1000 (G110408) was purchased from Glen Research. (I) coupling: phosphoramidites in dry acetonitrile (0.05 M) were activated by 0.25 M 5-(Ethylthio)-1H-tetrazole (ETT) in dry acetonitrile before coupling to the solid support with coupling times (120s for DNA and 180s for LNA); (II) Capping: (A) Ac₂O/2,6-Lutidine/THF, and (B) 16% 1-Methylimidazole in THF; (III) Oxidation (for phosphodiester linkages): 0.02 M I₂ in THF/Py/H₂O for 30s; or sulfurization (for phosphorothioate linkages) with 0.1M DDTT in pyridine for 180 s; (IV) Detritylation: 3% dichloroacetic acid in dichloromethane.

[0105] After synthesis, the synthesized MMTr-ON were removed from the solid support and deprotected by concentrated aqueous ammonia with 10% ammonium acetate at 65 °C for 12 hours. The crude MMTr-ON were then separated from CPG by washing with 100 mg/mL sodium chloride solution and subsequently purified on Glen-Pak™ DNA purification cartridge (Glen Research, cat#60-5100). Failing sequences were removed by washing with 15% MeCN in aqueous 100mg/mL NaCl, followed by MMTr-ON elution from cartridge with 50% MeCN/H₂O. Organics were removed by evaporation on a vacufuge concentrator. To monitor reactions, samples were analyzed on Waters Acquity H-class UPLC equipped with QDA detector.

[0106] **LC-MS analysis**

[0107] LC-MS analysis on a Waters Acquity H-Class UPLC was used to isolate and characterize the synthesized oligonucleotides. The following LC-MS conditions were used: Analytical LC/MS: Reverse Phase chromatography; Mobile phase A: 14.3 mM triethylamine, 114 mM hexafluoro-2-propanol, 2.5% methanol in water; Mobile phase B: 14.3 mM triethylamine, 114 mM hexafluoro-2-propanol, 60% methanol in water; Gradient elution of Mobile phase: 100% A to 100% B in 3.5 minutes; Column: Aquity UPLC® Oligonucleotide BEH C18, 130Å, 1.7µm, 2.1mm X 50mm (P#186003949); Column temperature: 65 °C; Amount of sample injection: ~1ug; Flow rate: 1.0 mL/min; UV detector: 260 nm.

[0108] **Results**

[0109] The exemplary 16-mer with a 5'-MMTr-protected 5' amino group, MMTr-ON #5 was synthesized according to the methods described above. The sequence (SEQ ID NO: 1) and mass of MMTr-ON #5 are as shown in Table 4. As shown by the sequence of SEQ ID NO: 1 depicted in Table 4, the MMTr-protected 5'-amino modified oligonucleotide of MMTr-ON #5 includes three locked nucleic acids at each of the 5' and 3' ends flanking a 10-mer of DNA, and all of the internucleotide linkages were phosphorothioates.

[0110] **TABLE 4:** Exemplary MMTr-ON #5

MMTr-ON #	Modified Oligonucleotide 5'→3' (nt sequence identifier)	Exact Mass	
		MMTr-ON	Deprotected ON
5	(C6N)-A*G*A* <u>t</u> * <u>t</u> *c*a*g*t*g*t*g*T*G*G (SEQ ID NO: 1)	5878.6	5590.5
(C6N) = MMTr-protected C6-amino linker; Uppercase letters indicate Locked Nucleic Acid (LNA); Lowercase letters indicate DNA; (*) = phosphorothioate.			

EXAMPLE 2: Acid-Catalyzed Versus Acid-Free Thermal Deprotection

[0111] This example illustrates a comparative study of the standard acid catalyzed deprotection reaction conditions recommended for removal of trityl-based protecting group from a 5'-amino group versus the acid-free thermal deprotection method of the present disclosure.

[0112] **Materials and Methods**

[0113] **Acid-catalyzed deprotection**

[0114] The industry standard protocol for the deprotection of a 5'-trityl protected 5'-amine-modified oligonucleotide was carried out. This protocol requires incubation in 20% acetic acid in water for 60 minutes at room temperature (RT). The exemplary MMTr-ON #5 (see Table 4) synthesized as described in Example 1 was treated according to the standard acid-catalyzed deprotection protocol. Additional acid-catalyzed deprotections of MMTr-ON #5 under varying conditions of time, % acetic acid, and temperature as follows: (i) 20% acetic acid, RT, 90 min;

(ii) 30% acetic acid, RT, 60 min; (iii) 20% acetic acid, 45 °C, 60 min; (iv) 80% acetic acid, RT, 60 min; and (v) 80% acetic acid, RT, 30 min.

[0115] Acid-free thermal deprotection

[0116] MMTr-ON #5 (see Table 4) was synthesized and purified on a 1 micromole scale according to Example 1. After purification, eluted oligonucleotide was concentrated *in vacuo* to remove acetonitrile, then diluted up to 1.0 mL with water. The solution was then heated to 60 °C in a dry heat block for 60 minutes to fully remove the 5'-MMTr-protecting group. A byproduct of the deprotection reaction, MMTr-OH, was observed to precipitate, and then was removed by extraction with ethyl acetate (3 x 1 mL). (Alternatively, the insoluble MMTr-OH byproduct can be removed by using a desalting method of choice.) Pure 5'-amine modified ON #5 was then confirmed by LC-MS and quantified by nanodrop (A260) yielding 400 nmol of ON #5.

[0117] Results

[0118] As shown by the LC plots over time depicted in **FIG. 1A**, the standard acid-catalyzed deprotection protocol resulted in incomplete deprotection of MMTr-ON #5. Additionally, the MS peaks for the acid-catalyzed deprotection (**FIG. 1B**) showed detection of >5% depurinated species, whereas such peaks were not detected following the acid-free thermal deprotection (**FIG. 1C**). As shown by the LC plots of **FIG. 1D**, the use of an increased percentage of acetic acid (30%, or 80%), and/or increased incubation time (90 min) at RT failed to result in any increased conversion to the deprotected product. Additionally, as illustrated by the MS results for the deprotection in 20% acetic acid, 45 °C, 60 min shown in **FIG. 1E**, the use of an increased temperature of 45 °C (rather than RT) resulted in increased overall conversion but with the trade-off of substantially increased levels of depurinated product oligonucleotide as compared to the reaction carried out in 20% acetic acid at RT (compare MS peaks in **FIG. 1D** to MS peaks in **FIG. 1B**). In contrast to the results shown in **FIG. 1A**, the use of the acid-free thermal deprotection conditions (i.e., heating at 60 °C for 60 min in non-buffered water) with MMTr-ON #5 resulted in near complete deprotection (**FIG. 1F**). These results demonstrate that following elution, a 5'-MMTr protected oligonucleotide can be nearly fully deprotected in elution buffer once acetonitrile is removed, by simple heating without the use of depurinating acidic conditions.

Example 3: Evaluation of Acid-Free Thermal Deprotection Reaction Variables

[0119] This example illustrates a study of the effect of different reaction variables (temperature, buffer system, solvent) on the acid-free thermal deprotection protocol of Example 2.

[0120] Materials and Methods

[0121] The acid-free thermal deprotection of the 5'-MMTr protected ON #5 as described in Example 2 was carried under a range of conditions of temperature, buffer/solvent and pH, and % conversion to the MMTr deprotected species and oligonucleotide depurination were

observed. Values of % conversion and presence of depurinated species were determined using LC-MS as described in Example 1.

[0122] Results

[0123] Results are summarized in Table 5, below (LC-MS traces not shown).

[0124] **TABLE 5:** Evaluation of acid-free thermal deprotection reaction parameters

Entry	Temp (°C)	Time (min)	Solvent (pH)	% Conversion	Depurination ?
Standard Acid	RT	60	20% acetic acid/water	92	Yes
Acid-Free Thermal	60	60	water	100	No
Variable Temperature					
1	RT	60	water	15	No
2	35	60	water	45	No
3	45	60	water	94	No
4	80	60	water	100	No
Variable Solvent (pH)					
5	60	60	50% CH ₃ CN/water	55	No
6	60	60	28% NH ₄ OH	1	No
7	60	60	PBS (pH 7.4)	100	No
8	60	60	TE buffer (pH 8.0)	100	No

[0125] While evaluating temperature, it was found that below 60 °C, full deprotection was not achieved (Table 5, entries 1-3). Accordingly, all other experiments were carried out at 60 °C. Various solutions commonly used in oligonucleotide purification and handling were evaluated.

[0126] Complete deprotection was observed in phosphate buffered saline (PBS, pH 7.4) and TE buffer (pH 8.0) indicating that pH need not be acidic to drive the reaction to completion (Table 5, entries 7-8). However, concentrated ammonium hydroxide (NH₄OH) led to only trace product formation (Table 5, entry 6).

[0127] A white precipitate was observed after incubation in the pH 7.0 and buffered samples (pH 7.4 and pH 8). As noted in Example 3, this white precipitate was confirmed by NMR to be the byproduct, MMTr-OH, as shown by comparison of a commercial sample spectrum (top, **FIG. 2A**) to the isolated white precipitate spectrum (bottom, **FIG. 2A**). It was postulated that the insolubility of the MMTr-OH byproduct helps drive the acid-free thermal deprotection reaction by minimizing the known reversibility of MMTr deprotection (see e.g., reference 17) through sequestration of the byproduct as an insoluble species. **FIG. 2B** depicts a schematic comparison of the two deprotection reactions illustrating how the insolubility of MMTr-OH in water relative to MMTr⁺ in 20% acetic acid can drive the deprotection reaction. Evidence supporting this is found in Table 5, entry 5, which shows that incubation of ON #5 in a 50:50 mix of CH₃CN/H₂O results in incomplete (55%) deprotection. This is likely due to the increased solubility of MMTr-OH in the 50% CH₃CN solvent thereby resulting in an equilibrium shift unfavorable for MMTr deprotection.

EXAMPLE 4: Effects of Oligonucleotide Sequence on Acid-Free Deprotection

[0128] This example illustrates a study of whether the oligonucleotide sequence affects the deprotection of the oligonucleotide using the acid-free thermal deprotection method of the present disclosure. More particularly, to rule out the possibility of that acid-free thermal deprotection is biased by a particular oligonucleotide sequence.

[0129] Materials and Methods

[0130] A total of eight different MMTr-ON with varying sequences relative to MMTr-ON #5 (see Table 4) were synthesized according to the method of Example 1. The varying sequences of the eight different MMTr-ONs included all possible bases at the first position, and all neighboring bases at the second position, as well as some changes at the third position. Additionally, an oligonucleotide with a 5'-DMTr-protected 5'-hydroxyl group was synthesized. The sequences (SEQ ID NOs: 2-10) and masses of these 5'-protected oligonucleotides are summarized in Table 6 below.

[0131] **TABLE 6:** Synthesized 5'-MMTr-protected oligonucleotides

#	Modified Oligonucleotide 5'→3' (nt sequence identifier)	Exact Mass	
		MMTr-ON	MMTr-OFF
6	(C6N)-G*G*A*t*t*c*a*g*t*g*t*g*g*T*G*G (SEQ ID NO: 2)	5852.6	5606.5
7	(C6N)-C*G*A*t*t*c*a*g*t*g*t*g*g*T*G*G (SEQ ID NO: 3)	5853.6	5580.5
8	(C6N)-T*G*A*t*t*c*a*g*t*g*t*g*g*T*G*G (SEQ ID NO: 4)	5846.6	5581.5
9	(C6N)-A*A*A*t*t*c*a*g*t*g*t*g*g*T*G*G (SEQ ID NO: 5)	5836.6	5574.5
10	(C6N)-A*C*A*t*t*c*a*g*t*g*t*g*g*T*G*G (SEQ ID NO: 6)	5837.6	5564.5
11	(C6N)-A*T*A*t*t*c*a*g*t*g*t*g*g*T*G*G (SEQ ID NO: 7)	5769.6	5565.5
12	(C6N)-C*G*T*t*a*a*c*t*a*g*c*t*T*A (SEQ ID NO: 8)	5723.7	5497.5
13	(C6N)-C*A*C*a*t*t*c*c*t*t*g*c*t*C*T*G (SEQ ID NO: 9)	5862.6	5451.5
14	DMTr-G*C*a*t*t*g*g*t*a*t*T*C*A* (SEQ ID NO: 10)	4624.6	4322.4
(C6N) = MMTr-protected C6-amino linker; Uppercase letters indicate Locked Nucleic Acid (LNA); Lowercase letters indicate DNA; (*) = phosphorothioate; C = 5-methyl deoxycytidine; DMTr = 4,4'-dimethoxytrityl-protected 5'.			

[0132] After synthesis and purification, each of the 5'-MMTr-protected ON #5 through #14 (Table 6) were concentrated *in vacuo* to remove acetonitrile, then diluted up to 1.0 mL with water. This solution was then heated to 60 °C in a dry heat block for 60 minutes to fully remove the 5'-MMTr-protecting group. Additionally, control samples of all of the 5'-MMTr-ON of Table 6 were subjected to standard acid-catalyzed deprotection conditions.

[0133] Results

[0134] Complete 5'-MMTr deprotection was achieved for all of the 5'-MMTr-ON #6-13 using the acid-free thermal deprotection protocol. In contrast, the acid-catalyzed deprotection of 5'-MMTr-ON #6-13 resulted in incomplete deprotection. **FIGS. 3A-3F** depict exemplary LC plots comparing the acid-catalyzed and acid-free thermal deprotection results for 5'-MMTr-ON #6, #7, #9, #11, #12, and #13. In each case, the acid-free thermal deprotection resulted in complete or nearly complete deprotection demonstrating the sequence independence of this method.

EXAMPLE 5: Thermal Deprotection of DMTr-O-protected oligonucleotides

[0135] This example illustrates a comparative study to determine whether an acid-free thermal deprotection protocol of the present disclosure can be used to deprotect 5'-DMTr-protected oligonucleotide, such as DMTr-ON #14 (Table 6), as well as or better than, an acid-catalyzed deprotection protocol. In this study, the 5'-DMTr protects a 5'-hydroxyl moiety rather than an amino group. **FIG. 4A** depicts the deprotection reaction conditions under comparison in the present example.

[0136] Materials and Methods

[0137] Deprotection of the 5'-DMTr-ON #14 (see Table 6) was carried out using either an acid-free thermal deprotection at 95 °C for 1.5 hours, or a standard 5% trifluoroacetic acid treatment at RT for 15 minutes.

[0138] Results

[0139] As shown by the LC plots depicted in **FIG. 4B**, full deprotection of 5'-DMTr-ON was achieved under the slightly more aggressive acid-free thermal deprotection conditions of 1.5 hours at 95°C to provide the 5'-OH ON.

[0140] Conclusions

[0141] The simplicity of the acid-free thermal deprotection method of the present disclosure and its compatibility with standard oligonucleotide purification techniques is demonstrated by the above-described examples. Following elution of MMTr-ON product from an oligonucleotide purification column, the resulting protected oligonucleotide can be deprotected in elution buffer once acetonitrile is removed, by simple heating. The insoluble MMTr-OH byproduct can be removed by, e.g., either extraction with ethyl acetate or by a desalting method of choice. The streamlined purification and deprotection process, for example, reduces the overall complexity and time required for amino-modified oligonucleotide purification while eliminating the use of corrosive acids and the corresponding waste streams. As such, the methods described herein increase access to oligonucleotide conjugate materials and expands the use of these species for use in human health and beyond.

[0142] While the foregoing disclosure of the present invention has been described in some detail by way of example and illustration for purposes of clarity and understanding, this disclosure including the examples, descriptions, and embodiments described herein are for illustrative purposes, are intended to be exemplary, and should not be construed as limiting the present disclosure. Additional embodiments of the invention are set forth in the appended claims.

[0143] It will be clear to one skilled in the art that various modifications or changes to the examples, descriptions, and embodiments described herein can be made and are to be included within the spirit and purview of this disclosure and the appended claims. Further, one of skill in the art will recognize a number of equivalent methods and procedure to those described herein. All such equivalents are to be understood to be within the scope of the present disclosure and are covered by the appended claims.

[0144] All publications, patents, patent applications, and other documents referenced in this disclosure are hereby incorporated by reference in their entireties for all purposes to the same extent as if each individual publication, patent, patent application or other document were individually indicated to be incorporated by reference herein for all purposes. In case of conflict, the present specification, including specified terms, will control.

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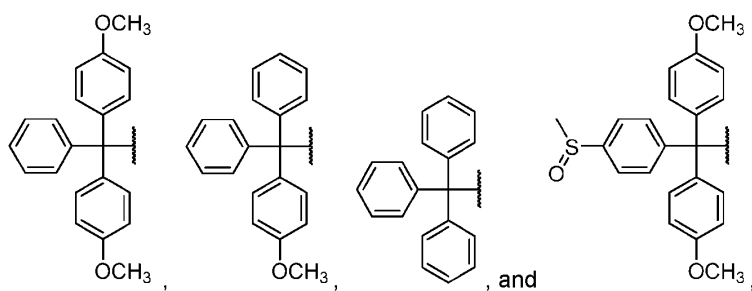
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<https://www.glenresearch.com/reports/gr19-14> (accessed 2023-08-21).

CLAIMS

What is claimed is:

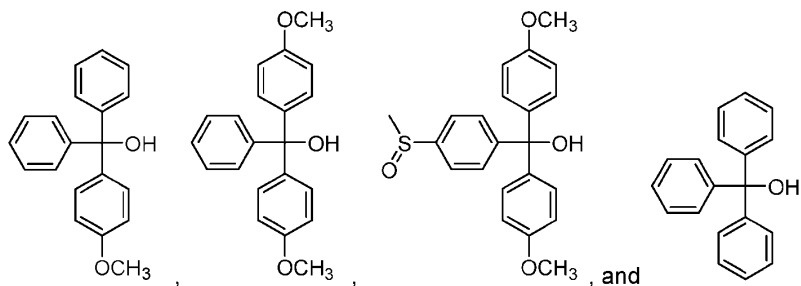
1. A method of deprotecting an oligonucleotide comprising an amine group or a hydroxyl group protected by a trityl-based protecting group, the method comprising heating the oligonucleotide in an acid-free aqueous solution at a temperature of at least 60 °C, thereby cleaving the trityl based protecting group to provide the oligonucleotide comprising the deprotected amine group or hydroxyl group.
2. The method of claim 1, wherein the trityl-based protecting group is selected from: trityl, monomethoxytrityl (MMTr), dimethoxytrityl (DMTr), and dimethoxymethylsulfonyltrityl (DMS(O)MTr).
3. The method of any one of claims 1-2, wherein the trityl-based protecting group has a structure selected from:



wherein represents a covalent bond to the nitrogen atom of the amine group, or the oxygen atom of the hydroxyl group.

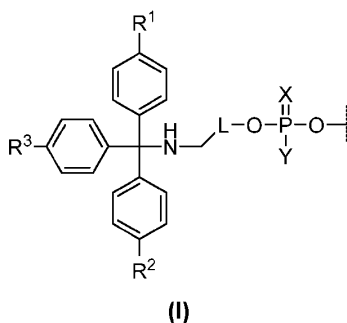
4. The method of any one of claims 1-3, wherein deprotection of the amine group or hydroxyl group produces an insoluble trityl byproduct, and the method further comprises separating the insoluble trityl byproduct from the deprotected oligonucleotide.
5. The method of claim 4, wherein the insoluble trityl byproduct is separated by extraction, desalting, or precipitation and filtration.
6. The method of claim 4 or claim 5, wherein the insoluble trityl byproduct is MMTr-OH, DMTr-OH, Tr-OH, or DMS(O)Tr-OH.

7. The method of any one of claims 4 to 6, wherein the insoluble trityl byproduct has a structure selected from:



8. The method of any one of claims 1-7, wherein the amine group or hydroxyl group is attached to the 5' phosphate group of the oligonucleotide.
9. The method of any one of claims 1-8, wherein the amine group or hydroxyl group is attached to the 5' phosphate group of the oligonucleotide through a linker group.
10. The method of claim 9, wherein the linker group comprises a 5-carbon to 12-carbon unbranched alkylene chain; optionally, wherein the alkylene chain further comprises at least one ether linkage.
11. The method of claim 9, wherein the linker group is selected from: a succinate linker, a PEG3 linker, a PEG4 linker, a sarcosine-glutarate linker, a hydrazone linker, a disulfide linker, a valine-citrulline linker, a valine-alanine linker, a tris-hexylamino linker, a hydroquinone-O,O'-diacetate ("Q") linker, a hexylamine linker, a hexyloxy linker, pentethyleneglycol linker, and a derivative thereof..
12. The method of any one of claims 1-11, wherein the amine group of the oligonucleotide protected by a trityl-based protecting group is a primary amine group.
13. The method of claim 12, further comprising conjugating a ligand to the oligonucleotide by reacting the deprotected primary amine group of the oligonucleotide with a ligand molecule comprising an amine-reactive group, thereby forming a ligand-oligonucleotide conjugate.
14. The method of claim 13, wherein the amine-reactive group is selected from an ester, an NHS ester, and a carboxylic acid.

15. The method of claim 13, wherein the ligand molecule is selected from from N-acetylgalactosamine (GalNAc), a triantennary cluster of N-acetylgalactosamine moieties, a lipid molecule, a small molecule peptide, a protein, and an antibody.
16. The method of any one of claims 1-15, wherein the percentage conversion of the oligonucleotide with trityl-based protecting group to the oligonucleotide with deprotected amine group or hydroxyl group is at least 95%, at least 97%, at least 99%, at least 99.5%, or at least 100%.
17. A method of deprotecting a 5'-amine modified oligonucleotide compound of Formula (I), the method comprising heating the 5'-amino modified oligonucleotide compound of Formula (I) in an acid-free aqueous solution at a temperature of at least 60 °C to form an oligonucleotide of Formula (II) and a byproduct compound of Formula (III):



or a salt thereof or a pharmaceutically acceptable salt thereof,

wherein:

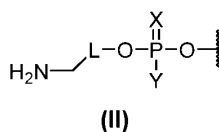
each of R¹, R², and R³ is independently hydrogen, -OCH₃, or -S(O)CH₃;

L is an optional linker;

X is O or S;

Y is OH or SH; and

— represents a covalent bond to the 5'-end of the oligonucleotide;



or a salt thereof, or a pharmaceutically acceptable salt thereof,

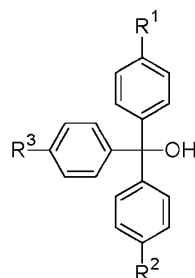
wherein:

X is O or S;

Y is -OH or -SH;

L is an optional linker; and

 represents a covalent bond to the 5'-end of the oligonucleotide;

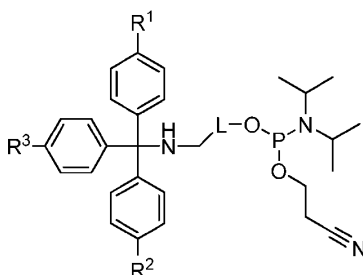


(III)

wherein:

each of R¹, R², and R³ is independently hydrogen, -OCH₃, or -S(O)CH₃.

18. The method of claim 17, further comprising separating the oligonucleotide compound of Formula (II) from the byproduct compound of Formula (III) by extraction, desalting, or precipitation and filtration.
19. The method of any one of claims 17-18, wherein the compound of Formula (I) is prepared by reacting a phosphoramidite compound of Formula (A) with an oligonucleotide compound of Formula (B):

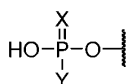


(A)

wherein:

each of R¹, R², and R³ is independently hydrogen, -OCH₃, or -S(O)CH₃; and

L is an optional linker;



(B)

or a salt thereof, or a pharmaceutically acceptable salt thereof,

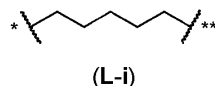
wherein:

X is O or S;

Y is -OH or -SH; and

 represents a covalent bond to the 5'-end of the oligonucleotide.

20. The method of any one of claims 17-19, wherein L is a divalent linker of Formula (L-i):



wherein:

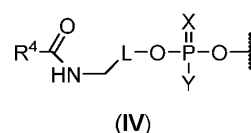
 represents a covalent bond to the nitrogen atom of the primary amine group; and

 represents a covalent bond to the oxygen atom of the phosphate group.

21. The method of any one of claims 17-19, wherein the linker L comprises: (i) a 5-carbon to 20-carbon branched or unbranched alkylene chain; or (ii) a 4-carbon to 20-carbon branched or unbranched alkylene chain with at least one ether linkage.

22. The method of any one of claims 17-19, wherein the linker L comprises: a succinate linker, a PEG3 linker, a PEG4 linker, a sarcosine-glutarate linker, a hydrazone linker, a disulfide linker, a valine-citrulline linker, a valine-alanine linker, a tris-hexylamino linker, a hydroquinone-O,O'-diacetate ("Q") linker, a hexylamine linker, a hexyloxy linker, pentethyleneglycol, or a derivative thereof.

23. The method of any one of claims 18-23, further comprising reacting the oligonucleotide compound of Formula (II) with a ligand molecule comprising an amine-reactive group to form a ligand-oligonucleotide conjugate compound of Formula (IV):



or a salt thereof, or a pharmaceutically acceptable salt thereof,

wherein:

L is a divalent linker;

X is S or O;

Y is -SH or -OH;

R⁴ is a ligand; and

 represents a covalent bond to the 5'-end of the oligonucleotide.

24. The method of claim 23, wherein the amine-reactive group is selected from an ester, an NHS ester, and a carboxylic acid.
25. The method of any one of claims 23-24, wherein the ligand R⁴ is selected from N-acetylgalactosamine (GalNAc), a triantennary cluster of N-acetylgalactosamine moieties, a lipid molecule, a small molecule peptide, a protein, and an antibody.
26. The method of any one of claims 1-25, wherein the acid-free aqueous solution is heated to a temperature of at least about 65°C, at least about 70°C, at least about 75°C, at least about 80°C, at least about 85°C, at least about 90°C, or at least about 95°C.
27. The method of any one of claims 1-26, wherein the acid-free aqueous solution is heated to a temperature of about 60°C to about 100°C, about 60°C to about 80°C, about 60°C to about 75°C, or about 60°C to about 70°C.
28. The method of any one of claims 1-27, wherein the acid-free aqueous solution is heated for at least about one hour, or at least about two hours.
29. The method of any one of claims 1-28, wherein the acid-free aqueous solution is heated for about one hour to about two hours.
30. The method of any one of claims 1-29, wherein the acid-free aqueous solution has a pH of about pH 6.5 to about pH 8.0, about pH 6.8 to about pH 7.8, about pH 6.8 to about pH 7.6, about pH 7.0, about pH 7.2, or about pH 7.4.
31. The method of any one of claims 17-30, wherein the percentage of oligonucleotide compound of Formula (I) converted to oligonucleotide compound of Formula (II) is at least 95%, at least 97%, at least 99%, at least 99.5%, or at least 100%.
32. The method of any one of claims 1-31, wherein the percentage of depurinated oligonucleotide relative to the total deprotected oligonucleotide detected by MS analysis after the deprotection is less than 5%, less than 2%, less than 1%, less than 0.5%, less than 0.1%, or less than 0.01%.
33. The method of any one of claims 1-32, wherein the oligonucleotide length is from 2-mer to 100-mer; optionally, wherein the oligonucleotide length is from 3-mer to 75-mer, from 5-mer to 50-mer, 15-mer to 50-mer, 15-mer to 40-mer, 15-mer to 30-mer, or 2-mer to 10-mer.