CHEMICAL LINKERS WITH SINGLE AMINO ACIDS AND CONJUGATES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority of US Provisional Patent Application No. 60/891,028 filed on February 21, 2007, the benefit of which is hereby claimed under 35 U.S.C. § 119 and the disclosure of which is herein incorporated by reference in their entirety.

FIELD OF THE INVENTION

The present invention provides linkers with a single amino acid that attach to a drug and a ligand and are cleaved in vivo. The linkers are of use in forming prodrugs and conjugates of the cytotoxins of the invention as well as other diagnostic and therapeutic moieties.

BACKGROUND OF THE INVENTION

Many therapeutic agents, particularly those that are especially effective in cancer chemotherapy, often exhibit acute toxicity *in vivo*, especially bone marrow and mucosal toxicity, as well as chronic cardiac and neurological toxicity. Such high toxicity can limit their applications. Development of more and safer specific therapeutic agents, particularly antitumor agents, is desirable for greater effectiveness against tumor cells and a decrease in the number and severity of the side effects of these products (toxicity, destruction of non-tumor cells, etc.). Another difficulty with some existing therapeutic agents is their less than optimal stability in plasma. Addition of functional groups to stabilize these compounds resulted in a significant lowering of the activity. Accordingly, it is desirable to identify ways to stabilize compounds while maintaining acceptable therapeutic activity levels.

The search for more selective cytotoxic agents has been extremely active for many decades, the dose limiting toxicity (i.e. the undesirable activity of the cytotoxins on normal tissues) being one of the major causes of failures in cancer therapy. For example, CC-1065 and the duocarmycins are known to be extremely potent cytotoxins.

CC-1065 was first isolated from *Streptomyces zelensis* in 1981 by the Upjohn Company (Hanka *et al., J. Antibiot.* **31**: 1211 (1978); Martin *et al., J. Antibiot.* **33**: 902 (1980); Martin *et al., J. Antibiot.* **34**: 1119 (1981)) and was found to have potent antitumor and antimicrobial activity both *in vitro* and in experimental animals (Li *et al., Cancer Res.*

42: 999 (1982)). CC-1065 binds to double-stranded B-DNA within the minor groove (Swenson *et al., Cancer Res.* **42**: 2821 (1982)) with the sequence preference of 5'-d(A/GNTTA)-3' and 5'-d(AAAAA)-3' and alkylates the N3 position of the 3'-adenine by its CPI left-hand unit present in the molecule (Hurley *et al., Science* **226**: 843 (1984)). Despite its potent and broad antitumor activity, CC-1065 cannot be used in humans because it causes delayed death in experimental animals.

Many analogues and derivatives of CC-1065 and the duocarmycins are known in the art. The research into the structure, synthesis and properties of many of the compounds has been reviewed. *See*, for example, Boger *et al., Angew. Chem. Int. Ed. Engl.* **35**: 1438 (1996); and Boger *et al., Chem. Rev.* **97**: 787 (1997).

A group at Kyowa Hakko Kogya Co., Ltd. has prepared a number of CC-1065 derivatives. See, for example, U.S. Pat. No. 5,101, 038; 5,641,780; 5,187,186; 5,070,092; 5,703,080; 5,070,092; 5,641,780; 5,101,038; and 5,084,468; and published PCT application, WO 96/10405 and published European application 0 537 575 A1.

The Upjohn Company (Pharmacia Upjohn) has also been active in preparing derivatives of CC-1065. See, for example, U.S. Patent No. 5,739,350; 4,978,757, 5,332, 837 and 4,912,227.

Research has also focused on the development of new therapeutic agents which are in the form of prodrugs, compounds that are capable of being converted to drugs (active therapeutic compounds) *in vivo* by certain chemical or enzymatic modifications of their structure. For purposes of reducing toxicity, this conversion is preferably confined to the site of action or target tissue rather than the circulatory system or non-target tissue. However, even prodrugs are problematic as many are characterized by a low stability in blood and serum, due to the presence of enzymes that degrade or activate the prodrugs before the prodrugs reach the desired sites within the patient's body.

Bristol-Myers Squibb has described particular lysosomal enzyme-cleavable antitumor drug conjugates. See, for example, U.S. Patent No. 6,214,345. This patent provides an aminobenzyl oxycarbonyl.

Seattle Genetics has published applications U.S. Pat. Appl. 2003/0096743 and U.S. Pat. Appl. 2003/0130189, which describe p-aminobenzylethers in drug delivery agents. The linkers described in these applications are limited to aminobenzyl ether compositions.

Other groups have also described linkers. See for example de Groot *et al., J. Med. Chem.* 42, 5277 (1999); de Groot *et al. J. Org. Chem.* 43, 3093 (2000); de Groot *et al., J.*

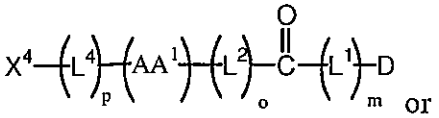
Med. Chem. 66, 8815, (2001); WO 02/083180; Carl *et al.*, *J. Med. Chem. Lett.* 24, 479, (1981); Dubowchik *et al., Bioorg & Med. Chem. Lett.* 8, 3347 (1998). These linkers include aminobenzyl ether spacer, elongated electronic cascade and cyclization spacer systems, cyclisation eliminations spacers, such as w-amino aminocarbonyls, and a *p* aminobenzy oxycarbonyl linker.

Stability of cytotoxin drugs, including *in vivo* stability, is still an important issue that needs to be addressed. In addition, the toxicity of many compounds makes them less useful, so compositions that will reduce drug toxicity, such as the formation of a cleaveable prodrug, are needed. Therefore, in spite of the advances in the art, there continues to be a need for the development of improved therapeutic agents for the treatment of mammals, and humans in particular, more specifically cytotoxins that exhibit high specificity of action, reduced toxicity, and improved stability in blood relative to known compounds of similar structure. The instant invention addresses those needs.

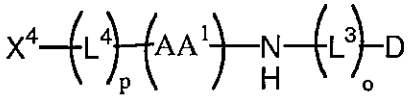
SUMMARY OF THE INVENTION

The present invention relates to drug-ligand conjugates where the drug and ligand are linked through a linker. These conjugates are potent cytotoxins that can be selectively delivered to a site of action of interest in an active form and then cleaved to release the active drug.

One embodiment is a compound of the formula



or



wherein

L^1 is substituted or unsubstituted alkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl or substituted or unsubstituted heteroalkyl group;

m is an integer 0, 1, 2, 3, 4, 5, or 6;

AA^1 is an amino acid selected from the group consisting of natural amino acids and unnatural α-amino acids;

L^2 is substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, unsubstituted heterocycloalkyl, substituted heterocycloalkyl, substituted or unsubstituted aryl, or substituted or unsubstituted heteroaryl;

L^3 is substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, or substituted or unsubstituted heterocycloalkyl;

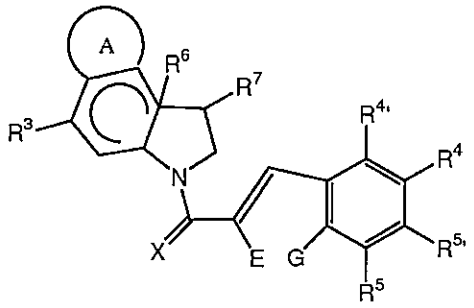
o is 0 or 1;

L^4 is a linker member;

p is 0 or 1;

X^4 is a member selected from the group consisting of protected reactive functional groups, unprotected reactive functional groups, detectable labels, and targeting agents; and

D comprises a structure:



wherein the ring system A is substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl or substituted or unsubstituted heterocycloalkyl;

E and G are members independently selected from H, substituted and unsubstituted alkyl, substituted and unsubstituted heteroalkyl, a heteroatom, and a single bond, or E and G are joined to form a ring system selected from substituted and unsubstituted aryl, substituted and unsubstituted heteroaryl and substituted and unsubstituted heterocycloalkyl;

X is a member selected from O, S and NR^{23};

R^{23} is H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, or acyl;

R^3 is OR^{11},

wherein R^{11} is H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, diphosphate, triphosphate, acyl, $C(O)R^{12}R^{13}$, $C(O)OR^{12}$, $C(O)NR^{12}R^{13}$, $P(O)(OR^{12})_2$, $C(O)CHR^{12}R^{13}$, SR^{12}, or $SiR^{12}R^{13}R^{14}$,

in which R^{12}, R^{13}, and R^{14} are members independently selected from H, substituted and unsubstituted alkyl, substituted and unsubstituted heteroalkyl and substituted and unsubstituted aryl, wherein R^{12} and R^{13} together with the nitrogen or carbon atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

R^4 , $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, SO_3, SO_2R^{15}, $NR^{15}R^{16}$, $NR^{16}C(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, $CR^{15}=NR^{16}$, and $O(CH_2)_nN(CH_3)_2$, or any adjacent pair of R^4, $R^{4'}$, R^5 and $R^{5'}$, together with the carbon atoms to which they are attached, are joined to form a substituted or unsubstituted cycloalkyl or heterocycloalkyl ring system having from 4 to 6 members;

wherein

n is an integer from 1 to 20;

R^{15} and R^{16} are independently selected from H, substituted and unsubstituted alkyl, substituted and unsubstituted heteroalkyl, substituted and unsubstituted aryl, substituted and unsubstituted heteroaryl, substituted and unsubstituted heterocycloalkyl, and substituted and unsubstituted peptidyl, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

R^6 is a single bond which is either present or absent and when present R^6 and R^7 are joined to form a cyclopropyl ring; and

R^7 is CH_2-X^1 or –CH_2- joined in said cyclopropyl ring with R^6, wherein

X^1 is a leaving group,

wherein at least one of R^4, $R^{4'}$, R^5, $R^{5'}$, R^{11}, R^{12}, R^{13}, R^{15} or R^{16} links D to the remainder of the compound;

or a pharmaceutically acceptable salt thereof.

Any of these compounds can be used as, or used to form, drug-ligand conjugates.

In yet another aspect, the invention pertains to pharmaceutical formulations. Such formulations typically comprise a conjugate compound of the invention and a pharmaceutically acceptable carrier.

In still a further aspect, the invention pertains to methods of using the conjugate compounds of the invention. For example, the invention provides a method of killing a cell, wherein a conjugate compound of the invention is administered to the cell an amount sufficient to kill the cell. In a preferred embodiment, the cell is a tumor cell. In another embodiment, the invention provides a method of retarding or stopping the growth of a tumor in a mammalian subject, wherein a conjugate compound of the invention is administered to the subject an amount sufficient to retard or stop growth of the tumor.

Other aspects, advantages and objects of the invention will be apparent from review of the detailed description below.

BRIEF DESCRIPTION OF THE DRAWINGS

Non-limiting and non-exhaustive embodiments of the present invention are described with reference to the following drawings. In the drawings, like reference numerals refer to like parts throughout the various figures unless otherwise specified.

For a better understanding of the present invention, reference will be made to the following Detailed Description, which is to be read in association with the accompanying drawings, wherein:

FIGS. 1-9 are graphs of mean tumor volume, median tumor volume, and median % body weight change, respectively, versus days past dosing for an *in vivo* study.

DETAILED DESCRIPTION OF THE INVENTION

Abbreviations

"Ala," refers to alanine.

"Boc," refers to *t*-butyloxycarbonyl.

"CPI," refers to cyclopropapyrroloindole.

"Cbz," refers to carbobenzoxy.

"DCM" refers to dichloromethane.

"DDQ" refers to 2,3-dichloro-5,6-dicyano-1,4-benzoquinone.

"DIPEA" refers to diisopropylethalamine

"DMDA" refers to N,N'-dimethylethylene diamine

"RBF" refers to a round bottom flask

"DMF" refers to N,B-dimethylformamide

"HATU" refers to N-[[(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridin-1-yl]methylene]-N-methylmethanaminium hexafluorophosphate N-oxide

The symbol "E," represents an enzymatically cleaveable group.

"EDCI" refers to 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide.

"FMOC" refers to 9-fluorenylmethyloxycarbonyl.

"HOAt" refers to 7-aza-1-hydroxybenzotriazole.

"Leu" refers to leucine.

"PABA" refers to *para*-aminobenzoic acid.

"PEG" refers to polyethylene glycol

"PMB" refers to *para*-methoxybenzyl.

"TBAF," refers to tetrabutylammonium fluoride.

"TBSO," refers to *t*-butyldimethylsilyl ether.

"TEA" refers to triethylamine.

"TFA" refers to trifluororoacetic acid.

"EDC" refers to (1-Ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride)

"TBTU" refers to (2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate)

"HOBT" refers to N-Hydroxybenzotriazole

The symbol "Q" refers to a therapeutic agent, diagnostic agent or detectable label.

Definitions

Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, organic chemistry and nucleic acid chemistry and hybridization described below are those well known and commonly employed in the art. Standard techniques are used for nucleic acid and peptide synthesis. Generally, enzymatic reactions and purification steps are performed according to the manufacturer's specifications.

The techniques and procedures are generally performed according to conventional methods in the art and various general references (*see generally*, Sambrook *et al.* MOLECULAR CLONING: A LABORATORY MANUAL, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., which is incorporated herein by reference), which are provided throughout this document. The nomenclature used herein and the laboratory procedures in analytical chemistry, and organic synthetic described below are those well known and commonly employed in the art. Standard techniques, or modifications thereof, are used for chemical syntheses and chemical analyses.

The term "therapeutic agent" is intended to mean a compound that, when present in a therapeutically effective amount, produces a desired therapeutic effect on a mammal. For treating carcinomas, it is desirable that the therapeutic agent also be capable of entering the target cell.

The term "cytotoxin" is intended to mean a therapeutic agent having the desired effect of being cytotoxic to cancer cells. Cytotoxic means that the agent arrests the growth of, or kills the cells. Exemplary cytotoxins include, by way of example and not limitation, combretastatins, duocarmycins, the CC-1065 anti-tumor antibiotics, anthracyclines, and related compounds. Other cytotoxins include mycotoxins, ricin and its analogues, calicheamycins, doxirubicin and maytansinoids.

The term "prodrug" and the term "drug conjugate" are used herein interchangeably. Both refer to a compound that is relatively innocuous to cells while still in the conjugated form but which is selectively degraded to a pharmacologically active form by conditions, e.g., enzymes, located within or in the proximity of target cells.

The term "marker" is intended to mean a compound useful in the characterization of tumors or other medical condition, for example, diagnosis, progression of a tumor, and assay of the factors secreted by tumor cells. Markers are considered a subset of "diagnostic agents."

The term "selective" as used in connection with enzymatic cleavage means that the rate of rate of cleavage of the linker moiety is greater than the rate of cleavage of a peptide having a random sequence of amino acids.

The terms "targeting group" and "targeting agent" are intended to mean a moiety that is (1) able to direct the entity to which it is attached (e.g., therapeutic agent or marker) to a target cell, for example to a specific type of tumor cell or (2) is preferentially activated at a target tissue, for example a tumor. The targeting group or targeting agent can be a small

molecule, which is intended to include both non-peptides and peptides. The targeting group can also be a macromolecule, which includes saccharides, lectins, receptors, ligand for receptors, proteins such as BSA, antibodies, and so forth. In a preferred embodiment of the current invention, the targeting group is an antibody or an antibody fragment, more preferably a monoclonal antibody or monoclonal antibody fragment

The term "self-immolative spacer" refers to a bifunctional chemical moiety that is capable of covalently linking two chemical moieties into a normally stable tripartate molecule. The self-immolative spacer is capable of spontaneously separating from the second moiety if the bond to the first moiety is cleaved.

The term "detectable label" is intended to mean a moiety having a detectable physical or chemical property.

The term "cleaveable group" is intended to mean a moiety that is unstable *in vivo*. Preferably the "cleaveable group" allows for activation of the marker or therapeutic agent by cleaving the marker or agent from the rest of the conjugate. Operatively defined, the linker is preferably cleaved *in vivo* by the biological environment. The cleavage may come from any process without limitation, e.g., enzymatic, reductive, pH, etc. Preferably, the cleaveable group is selected so that activation occurs at the desired site of action, which can be a site in or near the target cells (e.g., carcinoma cells) or tissues such as at the site of therapeutic action or marker activity. Such cleavage may be enzymatic and exemplary enzymatically cleaveable groups include natural amino acids or peptide sequences that end with a natural amino acid, and are attached at their carboxyl terminus to the linker. While the degree of cleavage rate enhancement is not critical to the invention, preferred examples of cleaveable linkers are those in which at least about 10% of the cleaveable groups are cleaved in the blood stream within 24 hours of administration, most preferably at least about 35%.

The term "ligand" means any molecule that specifically binds or reactively associates or complexes with a receptor, substrate, antigenic determinant, or other binding site on a target cell or tissue. Examples of ligands include antibodies and fragments thereof (e.g., a monoclonal antibody or fragment thereof), enzymes (e.g., fibrinolytic enzymes), biologic response modifiers (e.g., interleukins, interferons, erythropeoitin, or colony stimulating factors), peptide hormones, and antigen-binding fragments thereof.

The term "cyclization reaction," when referring to the cyclization of a linker or any portion thereof, indicates the cyclization of that linker into a ring and initiates the separation

of the drug-ligand complex. This rate can be measured *ex situ*, and is completed when at least 90%, 95%, or 100% of the product is formed.

The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymer. These terms also encompass the term "antibody."

The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. One amino acid that may be used in particular is citrulline, which is a precursor to arginine and is involved in the formation of urea in the liver. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but functions in a manner similar to a naturally occurring amino acid. The term "unnatural amino acid" is intended to represent the "D" stereochemical form of the twenty naturally occurring amino acids described above. It is further understood that the term unnatural amino acid includes homologues of the natural amino acids, and synthetically modified forms of the natural amino acids. The synthetically modified forms include, but are not limited to, amino acids having alkylene chains shortened or lengthened by up to two carbon atoms, amino acids comprising optionally substituted aryl groups, and amino acids comprised halogenated groups, preferably halogenated alkyl and aryl groups. When attached to a linker or conjugate of the invention, the amino acid is in the form of an "amino acid side chain", where the carboxylic acid group of the amino acid has been replaced with a keto (C(O)) group. Thus, for example, an alanine side chain is $-C(O)-CH(NH_2)-CH_3$, and so forth.

Amino acids and peptides may be protected by blocking groups. A blocking group is an atom or a chemical moiety that protects the N-terminus of an amino acid or a peptide from

undesired reactions and can be used during the synthesis of a drug-ligand conjugate. It should remain attached to the N-terminus throughout the synthesis, and may be removed after completion of synthesis of the drug conjugate by chemical or other conditions that selectively achieve its removal. The blocking groups suitable for N-terminus protection are well known in the art of peptide chemistry. Exemplary blocking groups include, but are not limited to, hydrogen, D-amino acid, and carbobenzoxy (Cbz) chloride.

"Nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs).

Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.* **19**:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.* **260**: 2605-2608 (1985); Rossolini *et al.*, *Mol. Cell. Probes* **8**: 91-98 (1994)). The term nucleic acid is used interchangeably with gene, cDNA, mRNA, oligonucleotide, and polynucleotide.

The symbol , whether utilized as a bond or displayed perpendicular to a bond indicates the point at which the displayed moiety is attached to the remainder of the molecule, solid support, etc.

The term "alkyl," by itself or as part of another substituent, means, unless otherwise stated, a straight or branched chain, or cyclic hydrocarbon radical, or combination thereof, which may be fully saturated, mono- or polyunsaturated and can include di- and multivalent radicals, having the number of carbon atoms designated (*i.e.* C_1-C_{10} means one to ten carbons). Examples of saturated hydrocarbon radicals include, but are not limited to, groups such as methyl, ethyl, n-propyl, isopropyl, n-butyl, t-butyl, isobutyl, sec-butyl, cyclohexyl, (cyclohexyl)methyl, cyclopropylmethyl, homologs and isomers of, for example, n-pentyl, n-

hexyl, n-heptyl, n-octyl, and the like. An unsaturated alkyl group is one having one or more double bonds or triple bonds. Examples of unsaturated alkyl groups include, but are not limited to, vinyl, 2-propenyl, crotyl, 2-isopentenyl, 2-(butadienyl), 2,4-pentadienyl, 3-(1,4-pentadienyl), ethynyl, 1- and 3-propynyl, 3-butynyl, and the higher homologs and isomers. The term "alkyl," unless otherwise noted, is also meant to include those derivatives of alkyl defined in more detail below, such as "heteroalkyl." Alkyl groups, which are limited to hydrocarbon groups are termed "homoalkyl".

The term "alkylene" by itself or as part of another substituent means a divalent radical derived from an alkane, as exemplified, but not limited, by $-CH_2CH_2CH_2CH_2-$, and further includes those groups described below as "heteroalkylene." Typically, an alkyl (or alkylene) group will have from 1 to 24 carbon atoms, with those groups having 10 or fewer carbon atoms being preferred in the present invention. A "lower alkyl" or "lower alkylene" is a shorter chain alkyl or alkylene group, generally having eight or fewer carbon atoms.

The term "heteroalkyl," by itself or in combination with another term, means, unless otherwise stated, a stable straight or branched chain, or cyclic hydrocarbon radical, or combinations thereof, consisting of the stated number of carbon atoms and at least one heteroatom selected from the group consisting of O, N, Si, and S, and wherein the nitrogen, carbon and sulfur atoms may optionally be oxidized and the nitrogen heteroatom may optionally be quaternized. The heteroatom(s) O, N, S, and Si may be placed at any interior position of the heteroalkyl group or at the position at which the alkyl group is attached to the remainder of the molecule. Examples include, but are not limited to, $-CH_2-CH_2-O-CH_3$, $-CH_2-CH_2-NH-CH_3$, $-CH_2-CH_2-N(CH_3)-CH_3$, $-CH_2-S-CH_2-CH_3$, $-CH_2-CH_2,-S(O)-CH_3$, $-CH_2-CH_2-S(O)_2-CH_3$, $-CH=CH-O-CH_3$, $-Si(CH_3)_3$, $-CH_2-CH=N-OCH_3$, and $-CH=CH-N(CH_3)-CH_3$. Up to two heteroatoms may be consecutive, such as, for example, $-CH_2-NH-OCH_3$ and $-CH_2-O-Si(CH_3)_3$. Similarly, the term "heteroalkylene" by itself or as part of another substituent means a divalent radical derived from heteroalkyl, as exemplified, but not limited by, $-CH_2-CH_2-S-CH_2-CH_2-$ and $-CH_2-S-CH_2-CH_2-NH-CH_2-$. For heteroalkylene groups, heteroatoms can also occupy either or both of the chain termini (*e.g.*, alkyleneoxy, alkylenedioxy, alkyleneamino, alkylenediamino, and the like). The terms "heteroalkyl" and "heteroalkylene" encompass poly(ethylene glycol) and its derivatives (see, for example, Shearwater Polymers Catalog, 2001). Still further, for alkylene and heteroalkylene linking groups, no orientation of the linking group is implied by the direction in which the formula of

the linking group is written. For example, the formula $-C(O)_2R'-$ represents both $-C(O)_2R'-$ and $-R'C(O)_2-$.

The term "lower" in combination with the terms "alkyl" or "heteroalkyl" refers to a moiety having from 1 to 6 carbon atoms.

The terms "alkoxy," "alkylamino," "alkylsulfonyl," and "alkylthio" (or thioalkoxy) are used in their conventional sense, and refer to those alkyl groups attached to the remainder of the molecule via an oxygen atom, an amino group, an SO_2 group or a sulfur atom, respectively. The term "arylsulfonyl" refers to an aryl group attached to the remainder of the molecule via an SO_2 group, and the term "sulfhydryl" refers to an SH group.

In general, an "acyl substituent" is also selected from the group set forth above. As used herein, the term "acyl substituent" refers to groups attached to, and fulfilling the valence of a carbonyl carbon that is either directly or indirectly attached to the polycyclic nucleus of the compounds of the present invention.

The terms "cycloalkyl" and "heterocycloalkyl", by themselves or in combination with other terms, represent, unless otherwise stated, cyclic versions of substituted or unsubstituted "alkyl" and substituted or unsubstituted "heteroalkyl", respectively. Additionally, for heterocycloalkyl, a heteroatom can occupy the position at which the heterocycle is attached to the remainder of the molecule. Examples of cycloalkyl include, but are not limited to, cyclopentyl, cyclohexyl, 1-cyclohexenyl, 3-cyclohexenyl, cycloheptyl, and the like. Examples of heterocycloalkyl include, but are not limited to, 1 –(1,2,5,6-tetrahydropyridyl), 1-piperidinyl, 2-piperidinyl, 3-piperidinyl, 4-morpholinyl, 3-morpholinyl, tetrahydrofuran-2-yl, tetrahydrofuran-3-yl, tetrahydrothien-2-yl, tetrahydrothien-3-yl, 1 –piperazinyl, 2-piperazinyl, and the like. The heteroatoms and carbon atoms of the cyclic structures are optionally oxidized.

The terms "halo" or "halogen," by themselves or as part of another substituent, mean, unless otherwise stated, a fluorine, chlorine, bromine, or iodine atom. Additionally, terms such as "haloalkyl," are meant to include monohaloalkyl and polyhaloalkyl. For example, the term "halo(C_1-C_4)alkyl" is mean to include, but not be limited to, trifluoromethyl, 2,2,2-trifluoroethyl, 4-chlorobutyl, 3-bromopropyl, and the like.

The term "aryl" means, unless otherwise stated, a substituted or unsubstituted polyunsaturated, aromatic, hydrocarbon substituent which can be a single ring or multiple rings (preferably from 1 to 3 rings) which are fused together or linked covalently. The term "heteroaryl" refers to aryl groups (or rings) that contain from one to four heteroatoms

selected from N, O, and S, wherein the nitrogen, carbon and sulfur atoms are optionally oxidized, and the nitrogen atom(s) are optionally quaternized. A heteroaryl group can be attached to the remainder of the molecule through a heteroatom. Non-limiting examples of aryl and heteroaryl groups include phenyl, 1-naphthyl, 2-naphthyl, 4-biphenyl, 1-pyrrolyl, 2-pyrrolyl, 3-pyrrolyl, 3-pyrazolyl, 2-imidazolyl, 4-imidazolyl, pyrazinyl, 2-oxazolyl, 4-oxazolyl, 2-phenyl-4-oxazolyl, 5-oxazolyl, 3-isoxazolyl, 4-isoxazolyl, 5-isoxazolyl, 2-thiazolyl, 4-thiazolyl, 5-thiazolyl, 2-furyl, 3-furyl, 2-thienyl, 3-thienyl, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-pyrimidyl, 4-pyrimidyl, 5-benzothiazolyl, purinyl, 2-benzimidazolyl, 5-indolyl, 1-isoquinolyl, 5-isoquinolyl, 2-quinoxalinyl, 5-quinoxalinyl, 3-quinolyl, and 6-quinolyl. Substituents for each of the above noted aryl and heteroaryl ring systems are selected from the group of acceptable substituents described below. "Aryl" and "heteroaryl" also encompass ring systems in which one or more non-aromatic ring systems are fused, or otherwise bound, to an aryl or heteroaryl system.

For brevity, the term "aryl" when used in combination with other terms (*e.g.*, aryloxy, arylthioxy, and arylalkyl) includes both aryl and heteroaryl rings as defined above. Thus, the term "arylalkyl" is meant to include those radicals in which an aryl group is attached to an alkyl group (*e.g.*, benzyl, phenethyl, pyridylmethyl and the like) including those alkyl groups in which a carbon atom (*e.g.*, a methylene group) has been replaced by, for example, an oxygen atom (*e.g.*, phenoxymethyl, 2-pyridyloxymethyl, 3-(1-naphthyloxy)propyl, and the like).

Each of the above terms (*e.g.*, "alkyl," "heteroalkyl," "aryl" and "heteroaryl") include both substituted and unsubstituted forms of the indicated radical. Preferred substituents for each type of radical are provided below.

Substituents for the alkyl, and heteroalkyl radicals (including those groups often referred to as alkylene, alkenyl, heteroalkylene, heteroalkenyl, alkynyl, cycloalkyl, heterocycloalkyl, cycloalkenyl, and heterocycloalkenyl) are generally referred to as "alkyl substituents" and "heteroalkyl substituents," respectively, and they can be one or more of a variety of groups selected from, but not limited to: -OR', =O, =NR', =N-OR', -NR'R", -SR', -halogen, -SiR'R"R"', -OC(O)R', -C(O)R', $-CO_2R'$, -CONR'R", -OC(O)NR'R", -NR"C(O)R', -NR'-C(O)NR"R"', -NR"$C(O)_2$R', -NR-C(NR'R"R"')=NR"", -NR-C(NR'R")=NR"', -S(O)R', $-S(O)_2R'$, $-S(O)_2NR'R"$, $-NRSO_2R'$, -CN and $-NO_2$ in a number ranging from zero to (2m'+1), where m' is the total number of carbon atoms in such radical. R', R", R"' and R"" each preferably independently refer to hydrogen, substituted or

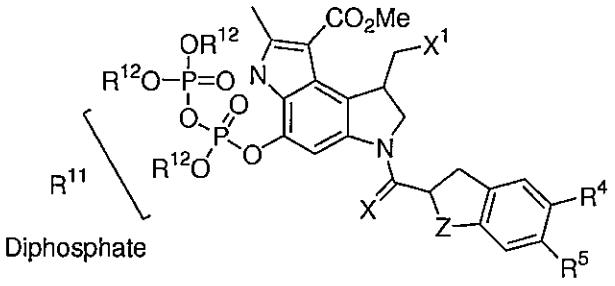
unsubstituted heteroalkyl, substituted or unsubstituted aryl, e.g., aryl substituted with 1-3 halogens, substituted or unsubstituted alkyl, alkoxy or thioalkoxy groups, or arylalkyl groups. When a compound of the invention includes more than one R group, for example, each of the R groups is independently selected as are each R', R", R'" and R"" groups when more than one of these groups is present. When R' and R" are attached to the same nitrogen atom, they can be combined with the nitrogen atom to form a 5-, 6-, or 7-membered ring. For example, -NR'R" is meant to include, but not be limited to, 1-pyrrolidinyl and 4-morpholinyl. From the above discussion of substituents, one of skill in the art will understand that the term "alkyl" is meant to include groups including carbon atoms bound to groups other than hydrogen groups, such as haloalkyl (*e.g.*, $-CF_3$ and $-CH_2CF_3$) and acyl (*e.g.*, $-C(O)CH_3$, $-C(O)CF_3$, $-C(O)CH_2OCH_3$, and the like).

Similar to the substituents described for the alkyl radical, the aryl substituents and heteroaryl substituents are generally referred to as "aryl substituents" and "heteroaryl substituents," respectively and are varied and selected from, for example: halogen, -OR', =O, =NR', =N-OR', -NR'R", -SR', -halogen, -SiR'R"R'", -OC(O)R', -C(O)R', $-CO_2R'$, -CONR'R", -OC(O)NR'R", -NR"C(O)R', -NR'-C(O)NR"R'", $-NR"C(O)_2R'$, -NR-C(NR'R")=NR'", $-S(O)R'$, $-S(O)_2R'$, $-S(O)_2NR'R"$, $-NRSO_2R'$, -CN and $-NO_2$, -R', $-N_3$, $-CH(Ph)_2$, fluoro(C_1-C_4)alkoxy, and fluoro(C_1-C_4)alkyl, in a number ranging from zero to the total number of open valences on the aromatic ring system; and where R', R", R'" and R"" are preferably independently selected from hydrogen, (C_1-C_8)alkyl and heteroalkyl, unsubstituted aryl and heteroaryl, (unsubstituted aryl)-(C_1-C_4)alkyl, and (unsubstituted aryl)oxy-(C_1-C_4)alkyl. When a compound of the invention includes more than one R group, for example, each of the R groups is independently selected as are each R', R", R'" and R"" groups when more than one of these groups is present.

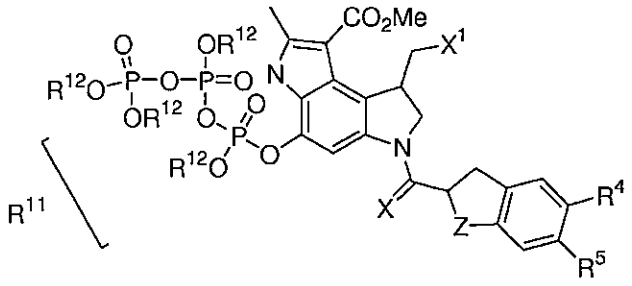
Two of the aryl substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula $-T-C(O)-(CRR')_q-U-$, wherein T and U are independently –NR-, -O-, -CRR'- or a single bond, and q is an integer of from 0 to 3. Alternatively, two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula $-A-(CH_2)_r-B-$, wherein A and B are independently –CRR'-, -O-, -NR-, -S-, -S(O)-, $-S(O)_2-$, $-S(O)_2NR'-$ or a single bond, and r is an integer of from 1 to 4. One of the single bonds of the new ring so formed may optionally be replaced with a double bond. Alternatively, two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula

-(CRR')$_s$-X-(CR"R'")$_d$-, where s and d are independently integers of from 0 to 3, and X is -O-, -NR'-, -S-, -S(O)-, -S(O)$_2$-, or –S(O)$_2$NR'-. The substituents R, R', R" and R'" are preferably independently selected from hydrogen or substituted or unsubstituted (C_1-C_6) alkyl.

As used herein, the term "diphosphate" includes but is not limited to an ester of phosphoric acid containing two phosphate groups. The term "triphosphate" includes but is not limited to an ester of phosphoric acid containing three phosphate groups. For example, particular drugs having a diphosphate or a triphosphate include:



Diphosphate



Triphosphate

As used herein, the term "heteroatom" includes oxygen (O), nitrogen (N), sulfur (S) and silicon (Si).

The symbol "R" is a general abbreviation that represents a substituent group that is selected from substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, and substituted or unsubstituted heterocyclyl groups.

The term "pharmaceutically acceptable carrier", as used herein means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting a chemical agent. Pharmaceutically acceptable carriers include pharmaceutically acceptable

salts, where the term "pharmaceutically acceptable salts" includes salts of the active compounds which are prepared with relatively nontoxic acids or bases, depending on the particular substituents found on the compounds described herein. When compounds of the present invention contain relatively acidic functionalities, base addition salts can be obtained by contacting the neutral form of such compounds with a sufficient amount of the desired base, either neat or in a suitable inert solvent. Examples of pharmaceutically acceptable base addition salts include sodium, potassium, calcium, ammonium, organic amino, or magnesium salt, or a similar salt. When compounds of the present invention contain relatively basic functionalities, acid addition salts can be obtained by contacting the neutral form of such compounds with a sufficient amount of the desired acid, either neat or in a suitable inert solvent. Examples of pharmaceutically acceptable acid addition salts include those derived from inorganic acids like hydrochloric, hydrobromic, nitric, carbonic, monohydrogencarbonic, phosphoric, monohydrogenphosphoric, dihydrogenphosphoric, sulfuric, monohydrogensulfuric, hydriodic, or phosphorous acids and the like, as well as the salts derived from relatively nontoxic organic acids like acetic, propionic, isobutyric, maleic, malonic, benzoic, succinic, suberic, fumaric, lactic, mandelic, phthalic, benzenesulfonic, p-tolylsulfonic, citric, tartaric, methanesulfonic, and the like. Also included are salts of amino acids such as arginate and the like, and salts of organic acids like glucuronic or galactunoric acids and the like (*see*, for example, Berge *et al.*, "Pharmaceutical Salts", *Journal of Pharmaceutical Science*, **1977**, *66*, 1-19). Certain specific compounds of the present invention contain both basic and acidic functionalities that allow the compounds to be converted into either base or acid addition salts.

The neutral forms of the compounds are preferably regenerated by contacting the salt with a base or acid and isolating the parent compound in the conventional manner. The parent form of the compound differs from the various salt forms in certain physical properties, such as solubility in polar solvents, but otherwise the salts are equivalent to the parent form of the compound for the purposes of the present invention.

In addition to salt forms, the present invention provides compounds, which are in a prodrug form. Prodrugs of the compounds described herein are those compounds that readily undergo chemical changes under physiological conditions to provide the compounds of the present invention. Additionally, prodrugs can be converted to the compounds of the present invention by chemical or biochemical methods in an *ex vivo* environment. For example,

prodrugs can be slowly converted to the compounds of the present invention when placed in a transdermal patch reservoir with a suitable enzyme or chemical reagent.

Certain compounds of the present invention can exist in unsolvated forms as well as solvated forms, including hydrated forms. In general, the solvated forms are equivalent to unsolvated forms and are encompassed within the scope of the present invention. Certain compounds of the present invention may exist in multiple crystalline or amorphous forms. In general, all physical forms are equivalent for the uses contemplated by the present invention and are intended to be within the scope of the present invention.

Certain compounds of the present invention possess asymmetric carbon atoms (optical centers) or double bonds; the racemates, diastereomers, geometric isomers and individual isomers are encompassed within the scope of the present invention.

The compounds of the present invention may also contain unnatural proportions of atomic isotopes at one or more of the atoms that constitute such compounds. For example, the compounds may be radiolabeled with radioactive isotopes, such as for example tritium (^{3}H), iodine-125 (^{125}I) or carbon-14 (^{14}C). All isotopic variations of the compounds of the present invention, whether radioactive or not, are intended to be encompassed within the scope of the present invention.

The term "attaching moiety" or "moiety for attaching a targeting group" refers to a moiety which allows for attachment of a targeting group to the linker. Typical attaching groups include, by way of illustration and not limitation, alkyl, aminoalkyl, aminocarbonylalkyl, carboxyalkyl, hydroxyalkyl, alkyl-maleimide, alkyl-N-hydroxylsuccinimide, poly(ethylene glycol)-maleimide and poly(ethylene glycol)-N-hydroxylsuccinimide, all of which may be further substituted. The linker can also have the attaching moiety be actually appended to the targeting group.

As used herein, the term "leaving group" refers to a portion of a substrate that is cleaved from the substrate in a reaction.

The term "antibody" as referred to herein includes whole antibodies and any antigen binding fragment (*i.e.*, "antigen-binding portion") or single chains thereof. An "antibody" refers to a glycoprotein comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds, or an antigen binding portion thereof. Each heavy chain is comprised of a heavy chain variable region (V_H) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, C_{H1}, C_{H2} and C_{H3}, and may be of the mu, delta, gamma, alpha or epsilon isotype. Each light chain is comprised of a light chain

variable region (V_L) and a light chain constant region. The light chain constant region is comprised of one domain, C_L, which may be of the kappa or lambda isotype. The V_H and V_L regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (*e.g.*, effector cells) and the first component (Clq) of the classical complement system.

The terms "antibody fragment" or "antigen-binding portion" of an antibody (or simply "antibody portion"), as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen. It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antibody fragment" or "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the V_L, V_H, C_L and C_{H1} domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the V_H and C_{H1} domains; (iv) a Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, (1989) *Nature* 341:544-546), which consists of a V_H domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, V_L and V_H, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules (known as single chain Fv (scFv); *see e.g.*, Bird *et al.* (1988) *Science* 242:423-426; and Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies.

The terms "monoclonal antibody" as used herein refers to a preparation of antibody molecules of single molecular composition. A monoclonal antibody composition displays a single binding specificity and affinity for a particular epitope.

For preparation of monoclonal or polyclonal antibodies, any technique known in the art can be used (*see, e.g.*, Kohler & Milstein, *Nature* 256:495-497 (1975); Kozbor *et al.*, *Immunology Today* 4: 72 (1983); Cole *et al.*, pp. 77-96 in MONOCLONAL ANTIBODIES AND CANCER THERAPY, Alan R. Liss, Inc. (1985)).

Methods of production of polyclonal antibodies are known to those of skill in the art. An inbred strain of mice (*e.g.*, BALB/C mice) or rabbits is immunized with the protein using a standard adjuvant, such as Freund's adjuvant, and a standard immunization protocol. The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the beta subunits. When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the protein can be done if desired.

Monoclonal antibodies may be obtained by various techniques familiar to those skilled in the art. Briefly, spleen cells from an animal immunized with a desired antigen are immortalized, commonly by fusion with a myeloma cell (*see* Kohler & Milstein, *Eur. J. Immunol.* **6**: 511-519 (1976)). Alternative methods of immortalization include transformation with Epstein Barr Virus, oncogenes, or retroviruses, or other methods well known in the art.

In a preferred embodiment, the antibody is a chimeric or humanized antibody. Chimeric or humanized antibodies of the present invention can be prepared based on the sequence of a murine monoclonal antibody. DNA encoding the heavy and light chain immunoglobulins can be obtained from the murine hybridoma of interest and engineered to contain non-murine (*e.g.*,. human) immunoglobulin sequences using standard molecular biology techniques. For example, to create a chimeric antibody, the murine variable regions can be linked to human constant regions using methods known in the art (see *e.g.*, U.S. Patent No. 4,816,567 to Cabilly *et al.*). To create a humanized antibody, the murine CDR regions can be inserted into a human framework using methods known in the art (see *e.g.*, U.S. Patent No. 5,225,539 to Winter, and U.S. Patent Nos. 5,530,101; 5,585,089; 5,693,762 and 6,180,370 to Queen *et al.*).

In another preferred embodiment, the antibody is a human antibody. Such human antibodies can be generated by immunizing transgenic or transchromosomic mice in which

the endogenous mouse immunoglobulin genes have been inactivated and exogenous human immunoglobulin genes have been introduced. Such mice are known in the art (see *e.g.*, U.S. Patent Nos. 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,789,650; 5,877,397; 5,661,016; 5,814,318; 5,874,299; and 5,770,429; all to Lonberg and Kay; U.S. Patent Nos. 5,939,598; 6,075,181; 6,114,598; 6,150,584 and 6,162,963 to Kucherlapati *et al.*; and PCT Publication WO 02/43478 to Ishida *et al.*) Human antibodies can also be prepared using phage display methods for screening libraries of human immunoglobulin genes. Such phage display methods for isolating human antibodies also are know in the art (see *e.g.*, U.S. Patent Nos. 5,223,409; 5,403,484; and 5,571,698 to Ladner *et al.*; U.S. Patent Nos. 5,427,908 and 5,580,717 to Dower *et al.*; U.S. Patent Nos. 5,969,108 and 6,172,197 to McCafferty *et al.*; and U.S. Patent Nos. 5,885,793; 6,521,404; 6,544,731; 6,555,313; 6,582,915 and 6,593,081 to Griffiths *et al.*).

"Solid support," as used herein refers to a material that is substantially insoluble in a selected solvent system, or which can be readily separated (*e.g.*, by precipitation) from a selected solvent system in which it is soluble. Solid supports useful in practicing the present invention can include groups that are activated or capable of activation to allow selected species to be bound to the solid support. A solid support can also be a substrate, for example, a chip, wafer or well, onto which an individual, or more than one compound, of the invention is bound.

"Reactive functional group," as used herein refers to groups including, but not limited to, olefins, acetylenes, alcohols, phenols, ethers, oxides, halides, aldehydes, ketones, carboxylic acids, esters, amides, cyanates, isocyanates, thiocyanates, isothiocyanates, amines, hydrazines, hydrazones, hydrazides, diazo, diazonium, nitro, nitriles, mercaptans, sulfides, disulfides, sulfoxides, sulfones, sulfonic acids, sulfinic acids, acetals, ketals, anhydrides, sulfates, sulfenic acids isonitriles, amidines, imides, imidates, nitrones, hydroxylamines, oximes, hydroxamic acids thiohydroxamic acids, allenes, ortho esters, sulfites, enamines, ynamines, ureas, pseudoureas, semicarbazides, carbodiimides, carbamates, imines, azides, azo compounds, azoxy compounds, and nitroso compounds. Reactive functional groups also include those used to prepare bioconjugates, e.g., N-hydroxysuccinimide esters, maleimides and the like (*see*, for example, Hermanson, BIOCONJUGATE TECHNIQUES, Academic press, San Diego, 1996). Methods to prepare each of these functional groups are well known in the art and their application to or modification for a particular purpose is within the ability of one of skill in the art (*see*, for example, Sandler and Karo, eds. ORGANIC FUNCTIONAL GROUP

PREPARATIONS, Academic Press, San Diego, 1989). The reactive functional groups may be protected or unprotected.

The compounds of the invention are prepared as a single isomer (*e.g.*, enantiomer, cis-trans, positional, diastereomer) or as a mixture of isomers. In a preferred embodiment, the compounds are prepared as substantially a single isomer. Methods of preparing substantially isomerically pure compounds are known in the art. For example, enantiomerically enriched mixtures and pure enantiomeric compounds can be prepared by using synthetic intermediates that are enantiomerically pure in combination with reactions that either leave the stereochemistry at a chiral center unchanged or result in its complete inversion. Alternatively, the final product or intermediates along the synthetic route can be resolved into a single stereoisomer. Techniques for inverting or leaving unchanged a particular stereocenter, and those for resolving mixtures of stereoisomers are well known in the art and it is well within the ability of one of skill in the art to choose and appropriate method for a particular situation. *See*, generally, Furniss *et al.* (eds.),VOGEL'S ENCYCLOPEDIA OF PRACTICAL ORGANIC CHEMISTRY 5TH ED., Longman Scientific and Technical Ltd., Essex, 1991, pp. 809-816; and Heller, *Acc. Chem. Res.* **23**: 128 (1990).

LINKERS

The present invention provides for drug-ligand conjugates where the drug is linked to the ligand through an amino acid linker, depicted herein as $(L^4)_p$—F— $(L^1)_m$. In addition to the linkers as being attached to the drug, the present invention also provides cleaveable linker arms that are appropriate for attachment to essentially any molecular species. The linker arm aspect of the invention is exemplified herein by reference to their attachment to a therapeutic moiety. It will, however, be readily apparent to those of skill in the art that the linkers can be attached to diverse species including, but not limited to, diagnostic agents, analytical agents, biomolecules, targeting agents, detectable labels and the like.

The use of peptidyl and other linkers in drug-ligand conjugates is described in U.S. Provisional Patent Applications Serial Nos. 60/295,196; 60/295,259; 60/295,342; 60/304,908; 60/572,667; 60/661,174; 60/669,871; 60/720,499; 60/730,804; 60/735,657; 60/882,461; and 60/991,300 and U.S. Patent Applications Serial Nos. 10/160,972; 10/161,234; 11/134,685; 11/134,826; and 11/398,854 and U.S. Patent No. 6,989,452 and PCT Patent Applications Nos. PCT/US2006/37793, PCT/US2006/60050, PCT/US2006/60711, and PCT/SU2007/89100, all of which are incorporated herein by reference.

In one aspect, the present invention relates to linkers that are useful to attach targeting groups to therapeutic agents and markers. In another aspect, the invention provides linkers that impart stability to compounds, reduce their *in vivo* toxicity, or otherwise favorably affect their pharmacokinetics, bioavailability and/or pharmacodynamics. It is generally preferred that in such embodiments, the linker is cleaved, releasing the active drug, once the drug is delivered to its site of action. Thus, in one embodiment of the invention, the linkers of the invention are traceless, such that once removed from the therapeutic agent or marker (such as during activation), no trace of the linker's presence remains.

In another embodiment of the invention, the linkers are characterized by their ability to be cleaved at a site in or near the target cell such as at the site of therapeutic action or marker activity. Such cleavage may be enzymatic in nature. This feature aids in reducing systemic activation of the therapeutic agent or marker, reducing toxicity and systemic side effects. In other embodiments, the linkers are sensitive to pH and are cleaved through changes in pH.

A surprising aspect of the present compounds that include a single amino acid in the linker is that these compounds can have activity similar to, or surpassing, linkers with peptidyl components. One would expect substantially lower activity because the single amino acid, as opposed to a peptide, is not necessarily a substrate for an enzyme.

The linkers also serve to stabilize the therapeutic agent or marker against degradation while in circulation. This feature provides a significant benefit since such stabilization results in prolonging the circulation half-life of the attached agent or marker. The linker also serves to attenuate the activity of the attached agent or marker so that the conjugate is relatively benign while in circulation and has the desired effect, for example is toxic, after activation at the desired site of action. For therapeutic agent conjugates, this feature of the linker serves to improve the therapeutic index of the agent.

The stabilizing groups are preferably selected to limit clearance and metabolism of the therapeutic agent or marker by enzymes that may be present in blood or non-target tissue and are further selected to limit transport of the agent or marker into the cells. The stabilizing groups serve to block degradation of the agent or marker and may also act in providing other physical characteristics of the agent or marker. The stabilizing group may also improve the agent or marker's stability during storage in either a formulated or non-formulated form.

Ideally, the stabilizing group is useful to stabilize a therapeutic agent or marker if it serves to protect the agent or marker from degradation when tested by storage of the agent or

marker in human blood at 37°C for 2 hours and results in less than 20%, preferably less than 10%, more preferably less than 5% and even more preferably less than 2%, cleavage of the agent or marker by the enzymes present in the human blood under the given assay conditions.

The present invention also relates to conjugates containing these linkers. More particularly, the invention relates to prodrugs that may be used for the treatment of disease, especially for cancer chemotherapy. Specifically, use of the linkers described herein provide for prodrugs that display a high specificity of action, a reduced toxicity, and an improved stability in blood relative to prodrugs of similar structure.

The linkers of the present invention as described herein may be present at a variety of positions within the cytotoxic conjugate.

Thus, there is provided a linker that may contain any of a variety of groups as part of its chain that will cleave *in vivo*, e.g., in the blood stream, at a rate which is enhanced relative to that of constructs that lack such groups. Also provided are conjugates of the linker arms with therapeutic and diagnostic agents. The linkers are useful to form prodrug analogs of therapeutic agents and to reversibly link a therapeutic or diagnostic agent to a targeting agent, a detectable label, or a solid support. The linkers may be incorporated into complexes that include the cytotoxins of the invention.

One or more self-immolative linker groups L^1 are optionally introduced between the cytotoxin and the targeting agent. These linker groups may also be described as spacer groups and contain at least two reactive functional groups. Typically, one chemical functionality of the spacer group bonds to a chemical functionality of the therapeutic agent, *e.g.*, cytotoxin, while the other chemical functionality of the spacer group is used to bond to a chemical functionality of the targeting agent or the cleaveable linker. Examples of chemical functionalities of spacer groups include hydroxy, mercapto, carbonyl, carboxy, amino, ketone, and mercapto groups.

The self-immolative linkers, represented by L^1, are generally a substituted or unsubstituted alkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl or substituted or unsubstituted heteroalkyl group. In one embodiment, the alkyl or aryl groups may comprise between 1 and 20 carbon atoms. They may also comprise a polyethylene glycol moiety.

Exemplary spacer groups include, for example, 6-aminohexanol, 6-mercaptohexanol, 10-hydroxydecanoic acid, glycine and other amino acids, 1,6-hexanediol, β-alanine, 2-aminoethanol, cysteamine (2-aminoethanethiol), 5-aminopentanoic acid, 6-aminohexanoic

acid, 3-maleimidobenzoic acid, phthalide, α-substituted phthalides, the carbonyl group, aminal esters, nucleic acids, peptides and the like.

The spacer can serve to introduce additional molecular mass and chemical functionality into the cytotoxin-targeting agent complex. Generally, the additional mass and functionality will affect the serum half-life and other properties of the complex. Thus, through careful selection of spacer groups, cytotoxin complexes with a range of serum half-lives can be produced.

The spacer(s) located directly adjacent to the drug moiety is also denoted as $(L^1)_m$, wherein m is an integer selected from 0, 1, 2, 3, 4, 5, and 6. When multiple L^1 spacers are present, either identical or different spacers may be used. L^1 may be any self-immolative group.

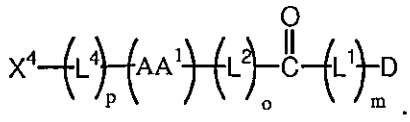
L^4 is a linker moiety that preferably imparts increased solubility or decreased aggregation properties to conjugates utilizing a linker that contains the moiety or modifies the hydrolysis rate of the conjugate. The L^4 linker does not have to be self immolative. In one embodiment, the L^4 moiety is substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroalkyl, or unsubstituted heteroalkyl, any of which may be straight, branched, or cyclic. The substitutions may be, for example, a lower (C^1-C^6) alkyl, alkoxy, aklylthio, alkylamino, or dialkylamino. In certain embodiments, L^4 comprises a non-cyclic moiety . In another embodiment, L^4 comprises any positively or negatively charged amino acid polymer, such as polylysine or polyargenine. L^4 can comprise a polymer such as a polyethylene glycol moiety. Additionally the L^4 linker can comprise, for example, both a polymer component and a small chemical moiety.

In a preferred embodiment, L^4 comprises a polyethylene glycol (PEG) moiety. The PEG portion of L^4 may be between 1 and 50 units long. Preferably, the PEG will have 1-12 repeat units, more preferably 3-12 repeat units, more preferably 2-6 repeat units, or even more preferably 3-5 repeat units and most preferably 4 repeat units. L^4 may consist solely of the PEG moiety, or it may also contain an additional substituted or unsubstituted alkyl or heteroalkyl. It is useful to combine PEG as part of the L^4 moiety to enhance the water solubility of the complex. Additionally, the PEG moiety reduces the degree of aggregation that may occur during the conjugation of the drug to the antibody.

As discussed above, the linkers of the invention can be represented by the general formula: $(L^4)_p$—F— $(L^1)_m$, wherein F represents the linker portion comprising the amino acid. In one embodiment, the F portion comprises an optional additional self-immolative

linker(s), L^2, and a carbonyl group. In another embodiment, the F portion comprises an amino group and an optional spacer group(s), L^3.

Accordingly, in one embodiment, the conjugate comprising the linker comprises a structure of the Formula 1:

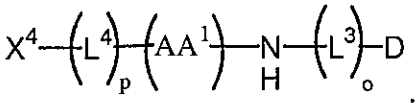


(1)

In this embodiment, L^1 is a self-immolative linker, as described above, and L^4 is a moiety that preferably imparts increased solubility, or decreased aggregation properties, or modifies the hydrolysis rate, as described above. L^2 represents a self-immolative linker(s). In addition, m is 0, 1, 2, 3, 4, 5, or 6; and o and p are independently 0 or 1. AA^1 represents a natural amino acid or unnatural α-amino acid.

In the linkers of the invention of the above Formula 1, AA^1 is linked, at its amino terminus, either directly to L^4 or, when L^4 is absent, directly to the X^4 group (*i.e.*, the targeting agent, detectable label, protected reactive functional group or unprotected reactive functional group).

In another embodiment, the conjugate comprising the linker comprises a structure of the Formula 2:



(2)

In this embodiment, L^4 is a moiety that preferably imparts increased solubility, or decreased aggregation properties, or modifies the hydrolysis rate, as described above; L^3 is a spacer group comprising a primary or secondary amine or a carboxyl functional group, and either the amine of L^3 forms an amide bond with a pendant carboxyl functional group of D or the carboxyl of L^3 forms an amide bond with a pendant amine functional group of D; and o and p are independently 0 or 1. AA^1 represents a natural amino acid or unnatural α-amino acid. In this embodiment, L^1 is absent (*i.e.*, m is 0 is the general formula).

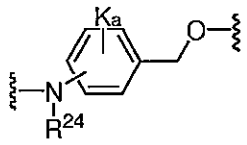
The self-immolative linker L^2

The self-immolative linker L^2 is a bifunctional chemical moiety which is capable of covalently linking together two spaced chemical moieties into a normally stable tripartate

molecule, releasing one of said spaced chemical moieties from the tripartate molecule by means of enzymatic cleavage; and following said enzymatic cleavage, spontaneously cleaving from the remainder of the molecule to release the other of said spaced chemical moieties. In accordance with the present invention, the self-immolative spacer is covalently linked at one of its ends to the amino acid AA^1 and covalently linked at its other end to the chemically reactive site of the drug moiety whose derivatization inhibits pharmacological activity, so as to space and covalently link together the amino acid AA^1 and the drug moiety into a tripartate molecule which is stable and pharmacologically inactive in the absence of the target enzyme, but which is enzymatically cleavable by such target enzyme at the bond covalently linking the spacer moiety and the amino acid AA^1 to thereby effect release of the amino acid AA^1 from the tripartate molecule. Such enzymatic cleavage, in turn, will activate the self-immolating character of the spacer moiety and initiate spontaneous cleavage of the bond covalently linking the spacer moiety to the drug moiety, to thereby effect release of the drug in pharmacologically active form.

The self-immolative linker L^2 may be any self-immolative group. Preferably L^2 is a substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, unsubstituted heterocycloalkyl, substituted heterocycloalkyl, substituted or unsubstituted aryl, or substituted and unsubstituted heteroaryl.

One particularly preferred self-immolative spacer L^2 may be represented by the Formula 3:



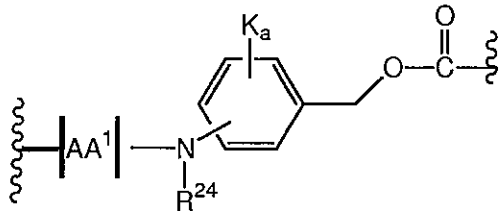
(3)

The aromatic ring of the aminobenzyl group may be substituted with one or more "K" groups. A "K" group is a substituent on the aromatic ring that replaces a hydrogen otherwise attached to one of the four non-substituted carbons that are part of the ring structure. The "K" group may be a single atom, such as a halogen, or may be a multi-atom group, such as alkyl, heteroalkyl, amino, nitro, hydroxy, alkoxy, haloalkyl, and cyano. Each K is independently selected from the group consisting of substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{21}R^{22}$, $NR^{21}COR^{22}$, $OCONR^{21}R^{22}$, $OCOR^{21}$, and OR^{21},

wherein R^{21} and R^{22} are independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl and unsubstituted heterocycloalkyl. Exemplary K substituents include, but are not limited to, F, Cl, Br, I, NO_2, OH, OCH_3, $NHCOCH_3$, $N(CH_3)_2$, $NHCOCF_3$ and methyl. For "K_a", a is an integer of 0, 1, 2, 3, or 4. In one preferred embodiment, a is 0.

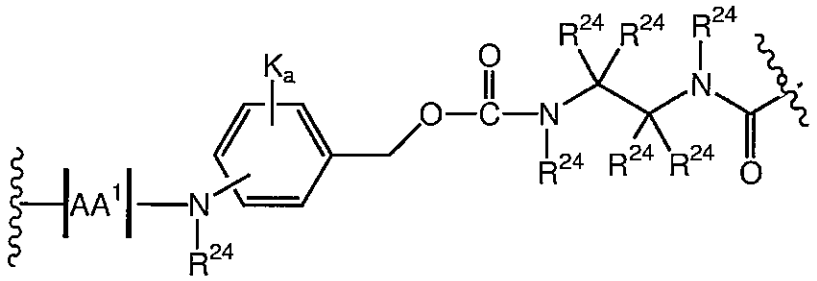
The ether oxygen atom of the structure shown above is connected to a carbonyl group. The line from the NR^{24} functionality into the aromatic ring indicates that the amine functionality may be bonded to any of the five carbons that both form the ring and are not substituted by the $-CH_2-O-$ group. Preferably, the NR^{24} functionality of X is covalently bound to the aromatic ring at the para position relative to the $-CH_2-O-$ group. R^{24} is a member selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, and unsubstituted heteroalkyl. In a specific embodiment, R^{24} is hydrogen.

In one embodiment, the invention provides a linker of Formula (1) above, wherein F comprises the structure:



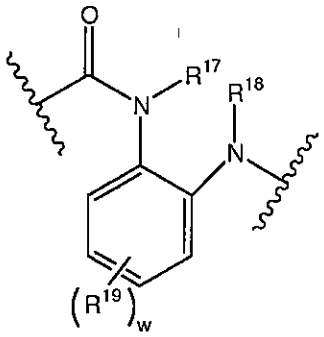
where R^{24} is selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, and unsubstituted heteroalkyl. Each K is a member independently selected from the group consisting of substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{21}R^{22}$, $NR^{21}COR^{22}$, $OCONR^{21}R^{22}$, $OCOR^{21}$, and OR^{21} where R^{21} and R^{22} are independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl; and a is an integer of 0,1, 2, 3, or 4.

In another embodiment, the linker of Formula (1) above comprises a $-F-(L^1)_m-$ that comprises the structure:



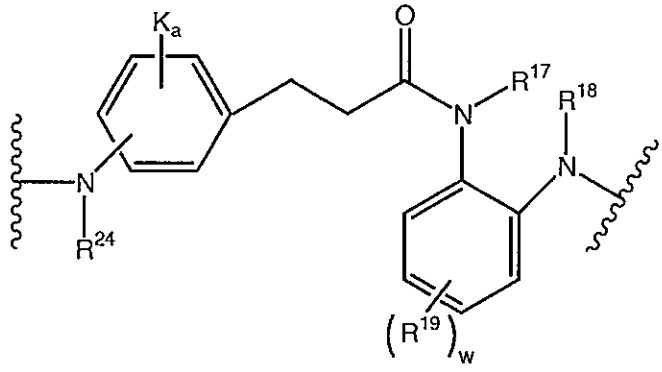
where each R^{24} is a member independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, and unsubstituted heteroalkyl.

In some embodiments, the self-immolative spacer L^2 includes



where each R^{17}, R^{18}, and R^{19} is independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl, and w is an integer from 0 to 4. In some embodiments, R^{17} and R^{18} are independently H or alkyl (preferably, unsubstituted C1-4 alkyl). Preferably, R^{17} and R^{18} are C1-4 alkyl, such as methyl or ethyl. In some embodiments, w is 0.

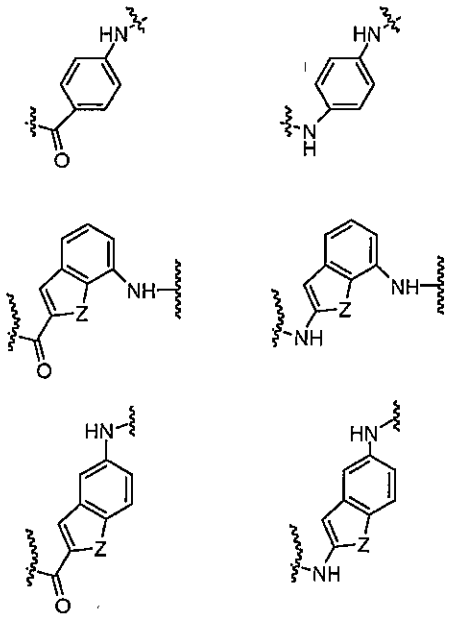
In some embodiments, L^2 includes



The Spacer Group L^3

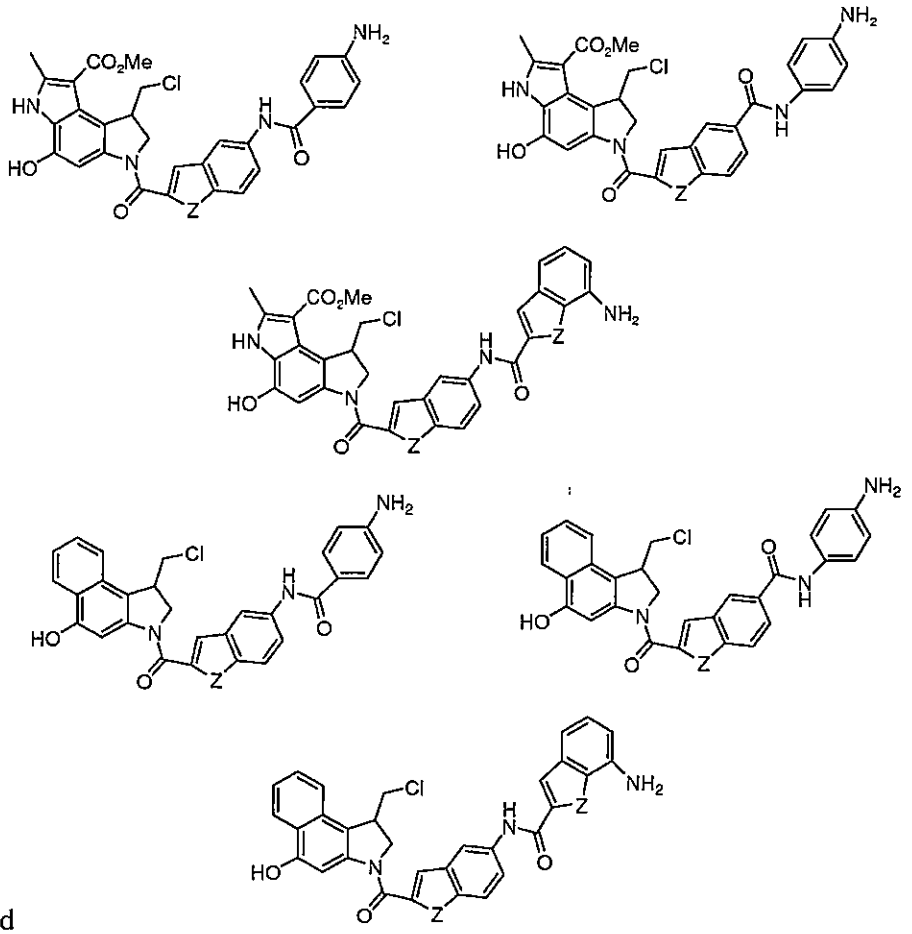
The spacer group L^3 is characterized in that it comprises a primary or secondary amine or a carboxyl functional group, and either the amine of the L^3 group forms an amide bond with a pendant carboxyl functional group of D or the carboxyl of L^3 forms an amide

bond with a pendant amine functional group of D. L^3 can be selected from the group consisting of substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, or substituted or unsubstituted heterocycloalkyl. In a preferred embodiment, L^3 comprises an aromatic group. More preferably, L^3 comprises a benzoic acid group, an aniline group or indole group. Non-limiting examples of structures that can serve as an -L^3-NH- spacer include the following structures:



where Z is a member selected from O, S and NR^{23}, and where R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl.

Upon cleavage of the linker of the invention containing L^3, the L^3 moiety remains attached to the drug, D. Accordingly, the L^3 moiety is chosen such that its presence attached to D does not significantly alter the activity of D. In another embodiment, a portion of the drug D itself functions as the L^3 spacer. For example, in one embodiment, the drug, D, is a duocarmycin derivative in which a portion of the drug functions as the L^3 spacer. Non-limiting examples of such embodiments include those in which NH_2-(L^3)-D has a structure selected from the group consisting of:



and

where Z is a member selected from O, S and NR^{23}, where R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl; and where the NH_2 group on each structure reacts with AA^1 to form $-AA^1$-NH-.

The Amino Acid AA^1

The group AA^1 represents a single amino acid and may be a natural amino acid or an unnatural α-amino acid. The amino acid may be in the L or the D configuration. The amino acid may be chosen based on their suitability for selective cleavage by particular molecules such as tumor-associated protease.

Without wishing to be limited to any specific mechanism of cleavage of the toxin from the antibody, it is believed that at least some of the compounds of this invention are cleaved by cathepsin B. Other mechanisms of cleavage or of releasing the toxin of the invention in the appropriate environment are also contemplated and included in the invention. In one embodiment, the amino acid AA^1 is chosen based on the linker's ability to be cleaved by a lysosomal proteases, non-limiting examples of which include cathepsins B,

C, D, H, L and S. In some embodiments, the linker containing amino acid AA^1 is capable of being cleaved by cathepsin B *in vitro*, which can be tested using *in vitro* protease cleavage assays known in the art.

In some embodiments, the linker containing amino acid AA^1 is not substantially cleaved by cathepsin B *in vitro* (e.g., no substantial cleavage in 24 hours) and yet is still cleavable *in vivo* to yield the active drug.

In another embodiment, the amino acid AA^1 is chosen based on the linker's ability to be cleaved by a tumor-associated protease, such as a protease that is found extracellularly in the vicinity of tumor cells, non-limiting examples of which include thimet oligopeptidase (TOP) and CD10. The ability of a linker to be cleaved by TOP or CD10 can be tested using *in vitro* protease cleavage assays known in the art.

In one embodiment, the amino acid is selected from the group consisting of: Ala, Arg, Asn, Asp, Cit, Cys, Gln, Glu, Gly, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val. In another embodiment, the amino acid is selected from the group consisting of Cit, Glu, Lys, and Ser.

Proteases have been implicated in cancer metastasis. Increased synthesis of the protease urokinase was correlated with an increased ability to metastasize in many cancers. Urokinase activates plasmin from plasminogen, which is ubiquitously located in the extracellular space and its activation can cause the degradation of the proteins in the extracellular matrix through which the metastasizing tumor cells invade. Plasmin can also activate the collagenases thus promoting the degradation of the collagen in the basement membrane surrounding the capillaries and lymph system thereby allowing tumor cells to invade into the target tissues (Dano, *et al. Adv. Cancer. Res.*, **44**: 139 (1985)). Thus, it is within the scope of the present invention to utilize a linker that is cleaved by urokinase.

The invention also provides the use of linkers that are sensitive to cleavage by tryptases. Human mast cells express at least four distinct tryptases, designated α βI, βII, and βIII. These enzymes are not controlled by blood plasma proteinase inhibitors and only cleave a few physiological substrates *in vitro*. The tryptase family of serine proteases has been implicated in a variety of allergic and inflammatory diseases involving mast cells because of elevated tryptase levels found in biological fluids from patients with these disorders. However, the exact role of tryptase in the pathophysiology of disease remains to be delineated. The scope of biological functions and corresponding physiological consequences of tryptase are substantially defined by their substrate specificity.

Tryptase is a potent activator of pro-urokinase plasminogen activator (uPA), the zymogen form of a protease associated with tumor metastasis and invasion. Activation of the plasminogen cascade, resulting in the destruction of extracellular matrix for cellular extravasation and migration, may be a function of tryptase activation of pro-urokinase plasminogen activator at the P4-P1 sequence of Pro-Arg-Phe-Lys (SEQ. ID NO: 1) (Stack, *et al., Journal of Biological Chemistry* **269** (13): 9416-9419 (1994)). Vasoactive intestinal peptide, a neuropeptide that is implicated in the regulation of vascular permeability, is also cleaved by tryptase, primarily at the Thr-Arg-Leu-Arg (SEQ. ID NO: 2) sequence (Tam, *et al., Am. J. Respir. Cell Mol. Biol.* **3**: 27-32 (1990)). The G-protein coupled receptor PAR-2 can be cleaved and activated by tryptase at the Ser-Lys-Gly-Arg (SEQ. ID NO: 3) sequence to drive fibroblast proliferation, whereas the thrombin activated receptor PAR-1 is inactivated by tryptase at the Pro-Asn-Asp-Lys (SEQ. ID NO: 4) sequence (Molino *et al., Journal of Biological Chemistry* **272**(7): 4043-4049 (1997)). Taken together, this evidence suggests a central role for tryptase in tissue remodeling as a consequence of disease. This is consistent with the profound changes observed in several mast cell-mediated disorders. One hallmark of chronic asthma and other long-term respiratory diseases is fibrosis and thickening of the underlying tissues that could be the result of tryptase activation of its physiological targets. Similarly, a series of reports have shown angiogenesis to be associated with mast cell density, tryptase activity and poor prognosis in a variety of cancers (Coussens *et al., Genes and Development* **13**(11): 1382-97 (1999)); Takanami *et al., Cancer* **88**(12): 2686-92 (2000); Toth-Jakatics *et al., Human Pathology* **31**(8): 955-960 (2000); Ribatti *et al., International Journal of Cancer* **85**(2): 171-5 (2000)).

The drug-ligand conjugate of the current invention may optionally contain two or more linkers. For example, one linker may be used to connect the drug to the ligand and a second linker may attach a diagnostic agent to the complex. Other uses for additional linkers include linking analytical agents, biomolecules, targeting agents, and detectable labels to the drug-ligand complex. The multiple linkers may be the same or different.

Also within the scope of the present invention are compounds of the invention that are poly- or multi-valent species, including, for example, species such as dimers, trimers, tetramers and higher homologs of the compounds of the invention or reactive analogues thereof. The poly- and multi-valent species can be assembled from a single species or more than one species of the invention. For example, a dimeric construct can be "homo-dimeric" or "heterodimeric." Moreover, poly- and multi-valent constructs in which a compound of the

invention or a reactive analogue thereof, is attached to an oligomeric or polymeric framework (*e.g.*, polylysine, dextran, hydroxyethyl starch and the like) are within the scope of the present invention. The framework is preferably polyfunctional (*i.e.* having an array of reactive sites for attaching compounds of the invention). Moreover, the framework can be derivatized with a single species of the invention or more than one species of the invention.

Moreover, the present invention includes compounds that are functionalized to afford compounds having water-solubility that is enhanced relative to analogous compounds that are not similarly functionalized. Thus, any of the substituents set forth herein can be replaced with analogous radicals that have enhanced water solubility. For example, it is within the scope of the invention to, for example, replace a hydroxyl group with a diol, or an amine with a quaternary amine, hydroxy amine or similar more water-soluble moiety. In a preferred embodiment, additional water solubility is imparted by substitution at a site not essential for the activity towards the ion channel of the compounds set forth herein with a moiety that enhances the water solubility of the parent compounds. Methods of enhancing the water-solubility of organic compounds are known in the art. Such methods include, but are not limited to, functionalizing an organic nucleus with a permanently charged moiety, e.g., quaternary ammonium, or a group that is charged at a physiologically relevant pH, e.g. carboxylic acid, amine. Other methods include, appending to the organic nucleus hydroxyl- or amine-containing groups, e.g. alcohols, polyols, polyethers, and the like. Representative examples include, but are not limited to, polylysine, polyethyleneimine, poly(ethyleneglycol) and poly(propyleneglycol). Suitable functionalization chemistries and strategies for these compounds are known in the art. *See*, for example, Dunn, R.L., *et al.*, Eds. POLYMERIC DRUGS AND DRUG DELIVERY SYSTEMS, ACS Symposium Series Vol. 469, American Chemical Society, Washington, D.C. 1991.

DRUGS

Drugs, depicted as "D" herein, are provided in the current invention as part of a drug-ligand conjugate, where the drug is linked to a ligand through a linker. The drug must possess a desired biological activity and contain a reactive functional group in order to link to the ligand. The desired biological activity includes the diagnosis, cure, mitigation, treatment, or prevention of disease in an animal such as a human. Thus, so long as it has the needed reactive functional group, the term "drug" refers to chemicals recognized as drugs in the official United States Pharmacopeia, official Homeopathic Pharmacopeia of the United

States, or official National Formulary, or any supplement thereof. Exemplary drugs are set forth in the Physician's Desk Reference (PDR) and in the Orange Book maintained by the U.S. Food and Drug Administration (FDA). New drugs are being continually being discovered and developed, and the present invention provides that these new drugs may also be incorporated into the drug-ligand complex of the current invention.

Preferred functional groups include primary or secondary amines, hydroxyls, sulfhydryls, carboxyls, aldehydes, and ketones. More preferred functional groups include hydroxyls, primary or secondary amines, sulfhydryls and carboxylic acid functional groups. Even more preferred functional groups include hydroxyls, primary and secondary amines and carboxylic acid functional groups. The drug must have at least one, but may have 2, 3, 4, 5, 6 or more reactive functional groups. Additionally, a self-immolative spacer, L^1, may be incorporated between the reactive functional group of the drug and the linker.

The drug-ligand conjugate is effective for the usual purposes for which the corresponding drugs are effective, but have superior efficacy because of the ability, inherent for at least some ligands, to transport the drug to the desired cell where it is of particular benefit.

Exemplary drugs include proteins, peptides, and small molecule drugs containing a functional group for linkage to the ligand. More specifically, these drugs include, for example, the enzyme inhibitors such as dihydrofolate reductase inhibitors, thymidylate synthase inhibitors, DNA intercalators, DNA cleavers, topoisomerase inhibitors, the anthracycline family of drugs, the vinca drugs, the mitomycins, the bleomycins, the cytotoxic nucleosides, the pteridine family of drugs, diynenes, the podophyllotoxins, differentiation inducers, and taxols.

Preferred drugs of the current invention include cytotoxic drugs useful in cancer therapy and other small molecules, proteins or polypeptides with desired biological activity, such as a toxin. The drug may be selected to be activated at a tumor cells by conjugation to a tumor-specific ligand. These tumor specific drug-ligand conjugates have tumor specificity arising from the specificity of the ligand. Examples of this are drug-ligand conjugates that are highly selective substrates for tumor specific enzymes, where these enzymes are present in the proximity of the tumor in sufficient amounts to generate cytotoxic levels of free drug in the vicinity of the tumor. One advantage of these tumor-specific drug-ligand complexes is that they are stable to adventitious proteases in the human serum. Another advantage of the drug-ligand complex is that they are less toxic than the corresponding free drug; additionally,

the specificity of the complex may allow for lower overall concentrations to be used relative to the free drug since the increased specificity will result in a higher percentage of the complex to be present at the tumor site.

Cytotoxins

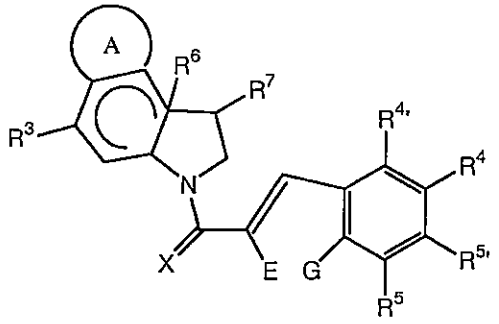
Cytotoxic drugs useful in the current invention include, for example, duocarmycins and CC-1065, and analogues thereof, including CBI (1,2,9,9a-tetrahydrocyclopropa[c]benz[e]indol-4-one)-based analogues, MCBI (7-methoxy-1,2,9,9a-tetra-hydrocyclopropa[c]benz[e]indol-4-one)-based analogues and CCBI (7-cyano-1,2,9,9a-tetra-hydrocyclo-propa[c]benz[e]-indol-4-one)-based analogues of the duocarmycins and CC-1065, doxorubicin and doxorubicin conjugates such as morpholino-doxorubicin and cyanomorpholino-doxorubicin, dolastatins such as dolastatin-10, combretastatin, calicheamicin, maytansine, maytansine analogs, DM-1, auristatin E, auristatin EB (AEB), auristatin EFP (AEFP), monomethyl auristatin E (MMAE), 5-benzoylvaleric acid-AE ester (AEVB), tubulysins, disorazole, epothilones , Paclitaxel, docetaxel, SN-38, Topotecan, rhizoxin, echinomycin, colchicine, vinblastin, vindesine, estramustine, cemadotin, eleutherobin, methotrexate, methopterin, dichloromethotrexate, 5-fluorouracil, 6-mercaptopurine, cytosine arabinoside, melphalan, leurosine, leurosideine, actinomycin, daunorubicin and daunorubicin conjugates, mitomycin C, mitomycin A, carminomycin, aminopterin, tallysomycin, podophyllotoxin and podophyllotoxin derivatives such as etoposide or etoposide phosphate, vincristine, taxol, taxotere retinoic acid, butyric acid, N^8 - acetyl spermidine, camptothecin, and their analogues. Other known drugs may be modified in order to provide a functional group for conjugation to the linker described herein. Such chemical modification is known in the art.

Preferred cytotoxins for use in the current invention include: duocarmycins, CC-1065, and CCBI-based and MCBI-based analogues thereof, morpholino-doxorubicin, cyanomorpholino-doxorubicin, dolastatin-10, combretastatin, calicheamicin, maytansine, DM-1, auristatin E, AEB, AEFP, MMAE, Tubulysin A, Disorazole, epothilone A and epothilone B.

Particularly preferred cytotoxins of the present invention are active, potent duocarmycin derivatives and CC-1065. The parent agents are exceptionally potent antitumor antibiotics that derive their biological effects through the reversible, stereoelectronically controlled sequence selective alkylation of DNA (Boger *et al. J. Org. Chem.* **55**: 4499 (1990);

Boger *et al. J. Am. Chem. Soc.* **112**: 8961 (1990); Boger *et al., J. Am. Chem. Soc.* **113**: 6645 (1991); Boger *et al. J. Am. Chem. Soc.* **115**: 9872 (1993); Boger *et al., Bioorg. Med. Chem. Lett.* **2**: 759 (1992)). Subsequent to the initial disclosure of the duocarmycins, extensive efforts have been devoted to elucidating the DNA alkylation selectivity of the duocarmycins and its structural origin.

A particularly preferred aspect of the current invention provides a cytotoxic compound having a structure according to Formula 4:



(4)

in which ring system A is a member selected from substituted or unsubstituted aryl substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl groups. Exemplary ring systems include phenyl and pyrrole.

The symbols E and G are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, a heteroatom, a single bond or E and G are optionally joined to form a ring system selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl.

The symbol X represents a member selected from O, S and NR^{23}. R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl.

The symbol R^3 represents a member selected from (=O), SR^{11}, NHR^{11} and OR^{11}, in which R^{11} is H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, diphosphates, triphosphates, acyl, $C(O)R^{12}$ R^{13}, $C(O)OR^{12}$, $C(O)NR^{12}R^{13}$, $P(O)(OR^{12})_2$, $C(O)CHR^{12}R^{13}$, SR^{12} or $SiR^{12}R^{13}R^{14}$. The symbols R^{12}, R^{13}, and R^{14} independently represent H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl, where R^{12} and R^{13} together with the nitrogen or carbon atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms.

R^4, $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, SO_3, SO_2R^{15}, CR^{15}=NR^{16}, and $O(CH_2)_nN(CH_3)_2$, where n is an integer from 1 to 20, or any adjacent pair of R^4, $R^{4'}$, R^5 and $R^{5'}$, together with the carbon atoms to which they are attached, are joined to form a substituted or unsubstituted cycloalkyl or heterocycloalkyl ring system having from 4 to 6 members. R^{15} and R^{16} independently represent H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl and substituted or unsubstituted peptidyl, where R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms. One exemplary structure is aniline.

At least one of R^4, $R^{4'}$, R^5, $R^{5'}$, R^{11}, R^{12}, R^{13}, R^{15} and R^{16} is used to join the drug to a linker of the present invention, as described herein, for example to L^1, if present or to F.

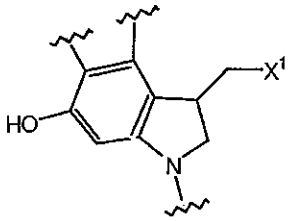
In one embodiment, at least one of R^4, $R^{4'}$, R^5, $R^{5'}$, R^{11}, R^{12}, R^{13}, R^{15} and R^{16} bears a reactive group appropriate for conjugating the compound. In a further exemplary embodiment, R^4, $R^{4'}$, R^5, $R^{5'}$, R^{11}, R^{12}, R^{13}, R^{15} and R^{16} are independently selected from H, substituted alkyl and substituted heteroalkyl and have a reactive functional group at the free terminus of the alkyl or heteroalkyl moiety. One or more of R^4, $R^{4'}$, R^5, $R^{5'}$, R^{11}, R^{12}, R^{13}, R^{15} and R^{16} may be conjugated to another species, *e.g.*, targeting agent, detectable label, solid support, etc.

R^6 is a single bond which is either present or absent. When R^6 is present, R^6 and R^7 are joined to form a cyclopropyl ring. R^7 is CH_2-X^1 or $-CH_2-$. When R^7 is $-CH_2-$ it is a component of the cyclopropane ring. The symbol X^1 represents a leaving group such as a halogen, for example Cl, Br or F. The combinations of R^6 and R^7 are interpreted in a manner that does not violate the principles of chemical valence.

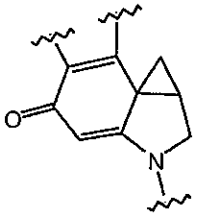
X^1 may be any leaving group. Useful leaving groups include, but are not limited to, halogens, azides, sulfonic esters (e.g., alkylsulfonyl, arylsulfonyl), oxonium ions, alkyl perchlorates, ammonioalkanesulfonate esters, alkylfluorosulfonates and fluorinated compounds (*e.g.*, triflates, nonaflates, tresylates) and the like. Particular halogens useful as leaving groups are F, Cl and Br. The choice of these and other leaving groups appropriate

for a particular set of reaction conditions is within the abilities of those of skill in the art (*see*, for example, March J, ADVANCED ORGANIC CHEMISTRY, 2nd Edition, John Wiley and Sons, 1992; Sandler SR, Karo W, ORGANIC FUNCTIONAL GROUP PREPARATIONS, 2nd Edition, Academic Press, Inc., 1983; and Wade LG, COMPENDIUM OF ORGANIC SYNTHETIC METHODS, John Wiley and Sons, 1980).

The curved line within the six-membered ring indicates that the ring may have one or more degrees of unsaturation, and it may be aromatic. Thus, ring structures such as those set forth below, and related structures, are within the scope of Formula (5):



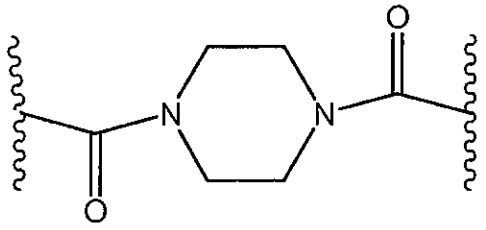
and



(5).

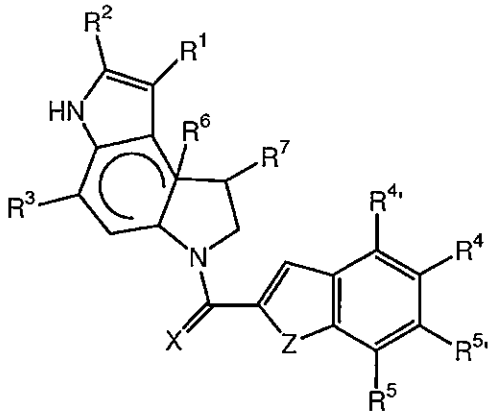
In some embodiments, at least one of R^4, $R^{4'}$, R^5, and $R^{5'}$ links said drug to L^1, if present, or to F.

In one embodiment, R^{11} includes a moiety, X^5, that does not self-cyclize and links the drug to L^1, if present, or to F. The moiety, X^5, is preferably cleavable using an enzyme and, when cleaved, provides the active drug. As an example, R^{11} can have the following structure (with the right side coupling to the remainder of the drug):



In an exemplary embodiment, ring system A of Formula (4) is a substituted or unsubstituted phenyl ring. Ring system A may be substituted with one or more aryl group substituents as set forth in the definitions section herein. In some embodiments, the phenyl ring is substituted with a CN or methoxy moiety.

In another exemplary embodiment, the invention provides a compound having a structure according to Formula 6:



(6).

In this embodiment, the identities of the substituents R^3, R^4, $R^{4'}$, R^5, $R^{5'}$, R^6, R^7 and X are substantially as described above for Formula 4, as well as preferences for particular embodiments. The symbol Z is a member independently selected from O, S and NR^{23}. The symbol R^{23} represents a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl. Each R^{23} is independently selected. The symbol R^1 represents H, substituted or unsubstituted lower alkyl, or $C(O)R^8$ or CO_2R^8. R^8 is a member selected from substituted alkyl, unsubstituted alkyl, NR^9R^{10}, NR^9NHR^{10} and OR^9. R^9 and R^{10} are independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl. R^2 is H, or substituted or unsubstituted lower alkyl. It is generally preferred that when R^2 is substituted alkyl, it is other than a perfluoroalkyl, e.g., CF_3. In one embodiment, R^2 is a substituted alkyl wherein the substitution is not a halogen. In another embodiment, R^2 is an unsubstituted alkyl.

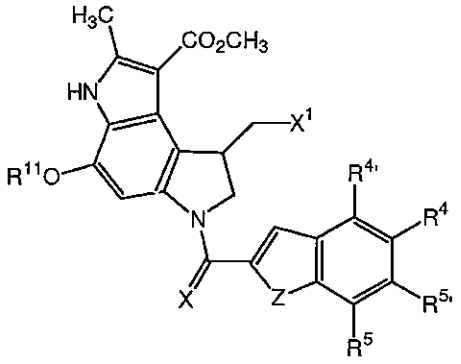
In some embodiments R^1 is an ester moiety, such as CO_2CH_3. In some embodiments, R^2 is a lower alkyl group, which may be substituted or unsubstituted. A presently preferred lower alkyl group is CH_3. In some preferred embodiments, R^1 is CO_2CH_3 and R^2 is CH_3.

In some embodiments, R^4, $R^{4'}$, R^5, and $R^{5'}$ are members independently selected from H, halogen, NH_2, OMe, $O(CH_2)_2N(R^{29})_2$ and NO_2. Each R^{29} is independently H or lower alkyl (e.g., methyl).

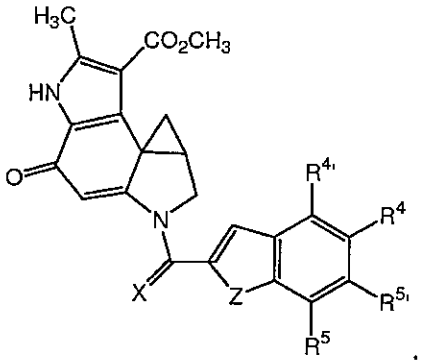
In some embodiments, the drug is selected such that the leaving group X^1 is a member selected from the group consisting of halogen, alkylsulfonyl, arylsulfonyl, and azide. In some embodiments, X^1 is F, Cl, or Br.

In some embodiments, Z is O or NH. In some embodiments, X is O.

In yet another exemplary embodiment, the invention provides compounds having a structure according to Formula 7 or 8:



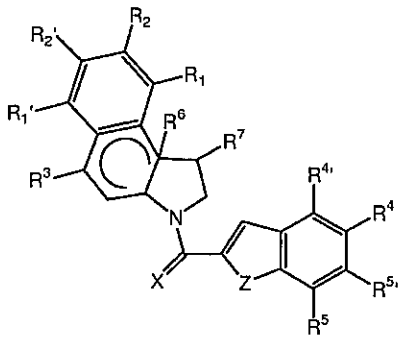
and



(7) (8)

Another preferred structure of the duocarmycin analog of Formula 4 is a structure in which the ring system A is an unsubstituted or substituted phenyl ring. The preferred substituents on the drug molecule described hereinabove for the structure of Formula 4 when the ring system A is a pyrrole are also preferred substituents when the ring system A is an unsubstituted or substituted phenyl ring.

For example, in a preferred embodiment, the drug (D) comprises a structure of Formula (9):



(9)

In this structure, R^3, R^6, R^7, X are as described above for Formula 4. Furthermore, Z is a member selected from O, S and NR^{23}, wherein R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

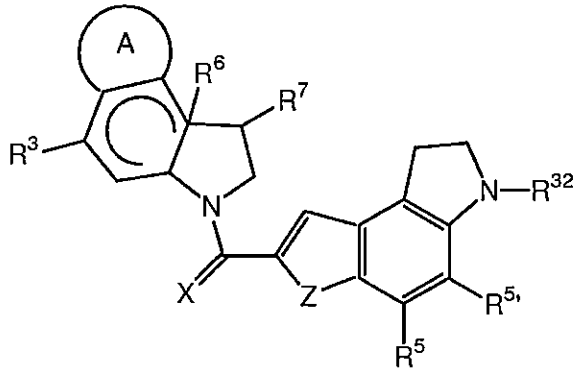
R^1 is H, substituted or unsubstituted lower alkyl, $C(O)R^8$, or CO_2R^8, wherein R^8 is a member selected from NR^9R^{10} and OR^9, in which R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

$R^{1'}$ is H, substituted or unsubstituted lower alkyl, or $C(O)R^8$, wherein R^8 is a member selected from NR^9R^{10} and OR^9, in which R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

R^2 is H, or substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl or cyano or alkoxy; and $R^{2'}$ is H, or substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl.

At least one of R^4, $R^{4'}$, R^5, $R^{5'}$, R^{11}, R^{12}, R^{13}, R^{15} or R^{16} links the drug to L^1, if present, or to F.

Another embodiment of the drug (D) comprises a structure (13) where R^4 and $R^{4'}$ have been joined to from a heterocycloalkyl:



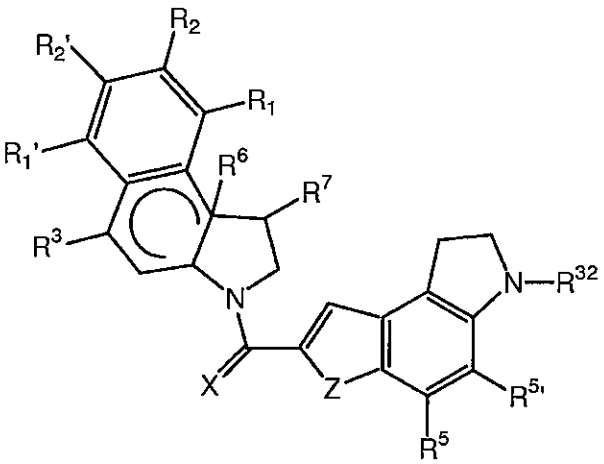
(13)

In this structure, R^3, R^5, $R^{5'}$, R^6, R^7, X are as described above for Formula 4. Furthermore, Z is a member selected from O, S and NR^{23}, wherein R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^{32} is selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, $CR^{15}{=}NR^{16}$, and $O(CH_2)_nN(CH_3)_2$, where n is an integer from 1 to 20. R^{15} and R^{16} independently represent H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl and substituted or unsubstituted peptidyl, where R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms.

At least one of R^5, $R^{5'}$, R^{11}, R^{12}, R^{13}, R^{15}, R^{16}, or R^{32} links the drug to L^1, if present, or to F. In at least some embodiments, R^{32} links the drug to L^1, if present, or to F.

One preferred embodiment of this compound is:

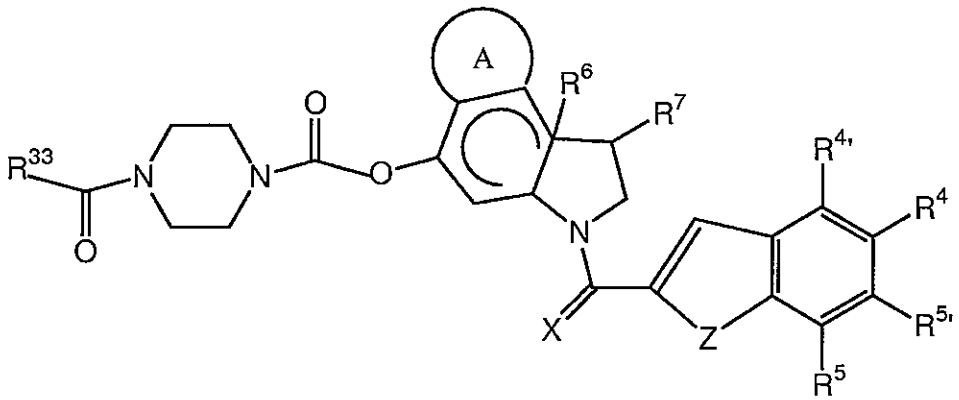


R^1 is H, substituted or unsubstituted lower alkyl, $C(O)R^8$, or CO_2R^8, wherein R^8 is a member selected from NR^9R^{10} and OR^9, in which R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

$R^{1'}$ is H, substituted or unsubstituted lower alkyl, or $C(O)R^8$, wherein R^8 is a member selected from NR^9R^{10} and OR^9, in which R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

R^2 is H, or substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl or cyano or alkoxy; and $R^{2'}$ is H, or substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl.

A further embodiment has the formula:



In this structure, A, R^6, R^7, X, R^4, $R^{4'}$, R^5, and $R^{5'}$ are as described above for Formula 4. Furthermore, Z is a member selected from O, S and NR^{23}, where R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^{33} is selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl,

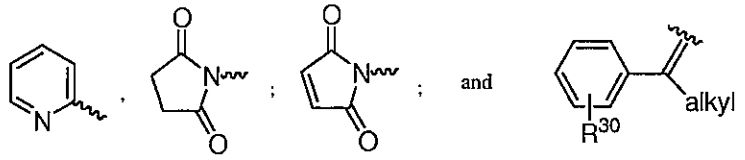
halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, $CR^{15}=NR^{16}$, and $O(CH_2)_nN(CH_3)_2$, where n is an integer from 1 to 20. R^{15} and R^{16} independently represent H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl and substituted or unsubstituted peptidyl, where R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms. R^{33} links the drug to L^1, if present, or to F.

Preferably, A is substituted or unsubstituted phenyl or substituted or unsubstituted pyrrole. Moreover, any selection of substituents described herein for R^{11} is also applicable to R^{33}.

LIGANDS

X^4 represents a ligand selected from the group consisting of protected reactive functional groups, unprotected reactive functional groups, detectable labels, and targeting agents. Preferred ligands are targeting agents, such as antibodies and fragments thereof.

In some embodiments, the group X^4 can be described as a member selected from R^{29}, $COOR^{29}$, $C(O)NR^{29}$, and $C(O)NNR^{29}$ wherein R^{29} is a member selected from substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted heteroaryl. In yet another exemplary embodiment, R^{29} is a member selected from H; OH; $NHNH_2$;



where R^{30} represents substituted or unsubstituted alkyl terminated with a reactive functional group, substituted or unsubstituted heteroaryl terminated with a functional group. The above structures act as reactive protective groups that can be reacted with, for example, a side chain of an amino acid of a targeting agent, such as an antibody, to thereby link the targeting agent to the linker-drug moiety.

Targeting Agents

The linker arms and cytotoxins of the invention can be linked to targeting agents that selectively deliver a payload to a cell, organ or region of the body. Exemplary targeting agents such as antibodies (e.g., chimeric, humanized and human), ligands for receptors, lectins, saccharides, antibodies, and the like are recognized in the art and are useful without limitation in practicing the present invention. Other targeting agents include a class of compounds that do not include specific molecular recognition motifs include macromolecules such as poly(ethylene glycol), polysaccharide, polyamino acids and the like, which add molecular mass to the cytotoxin. The additional molecular mass affects the pharmacokinetics of the cytotoxin, e.g., serum half-life.

In an exemplary embodiment, the invention provides a cytotoxin, linker or cytotoxin-linker conjugate with a targeting agent that is a biomolecule, e.g., an antibody, receptor, peptide, lectin, saccharide, nucleic acid or a combination thereof.

Biomolecules useful in practicing the present invention can be derived from any source. The biomolecules can be isolated from natural sources or can be produced by synthetic methods. Proteins can be natural proteins or mutated proteins. Mutations can be effected by chemical mutagenesis, site-directed mutagenesis or other means of inducing mutations known to those of skill in the art. Proteins useful in practicing the instant invention include, for example, enzymes, antigens, antibodies and receptors. Antibodies can be either polyclonal or monoclonal, but most preferably are monoclonal. Peptides and nucleic acids can be isolated from natural sources or can be wholly or partially synthetic in origin.

In a preferred embodiment, the targeting agent is an antibody, or antibody fragment, that is selected based on its specificity for an antigen expressed on a target cell, or at a target site, of interest. A wide variety of tumor-specific or other disease-specific antigens have been identified and antibodies to those antigens have been used or proposed for use in the treatment of such tumors or other diseases. The antibodies that are known in the art can be used in the conjugates of the invention, in particular for the treatment of the disease with which the target antigen is associated. Non-limiting examples of target antigens (and their associated diseases) to which an antibody-linker-drug conjugate of the invention can be targeted include: Her2 (breast cancer), CD20 (lymphomas), EGFR (solid tumors), CD22 (lymphomas, including non-Hodgkin's lymphoma), CD52 (chronic lymphocytic leukemia), CD33 (acute myelogenous leukemia), CD4 (lymphomas, autoimmune diseases, including rheumatoid arthritis), CD30 (lymphomas, including non-Hodgkin's lymphoma), Muc18

(melanoma), integrins (solid tumors), PSMA (prostate cancer, benign prostatic hyperplasia), CEA (colorectal cancer), CD11a (psoriasis), CD80 (psoriasis), CD23 (asthma), CD40L (immune thromobcytopenic purpura), CTLA4 (T cell lymphomas) and BLys (autoimmune diseases, including systemic lupus erythematosus).

In those embodiments wherein the recognition moiety is a protein or antibody, the protein can be tethered to a surface or a self assembled monolayer (SAM) component or connected through a spacer arm by any reactive peptide residue available on the surface of the protein. In preferred embodiments, the reactive groups are amines or carboxylates. In particularly preferred embodiments, the reactive groups are the ε-amine groups of lysine residues. Furthermore, these molecules can be adsorbed onto the surface of the substrate or SAM by non-specific interactions (*e.g.*, chemisorption, physisorption).

Recognition moieties which are antibodies can be used to recognize analytes which are proteins, peptides, nucleic acids, saccharides or small molecules such as drugs, herbicides, pesticides, industrial chemicals and agents of war. Methods of raising antibodies for specific molecules are well-known to those of skill in the art. *See*, for example, U.S. Patents Nos. 5,147,786; 5,334,528; 5,686,237; and 5,573,922. Methods for attaching antibodies to surfaces are also art-known. *See*, for example, Delamarche *et al. Langmuir* **12:**1944-1946 (1996).

Targeting agents can be attached to the linkers of the invention by any available reactive group. For example, peptides can be attached through an amine, carboxyl, sulfhydryl, or hydroxyl group. Such a group can reside at a peptide terminus or at a site internal to the peptide chain. Nucleic acids can be attached through a reactive group on a base (*e.g.*, exocyclic amine) or an available hydroxyl group on a sugar moiety (*e.g.*, 3'- or 5'-hydroxyl). The peptide and nucleic acid chains can be further derivatized at one or more sites to allow for the attachment of appropriate reactive groups onto the chain. *See*, for example, Chrisey *et al. Nucleic Acids Res.* **24:**3031-3039 (1996).

When the peptide or nucleic acid is a fully or partially synthetic molecule, a reactive group or masked reactive group can be incorporated during the process of the synthesis. Many derivatized monomers appropriate for reactive group incorporation in both peptides and nucleic acids are know to those of skill in the art. *See*, for example, THE PEPTIDES: ANALYSIS, SYNTHESIS, BIOLOGY, Vol. 2: "Special Methods in Peptide Synthesis," Gross, E. and Melenhofer, J., Eds., Academic Press, New York (1980). Many useful monomers are commercially available (Bachem, Sigma, *etc.*). This masked group can then be unmasked

following the synthesis, at which time it becomes available for reaction with a component of a compound of the invention.

Exemplary nucleic acid targeting agents include aptamers, antisense compounds, and nucleic acids that form triple helices. Typically, a hydroxyl group of a sugar residue, an amino group from a base residue, or a phosphate oxygen of the nucleotide is utilized as the needed chemical functionality to couple the nucleotide-based targeting agent to the cytotoxin. However, one of skill in the art will readily appreciate that other "non-natural" reactive functionalities can be appended to a nucleic acid by conventional techniques. For example, the hydroxyl group of the sugar residue can be converted to a mercapto or amino group using techniques well known in the art.

Aptamers (or nucleic acid antibody) are single- or double-stranded DNA or single-stranded RNA molecules that bind specific molecular targets. Generally, aptamers function by inhibiting the actions of the molecular target, *e.g.*, proteins, by binding to the pool of the target circulating in the blood. Aptamers possess chemical functionality and thus, can covalently bond to cytotoxins, as described herein.

Although a wide variety of molecular targets are capable of forming non-covalent but specific associations with aptamers, including small molecules drugs, metabolites, cofactors, toxins, saccharide-based drugs, nucleotide-based drugs, glycoproteins, and the like, generally the molecular target will comprise a protein or peptide, including serum proteins, kinins, eicosanoids, cell surface molecules, and the like. Examples of aptamers include Gilead's antithrombin inhibitor GS 522 and its derivatives (Gilead Science, Foster City, Calif.). *See also*, Macaya *et al. Proc. Natl. Acad. Sci. USA* **90**:3745-9 (1993); Bock *et al. Nature (London)* **355**:564-566 (1992) and Wang *et al. Biochem.* **32**:1899-904 (1993).

Aptamers specific for a given biomolecule can be identified using techniques known in the art. *See, e.g.*, Toole *et al.* (1992) PCT Publication No. WO 92/14843; Tuerk and Gold (1991) PCT Publication No. WO 91/19813; Weintraub and Hutchinson (1992) PCT Publication No. 92/05285; and Ellington and Szostak, *Nature* **346**:818 (1990). Briefly, these techniques typically involve the complexation of the molecular target with a random mixture of oligonucleotides. The aptamer-molecular target complex is separated from the uncomplexed oligonucleotides. The aptamer is recovered from the separated complex and amplified. This cycle is repeated to identify those aptamer sequences with the highest affinity for the molecular target.

For diseases that result from the inappropriate expression of genes, specific prevention or reduction of the expression of such genes represents an ideal therapy. In principle, production of a particular gene product may be inhibited, reduced or shut off by hybridization of a single-stranded deoxynucleotide or ribodeoxynucleotide complementary to an accessible sequence in the mRNA, or a sequence within the transcript that is essential for pre-mRNA processing, or to a sequence within the gene itself. This paradigm for genetic control is often referred to as antisense or antigene inhibition. Additional efficacy is imparted by the conjugation to the nucleic acid of an alkylating agent, such as those of the present invention.

Antisense compounds are nucleic acids designed to bind and disable or prevent the production of the mRNA responsible for generating a particular protein. Antisense compounds include antisense RNA or DNA, single or double stranded, oligonucleotides, or their analogs, which can hybridize specifically to individual mRNA species and prevent transcription and/or RNA processing of the mRNA species and/or translation of the encoded polypeptide and thereby effect a reduction in the amount of the respective encoded polypeptide. Ching *et al. Proc. Natl. Acad. Sci. U.S.A.* **86**:10006-10010 (1989); Broder *et al. Ann. Int. Med.* **113**:604-618 (1990); Loreau *et al. FEBS Letters* **274**:53-56 (1990); Holcenberg *et al.* WO91/11535; WO91/09865; WO91/04753; WO90/13641; WO 91/13080, WO 91/06629, and EP 386563). Due to their exquisite target sensitivity and selectivity, antisense oligonucleotides are useful for delivering therapeutic agents, such as the cytotoxins of the invention to a desired molecular target.

Others have reported that nucleic acids can bind to duplex DNA via triple helix formation and inhibit transcription and/or DNA synthesis. Triple helix compounds (also referred to as triple strand drugs) are oligonucleotides that bind to sequences of double-stranded DNA and are intended to inhibit selectively the transcription of disease-causing genes, such as viral genes, *e.g.*, HIV and herpes simplex virus, and oncogenes, *i.e.*, they stop protein production at the cell nucleus. These drugs bind directly to the double stranded DNA in the cell's genome to form a triple helix and prevent the cell from making a target protein. *See, e.g.*, PCT publications Nos. WO 92/10590, WO 92/09705, WO91/06626, and U.S. Pat. No. 5,176,996. Thus, the cytotoxins of the present invention are also conjugated to nucleic acid sequences that form triple helices.

The site specificity of nucleic acids (*e.g.*, antisense compounds and triple helix drugs) is not significantly affected by modification of the phosphodiester linkage or by chemical

modification of the oligonucleotide terminus. Consequently, these nucleic acids can be chemically modified; enhancing the overall binding stability, increasing the stability with respect to chemical degradation, increasing the rate at which the oligonucleotides are transported into cells, and conferring chemical reactivity to the molecules. The general approach to constructing various nucleic acids useful in antisense therapy has been reviewed by van der Krol *et al.*, *Biotechniques* **6**:958-976 (1988) and Stein *et al. Cancer Res.* **48**:2659-2668 (1988). Therefore, in an exemplary embodiment, the cytotoxins of the invention are conjugated to a nucleic acid by modification of the phosphodiester linkage.

Moreover, aptamers, antisense compounds and triple helix drugs bearing cytotoxins of the invention can also can include nucleotide substitutions, additions, deletions, or transpositions, so long as specific hybridization to or association with the relevant target sequence is retained as a functional property of the oligonucleotide. For example, some embodiments will employ phosphorothioate analogs which are more resistant to degradation by nucleases than their naturally occurring phosphate diester counterparts and are thus expected to have a higher persistence in vivo and greater potency (*see, e.g.*, Campbell *et al.*, *J. Biochem. Biophys. Methods* **20**:259-267(1990)). Phosphoramidate derivatives of oligonucleotides also are known to bind to complementary polynucleotides and have the additional capability of accommodating covalently attached ligand species and will be amenable to the methods of the present invention. *See*, for example, Froehler *et al., Nucleic Acids Res.* **16**(11):4831 (1988).

In some embodiments the aptamers, antisense compounds and triple helix drugs will comprise O-methylribonucleotides (EP Publication No. 360609). Chimeric oligonucleotides may also be used (Dagle *et al., Nucleic Acids Res.* **18**: 4751 (1990)). For some applications, antisense oligonucleotides and triple helix may comprise polyamide nucleic acids (Nielsen *et al., Science* **254**: 1497 (1991) and PCT publication No. WO 90/15065) or other cationic derivatives (Letsinger *et al., J. Am. Chem. Soc.* **110**: 4470-4471 (1988)). Other applications may utilize oligonucleotides wherein one or more of the phosphodiester linkages has been substituted with an isosteric group, such as a 2-4 atom long internucleoside linkage as described in PCT publication Nos. WO 92/05186 and 91/06556, or a formacetal group (Matteucci *et al., J. Am. Chem. Soc.* **113**: 7767-7768 (1991)) or an amide group (Nielsen *et al., Science* **254**: 1497-1500 (1991)).

In addition, nucleotide analogs, for example wherein the sugar or base is chemically modified, can be employed in the present invention. "Analogous" forms of purines and

pyrimidines are those generally known in the art, many of which are used as chemotherapeutic agents. An exemplary but not exhaustive list includes 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl- 2-thiouracil, 5-carboxymethylaminomethyluracil, dihydrouracil, inosine, N^6 -isopentenyladenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N^6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, β-D-mannosylqueosine, 5'methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N^6 -isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), pseudouracil, queosine, 2-thiocytosine, and 2,6-diaminopurine. In addition, the conventional bases by halogenated bases. Furthermore, the 2'-furanose position on the base can have a non-charged bulky group substitution. Examples of non-charged bulky groups include branched alkyls, sugars and branched sugars.

Terminal modification also provides a useful procedure to conjugate the cytotoxins to the nucleic acid, modify cell type specificity, pharmacokinetics, nuclear permeability, and absolute cell uptake rate for oligonucleotide pharmaceutical agents. For example, an array of substitutions at the 5' and 3' ends to include reactive groups are known, which allow covalent attachment of the cytotoxins. See, *e.g.*, OLIGODEOXYNUCLEOTIDES: ANTISENSE INHIBITORS OF GENE EXPRESSION, (1989) Cohen, Ed., CRC Press; PROSPECTS FOR ANTISENSE NUCLEIC ACID THERAPEUTICS FOR CANCER AND AIDS, (1991), Wickstrom, Ed., Wiley-Liss; GENE REGULATION: BIOLOGY OF ANTISENSE RNA AND DNA, (1992) Erickson and Izant, Eds., Raven Press; and ANTISENSE RNA AND DNA, (1992), Murray, Ed., Wiley-Liss. For general methods relating to antisense compounds, *see*, ANTISENSE RNA AND DNA, (1988), D. A. Melton, Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.).

Detectable Labels

The particular label or detectable group used in conjunction with the compounds and methods of the invention is generally not a critical aspect of the invention, as long as it does not significantly interfere with the activity or utility of the compound of the invention. The detectable group can be any material having a detectable physical or chemical property. Such detectable labels have been well developed in the field of immunoassays and, in general,

most any label useful in such methods can be applied to the present invention. Thus, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include magnetic beads (e.g., DYNABEADS™), fluorescent dyes (*e.g.*, fluorescein isothiocyanate, Texas red, rhodamine, and the like), radiolabels (*e.g.*, ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P), enzymes (*e.g.*, horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic beads (*e.g.*, polystyrene, polypropylene, latex, etc.).

The label may be coupled directly or indirectly to a compound of the invention according to methods well known in the art. As indicated above, a wide variety of labels may be used, with the choice of label depending on sensitivity required, ease of conjugation with the compound, stability requirements, available instrumentation, and disposal provisions.

When the compound of the invention is conjugated to a detectable label, the label is preferably a member selected from the group consisting of radioactive isotopes, fluorescent agents, fluorescent agent precursors, chromophores, enzymes and combinations thereof. Methods for conjugating various groups to antibodies are well known in the art. For example, a detectable label that is frequently conjugated to an antibody is an enzyme, such as horseradish peroxidase, alkaline phosphatase, β-galactosidase, and glucose oxidase.

Non-radioactive labels are often attached by indirect means. Generally, a ligand molecule (*e.g.*, biotin) is covalently bound to a component of the conjugate. The ligand then binds to another molecules (*e.g.*, streptavidin) molecule, which is either inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound.

Components of the conjugates of the invention can also be conjugated directly to signal generating compounds, *e.g.*, by conjugation with an enzyme or fluorophore. Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidotases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, *etc.* Chemiluminescent compounds include luciferin, and 2,3-dihydrophthalazinediones, *e.g.*, luminol. For a review of various labeling or signal producing systems that may be used, *see*, U.S. Patent No. 4,391,904.

Methods of detecting labels are well known to those of skill in the art. Thus, for example, where the label is a radioactive label, methods for detection include use of a

scintillation counter or photographic film as in autoradiography. Where the label is a fluorescent label, it may be detected by exciting the fluorochrome with the appropriate wavelength of light and detecting the resulting fluorescence. The fluorescence may be detected visually, by photographic film, by the use of electronic detectors such as charge coupled devices (CCDs) or photomultipliers and the like. Similarly, enzymatic labels may be detected by providing the appropriate substrates for the enzyme and detecting the resulting reaction product. Finally simple colorimetric labels may be detected simply by observing the color associated with the label. Thus, in various dipstick assays, conjugated gold often appears pink, while various conjugated beads appear the color of the bead.

Fluorescent labels are presently preferred as they have the advantage of requiring few precautions in handling, and being amenable to high-throughput visualization techniques (optical analysis including digitization of the image for analysis in an integrated system comprising a computer). Preferred labels are typically characterized by one or more of the following: high sensitivity, high stability, low background, low environmental sensitivity and high specificity in labeling. Many fluorescent labels are commercially available from the SIGMA chemical company (Saint Louis, MO), Molecular Probes (Eugene, OR), R&D systems (Minneapolis, MN), Pharmacia LKB Biotechnology (Piscataway, NJ), CLONTECH Laboratories, Inc. (Palo Alto, CA), Chem Genes Corp., Aldrich Chemical Company (Milwaukee, WI), Glen Research, Inc., GIBCO BRL Life Technologies, Inc. (Gaithersburg, MD), Fluka Chemica- Biochemika Analytika (Fluka Chemie AG, Buchs, Switzerland), and Applied Biosystems (Foster City, CA), as well as many other commercial sources known to one of skill. Furthermore, those of skill in the art will recognize how to select an appropriate fluorophore for a particular application and, if it not readily available commercially, will be able to synthesize the necessary fluorophore de novo or synthetically modify commercially available fluorescent compounds to arrive at the desired fluorescent label.

In addition to small molecule fluorophores, naturally occurring fluorescent proteins and engineered analogues of such proteins are useful in the present invention. Such proteins include, for example, green fluorescent proteins of cnidarians (Ward *et al., Photochem. Photobiol.* **35**:803-808 (1982); Levine *et al., Comp. Biochem. Physiol.*, **72B**:77-85 (1982)), yellow fluorescent protein from Vibrio *fischeri* strain (Baldwin *et al., Biochemistry* **29**:5509-15 (1990)), Peridinin-chlorophyll from the dinoflagellate Symbiodinium sp. (Morris *et al., Plant Molecular Biology* **24**:673:77 (1994)), phycobiliproteins from marine cyanobacteria,

such as Synechococcus, *e.g.*, phycoerythrin and phycocyanin (Wilbanks *et al., J. Biol. Chem.* **268**:1226-35 (1993)), and the like.

Generally, prior to forming the linkage between the cytotoxin and the targeting (or other) agent, and optionally, the spacer group, at least one of the chemical functionalities will be activated. One skilled in the art will appreciate that a variety of chemical functionalities, including hydroxy, amino, and carboxy groups, can be activated using a variety of standard methods and conditions. For example, a hydroxyl group of the cytotoxin or targeting agent can be activated through treatment with phosgene to form the corresponding chloroformate, or p-nitrophenylchloroformate to form the corresponding carbonate.

In an exemplary embodiment, the invention makes use of a targeting agent that includes a carboxyl functionality. Carboxyl groups may be activated by, for example, conversion to the corresponding acyl halide or active ester. This reaction may be performed under a variety of conditions as illustrated in March, supra pp. 388-89. In an exemplary embodiment, the acyl halide is prepared through the reaction of the carboxyl-containing group with oxalyl chloride. The activated agent is reacted with a cytotoxin or cytotoxin-linker arm combination to form a conjugate of the invention. Those of skill in the art will appreciate that the use of carboxyl-containing targeting agents is merely illustrative, and that agents having many other functional groups can be conjugated to the linkers of the invention.

Reactive Functional Groups

For clarity of illustration the following discussion focuses on the conjugation of a cytotoxin of the invention to a targeting agent. The focus exemplifies one embodiment of the invention from which, others are readily inferred by one of skill in the art. No limitation of the invention is implied, by focusing the discussion on a single embodiment.

Exemplary compounds of the invention bear a reactive functional group, which is generally located on a substituted or unsubstituted alkyl or heteroalkyl chain, allowing their facile attachment to another species. A convenient location for the reactive group is the terminal position of the chain.

Reactive groups and classes of reactions useful in practicing the present invention are generally those that are well known in the art of bioconjugate chemistry. The reactive functional group may be protected or unprotected, and the protected nature of the group may be changed by methods known in the art of organic synthesis. Currently favored classes of reactions available with reactive cytotoxin analogues are those which proceed under

relatively mild conditions. These include, but are not limited to nucleophilic substitutions (*e.g.*, reactions of amines and alcohols with acyl halides, active esters), electrophilic substitutions (*e.g.*, enamine reactions) and additions to carbon-carbon and carbon-heteroatom multiple bonds (*e.g.*, Michael reaction, Diels-Alder addition). These and other useful reactions are discussed in, for example, March, ADVANCED ORGANIC CHEMISTRY, 3rd Ed., John Wiley & Sons, New York, 1985; Hermanson, BIOCONJUGATE TECHNIQUES, Academic Press, San Diego, 1996; and Feeney *et al.*, MODIFICATION OF PROTEINS; Advances in Chemistry Series, Vol. 198, American Chemical Society, Washington, D.C., 1982.

Exemplary reaction types include the reaction of carboxyl groups and various derivatives thereof including, but not limited to, N-hydroxysuccinimide esters, N-hydroxybenztriazole esters, acid halides, acyl imidazoles, thioesters, p-nitrophenyl esters, alkyl, alkenyl, alkynyl and aromatic esters. Hydroxyl groups can be converted to esters, ethers, aldehydes, *etc.* Haloalkyl groups are converted to new species by reaction with, for example, an amine, a carboxylate anion, thiol anion, carbanion, or an alkoxide ion. Dienophile (e.g., maleimide) groups participate in Diels-Alder. Aldehyde or ketone groups can be converted to imines, hydrazones, semicarbazones or oximes, or via such mechanisms as Grignard addition or alkyllithium addition. Sulfonyl halides react readily with amines, for example, to form sulfonamides. Amine or sulfhydryl groups are, for example, acylated, alkylated or oxidized. Alkenes, can be converted to an array of new species using cycloadditions, acylation, Michael addition, *etc.* Epoxides react readily with amines and hydroxyl compounds.

One skilled in the art will readily appreciate that many of these linkages may be produced in a variety of ways and using a variety of conditions. For the preparation of esters, *see, e.g.*, March *supra* at 1157; for thioesters, *see*, March, *supra* at 362-363, 491, 720-722, 829, 941, and 1172; for carbonates, *see*, March, *supra* at 346-347; for carbamates, *see*, March, *supra* at 1156-57; for amides, *see*, March *supra* at 1152; for ureas and thioureas, *see*, March *supra* at 1174; for acetals and ketals, *see*, Greene *et al. supra* 178-210 and March *supra* at 1146; for acyloxyalkyl derivatives, *see*, PRODRUGS: TOPICAL AND OCULAR DRUG DELIVERY, K. B. Sloan, ed., Marcel Dekker, Inc., New York, 1992; for enol esters, *see*, March supra at 1160; for N-sulfonylimidates, *see*, Bundgaard *et al.*, *J. Med. Chem.*, **31**:2066 (1988); for anhydrides, *see*, March *supra* at 355-56, 636-37, 990-91, and 1154; for N-acylamides, *see*, March *supra* at 379; for N-Mannich bases, *see*, March *supra* at 800-02, and 828; for hydroxymethyl ketone esters, *see*, Petracek *et al. Annals NY Acad. Sci.*, **507**:353-54

(1987); for disulfides, *see*, March *supra* at 1160; and for phosphonate esters and phosphonamidates.

The reactive functional groups can be unprotected and chosen such that they do not participate in, or interfere with, the reactions. Alternatively, a reactive functional group can be protected from participating in the reaction by the presence of a protecting group. Those of skill in the art will understand how to protect a particular functional group from interfering with a chosen set of reaction conditions. For examples of useful protecting groups, *see* Greene *et al.*, PROTECTIVE GROUPS IN ORGANIC SYNTHESIS, John Wiley & Sons, New York, 1991.

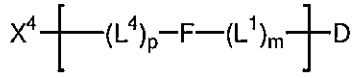
Typically, the targeting agent is linked covalently to a cytotoxin using standard chemical techniques through their respective chemical functionalities. Optionally, the linker or agent is coupled to the agent through one or more spacer groups. The spacer groups can be equivalent or different when used in combination.

Generally, prior to forming the linkage between the cytotoxin and the reactive functional group, and optionally, the spacer group, at least one of the chemical functionalities will be activated. One skilled in the art will appreciate that a variety of chemical functionalities, including hydroxy, amino, and carboxy groups, can be activated using a variety of standard methods and conditions. In an exemplary embodiment, the invention comprises a carboxyl functionality as a reactive functional group. Carboxyl groups may be activated as described hereinabove.

EXAMPLES OF CONJUGATES

The linkers of the invention can be used in conjugates containing duocarmycin or CBI analogs as cytotoxic agents. Examples of conjugates of the invention are described in further detail below. Unless otherwise indicated, substituents are defined as set forth above in the sections regarding cytotoxins and linkers.

One example of a suitable conjugate is a compound of the formula

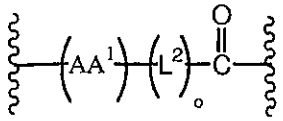


where

L^1 is a self-immolative linker;

m is an integer 0, 1, 2, 3, 4, 5, or 6;

F is a linker comprising the structure:



where

AA^1 is an amino acid selected from the group consisting of natural amino acids and unnatural α-amino acids;

L^2 is a self-immolative linker

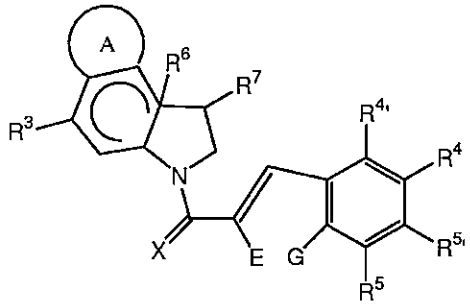
o is 0 or 1;

L^4 is a linker member;

p is 0 or 1;

X^4 is a member selected from the group consisting of protected reactive functional groups, unprotected reactive functional groups, detectable labels, and targeting agents; and

D comprises a structure:



where the ring system A is substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl or substituted or unsubstituted heterocycloalkyl groups;

E and G are members independently selected from H, substituted and unsubstituted alkyl, substituted and unsubstituted heteroalkyl, a heteroatom, and a single bond, or E and G are joined to form a ring system selected from substituted and unsubstituted aryl, substituted and unsubstituted heteroaryl and substituted and unsubstituted heterocycloalkyl;

X is a member selected from O, S and NR^{23};

R^{23} is H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, or acyl;

R^3 is OR^{11},

wherein R^{11} is a member selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, diphosphates, triphosphates, acyl, $C(O)R^{12}R^{13}$, $C(O)OR^{12}$, $C(O)NR^{12}R^{13}$, $P(O)(OR^{12})_2$, , $C(O)CHR^{12}R^{13}$, SR^{12} and $SiR^{12}R^{13}R^{14}$,

in which R^{12}, R^{13}, and R^{14} are members independently selected from H, substituted and unsubstituted alkyl, substituted and unsubstituted heteroalkyl and substituted and unsubstituted aryl, wherein R^{12} and R^{13} together with the nitrogen or carbon atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

R^4 , $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, SO_3, SO_2R^{15}, $CR^{15}{=}NR^{16}$, and $O(CH_2)_nN(CH_3)_2$, or any adjacent pair of R^4, $R^{4'}$, R^5 and $R^{5'}$, together with the carbon atoms to which they are attached, are joined to form a substituted or unsubstituted cycloalkyl or heterocycloalkyl ring system having from 4 to 6 members;

where

n is an integer from 1 to 20;

R^{15} and R^{16} are independently selected from H, substituted and unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted and unsubstituted aryl, substituted and unsubstituted heteroaryl, substituted and unsubstituted heterocycloalkyl, and substituted and unsubstituted peptidyl, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

R^6 is a single bond which is either present or absent and when present R^6 and R^7 are joined to form a cyclopropyl ring; and

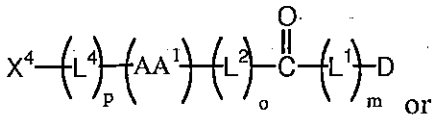
R^7 is CH_2-X^1 or $-CH_2$- joined in said cyclopropyl ring with R^6, wherein

X^1 is a leaving group,

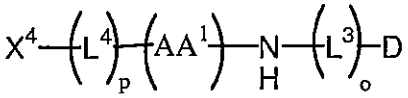
where at least one of R^4, $R^{4'}$, R^5, $R^{5'}$, R^{11}, R^{12}, R^{13}, R^{15} or R^{16} links said drug to L^1, if present, or to F;

or a pharmaceutically acceptable salt thereof.

Another embodiment is a compound of the formula



or



wherein

L^1 is substituted or unsubstituted alkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl or substituted or unsubstituted heteroalkyl group;

m is an integer 0, 1, 2, 3, 4, 5, or 6;

AA^1 is an amino acid selected from the group consisting of natural amino acids and unnatural α-amino acids;

L^2 is substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, unsubstituted heterocycloalkyl, substituted heterocycloalkyl, substituted or unsubstituted aryl, or substituted or unsubstituted heteroaryl;

L^3 is substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, or substituted or unsubstituted heterocycloalkyl;

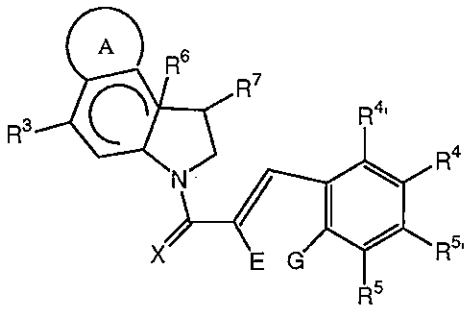
o is 0 or 1;

L^4 is a linker member;

p is 0 or 1;

X^4 is a member selected from the group consisting of protected reactive functional groups, unprotected reactive functional groups, detectable labels, and targeting agents; and

D comprises a structure:



wherein the ring system A is substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl or substituted or unsubstituted heterocycloalkyl;

E and G are members independently selected from H, substituted and unsubstituted alkyl, substituted and unsubstituted heteroalkyl, a heteroatom, and a single bond, or E and G are joined to form a ring system selected from substituted and unsubstituted aryl, substituted and unsubstituted heteroaryl and substituted and unsubstituted heterocycloalkyl;

X is a member selected from O, S and NR^{23};

R^{23} is H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, or acyl;

R^3 is OR^{11},

wherein R^{11} is H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, diphosphate, triphosphate, acyl, $C(O)R^{12}R^{13}$, $C(O)OR^{12}$, $C(O)NR^{12}R^{13}$, $P(O)(OR^{12})_2$, $C(O)CHR^{12}R^{13}$, SR^{12}, or $SiR^{12}R^{13}R^{14}$,

in which R^{12}, R^{13}, and R^{14} are members independently selected from H, substituted and unsubstituted alkyl, substituted and unsubstituted heteroalkyl and substituted and unsubstituted aryl, wherein R^{12} and R^{13} together with the nitrogen or carbon atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

R^4 , $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, SO_3, SO_2R^{15}, $NR^{15}R^{16}$, $NR^{16}C(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, $CR^{15}=NR^{16}$, and $O(CH_2)_nN(CH_3)_2$, or any adjacent pair of R^4, $R^{4'}$, R^5 and $R^{5'}$, together with the carbon atoms to which they are attached, are joined to form a substituted or unsubstituted cycloalkyl or heterocycloalkyl ring system having from 4 to 6 members;

wherein

n is an integer from 1 to 20;

R^{15} and R^{16} are independently selected from H, substituted and unsubstituted alkyl, substituted and unsubstituted heteroalkyl, substituted and

unsubstituted aryl, substituted and unsubstituted heteroaryl, substituted and unsubstituted heterocycloalkyl, and substituted and unsubstituted peptidyl, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

R^6 is a single bond which is either present or absent and when present R^6 and R^7 are joined to form a cyclopropyl ring; and

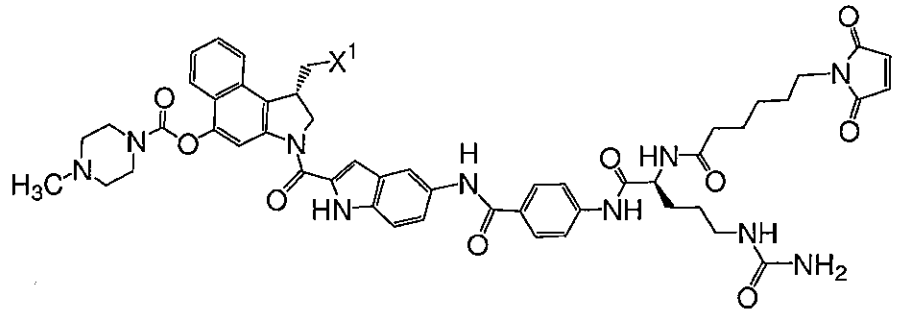
R^7 is CH_2-X^1 or –CH_2- joined in said cyclopropyl ring with R^6, wherein

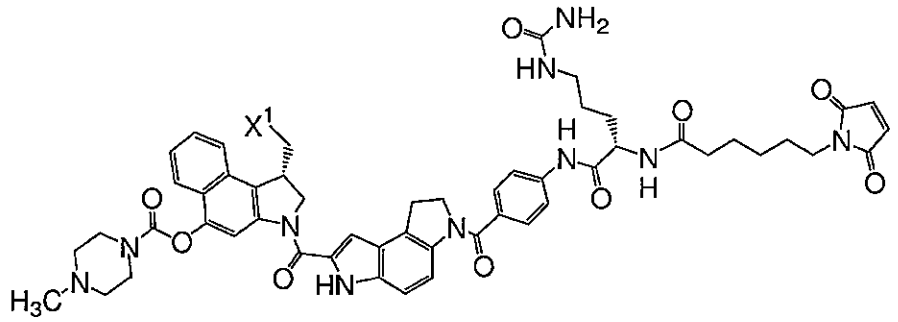
X^1 is a leaving group,

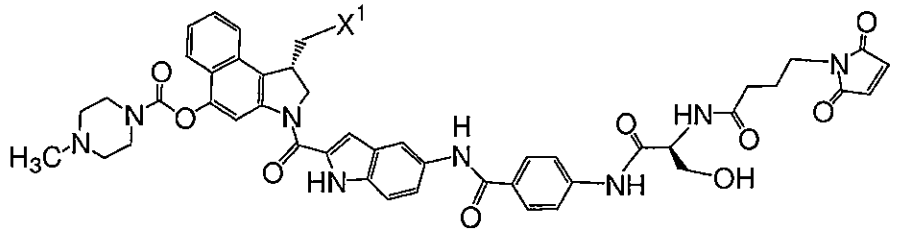
wherein at least one of R^4, $R^{4'}$, R^5, $R^{5'}$, R^{11}, R^{12}, R^{13}, R^{15} or R^{16} links D to the remainder of the compound;

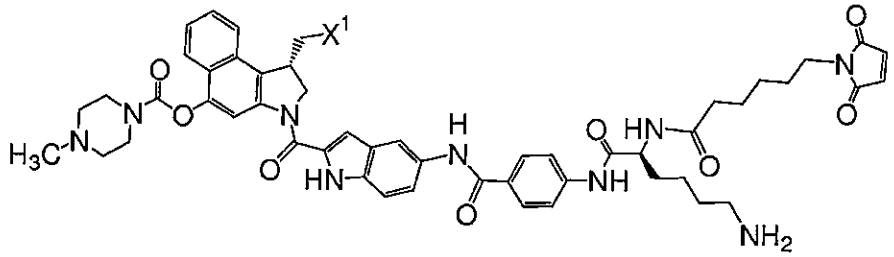
or a pharmaceutically acceptable salt thereof.

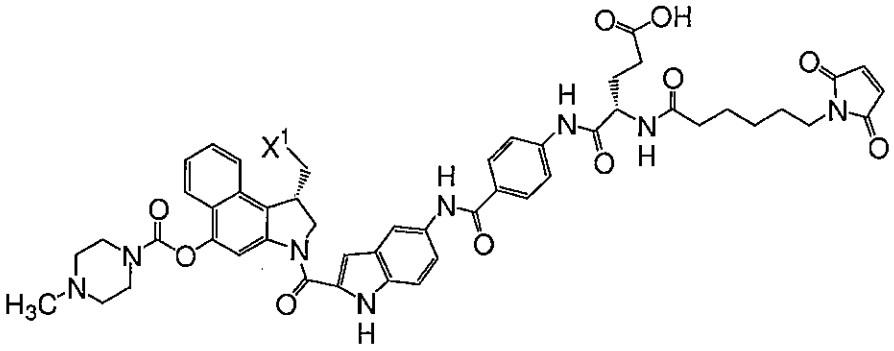
Specific examples of compounds suitable for use as conjugates include

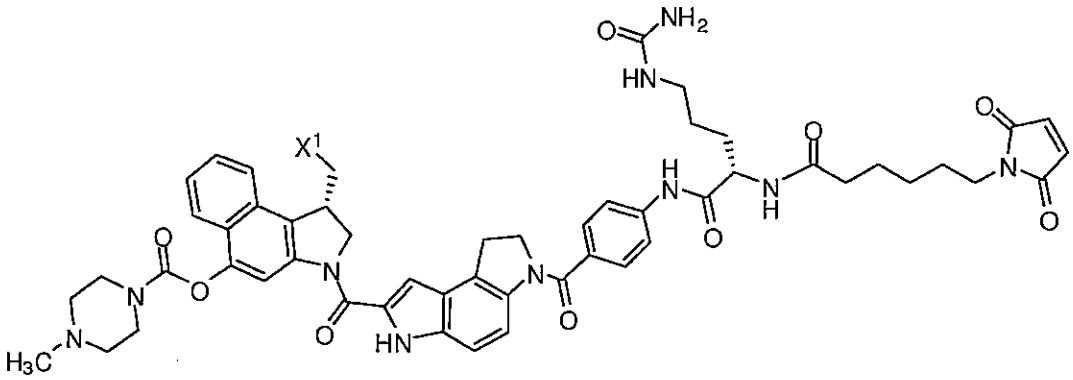


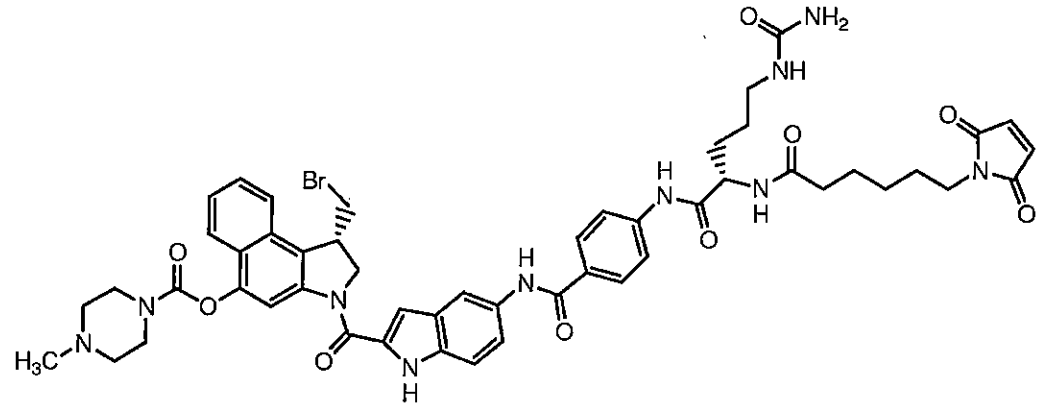


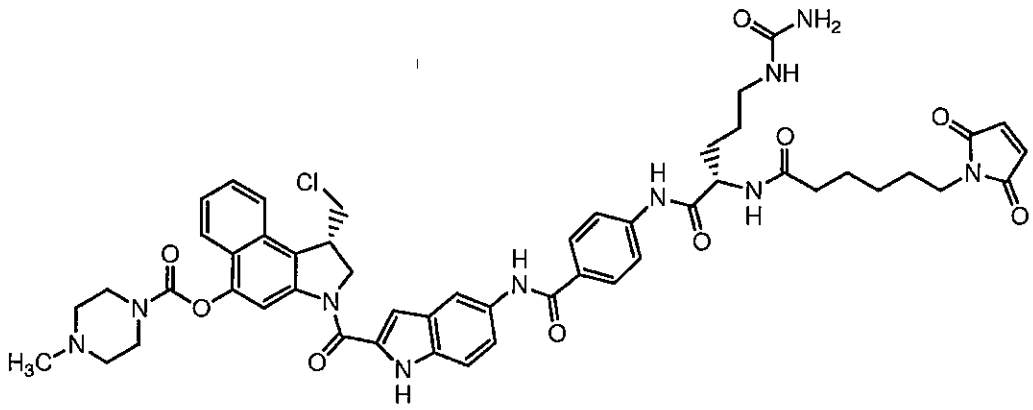


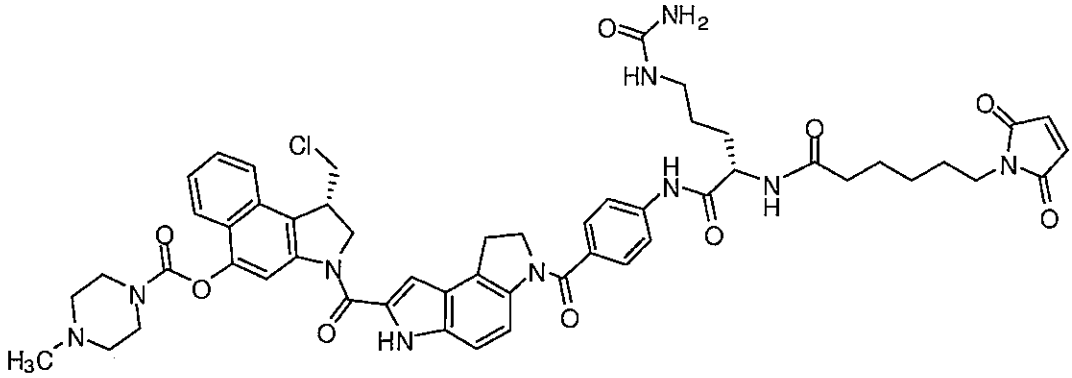


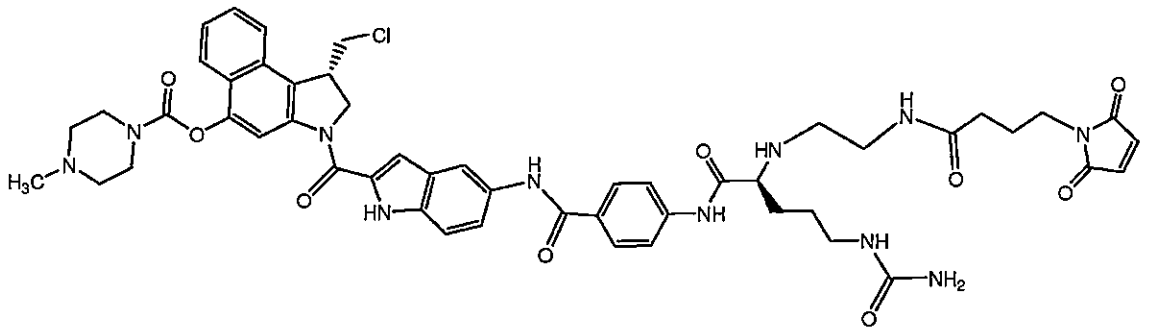


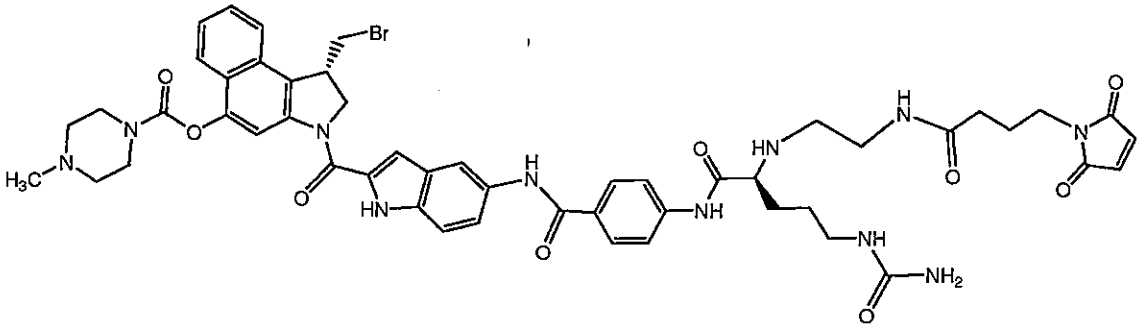












where X^1 is Cl or Br. In some preferred embodiments, X^1 is Cl.

PHARMACEUTICAL FORMULATIONS AND ADMINISTRATION

In another preferred embodiment, the present invention provides a pharmaceutical formulation comprising a compound of the invention and a pharmaceutically acceptable carrier.

The compounds described herein including pharmaceutically acceptable carriers such as addition salts or hydrates thereof, can be delivered to a patient using a wide variety of routes or modes of administration. Suitable routes of administration include, but are not limited to, inhalation, transdermal, oral, rectal, transmucosal, intestinal and parenteral administration, including intramuscular, subcutaneous and intravenous injections. Preferably, the conjugates of the invention are administered parenterally, more preferably intravenously.

As used herein, the terms "administering" or "administration" are intended to encompass all means for directly and indirectly delivering a compound to its intended site of action.

The compounds described herein, or pharmaceutically acceptable salts and/or hydrates thereof, may be administered singly, in combination with other compounds of the invention, and/or in cocktails combined with other therapeutic agents. Of course, the choice

of therapeutic agents that can be co-administered with the compounds of the invention will depend, in part, on the condition being treated.

For example, when administered to patients suffering from a disease state caused by an organism that relies on an autoinducer, the compounds of the invention can be administered in cocktails containing agents used to treat the pain, infection and other symptoms and side effects commonly associated with the disease. Such agents include, *e.g.*, analgesics, antibiotics, *etc.*

When administered to a patient undergoing cancer treatment, the compounds may be administered in cocktails containing anti-cancer agents and/or supplementary potentiating agents. The compounds may also be administered in cocktails containing agents that treat the side-effects of radiation therapy, such as anti-emetics, radiation protectants, *etc.*

Supplementary potentiating agents that can be co-administered with the compounds of the invention include, *e.g.*, tricyclic anti-depressant drugs (*e.g.*, imipramine, desipramine, amitriptyline, clomipramine, trimipramine, doxepin, nortriptyline, protriptyline, amoxapine and maprotiline); non-tricyclic and anti-depressant drugs (*e.g.*, sertraline, trazodone and citalopram); Ca^{+2} antagonists (*e.g.*, verapamil, nifedipine, nitrendipine and caroverine); amphotericin; triparanol analogues (*e.g.*, tamoxifen); antiarrhythmic drugs (*e.g.*, quinidine); antihypertensive drugs (*e.g.*, reserpine); thiol depleters (*e.g.*, buthionine and sulfoximine); and calcium leucovorin.

The active compound(s) of the invention are administered *per se* or in the form of a pharmaceutical composition wherein the active compound(s) is in admixture with one or more pharmaceutically acceptable carriers, excipients or diluents. Pharmaceutical compositions for use in accordance with the present invention are typically formulated in a conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active compounds into preparations which, can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the compounds can be formulated readily by combining the active compound(s) with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a

patient to be treated. Pharmaceutical preparations for oral use can be obtained solid excipient, 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 such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxyniethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations, which 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. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for such administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of *e.g.*, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Injection is a preferred method of administration for the compositions of the current invention. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents, which increase the solubility of the compounds to allow for the preparation of highly, concentrated solutions. For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation or transcutaneous delivery (*e.g.*, subcutaneously or intramuscularly), intramuscular injection or a transdermal patch. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (*e.g.*, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to

calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

A preferred pharmaceutical composition is a composition formulated for injection such as intravenous injection and includes about 0.01% to about 100% by weight of the drug conjugate, based upon 100% weight of total pharmaceutical composition. The drug conjugate may be an antibody-cytotoxin conjugate where the antibody has been selected to target a particular cancer.

LIBRARIES

Also within the scope of the present invention are libraries of the cytotoxin, cytotoxin-linker, and agent-linker conjugates of the cytotoxins and linkers of the invention. Exemplary libraries include at least 10 compounds, more preferably at least 100 compound, even more preferably at least 1000 compounds and still more preferably at least 100,000 compounds. The libraries are in a form that is readily queried for a particular property, e.g., cytotoxicity, cleavage of a linker by an enzyme or other cleavage reagent. Exemplary forms include chip formats, microarrays, and the like.

Parallel, or combinatorial, synthesis has as its primary objective the generation of a library of diverse molecules which all share a common feature, referred to throughout this description as a scaffold. By substituting different moieties at each of the variable parts of the scaffold molecule, the amount of space explorable in a library grows. Theories and modern medicinal chemistry advocate the concept of occupied space as a key factor in determining the efficacy of a given compound against a given biological target. By creating a diverse library of molecules, which explores a large percentage of the targeted space, the odds of developing a highly efficacious lead compound increase dramatically.

Parallel synthesis is generally conducted on a solid phase support, such as a polymeric resin. The scaffold, or other suitable intermediate is cleavably tethered to the resin by a chemical linker. Reactions are carried out to modify the scaffold while tethered to the particle. Variations in reagents and/or reaction conditions produce the structural diversity, which is the hallmark of each library.

Parallel synthesis of "small" molecules (non-oligomers with a molecular weight of 200-1000) was rarely attempted prior to 1990. *See*, for example, Camps. *et al.*, *Annaks de Quimica,* **70**: 848 (1990). Recently, Ellmann disclosed the solid phase-supported parallel (also referred to as "combinatorial") synthesis of eleven benzodiazepine analogs along with

some prostaglandins and beta-turn mimetics. These disclosures are exemplified in U.S. Pat. No. 5,288,514. Another relevant disclosure of parallel synthesis of small molecules may be found in U.S. Pat. No. 5,324,483. This patent discloses the parallel synthesis of between 4 and 40 compounds in each of sixteen different scaffolds. Chen *et al.* have also applied organic synthetic strategies to develop non-peptide libraries synthesized using multi-step processes on a polymer support. (Chen *et al., J. Am. Chem. Soc.*, **116**: 2661-2662 (1994)).

Once a library of unique compounds is prepared, the preparation of a library of immunoconjugates, or antibodies can be prepared using the library of autoinducers as a starting point and using the methods described herein.

KITS

In another aspect, the present invention provides kits containing one or more of the compounds or compositions of the invention and directions for using the compound or composition. In an exemplary embodiment, the invention provides a kit for conjugating a linker of the invention to another molecule. The kit includes the linker, and directions for attaching the linker to a particular functional group. The kit may also, or alternatively, include one or more of a cytotoxic drug, a targeting agent, a detectable label, and pharmaceutical salts or buffers. The kit may also include a container and optionally one or more vial, test tube, flask, bottle, or syringe. Other formats for kits will be apparent to those of skill in the art and are within the scope of the present invention.

PURIFICATION

In another exemplary embodiment, the present invention provides a method for isolating a molecular target for a ligand-cytotoxin of the invention, which binds to ligand X^4. The method preferably comprises, contacting a cellular preparation that includes the target with an immobilized compound, thereby forming a complex between the receptor and the immobilized compound.

The cytotoxin of the invention can be immobilized on an affinity support by any art-recognized means. Alternatively, the cytotoxin can be immobilized using one or more of the linkers of the invention.

In yet another exemplary embodiment, the invention provides an affinity purification matrix.

The method of the invention for isolating a target will typically utilize one or more affinity chromatography techniques. Affinity chromatography enables the efficient isolation of species such as biological molecules or biopolymers by utilizing their recognition sites for certain supported chemical structures with a high degree of selectivity. The literature is replete with articles, monographs, and books on the subject of affinity chromatography, including such topics as affinity chromatography supports, crosslinking members, ligands and their preparation and use. A sampling of those references includes: Ostrove, *Methods Enzymol.* **182**: 357-71 (1990); Ferment, *Bioeng.* **70**: 199-209 (1990). Huang *et al.*, *J. Chromatogr.* **492**: 431-69 (1989); "Purification of enzymes by heparin-Sepharose affinity chromatography," *J. Chromatogr.*, **184**: 335-45 (1980); Farooqi, *Enzyme Eng.*, **4**: 441-2 (1978); Nishikawa, *Chem. Technol.*, **5(9)**: 564-71 (1975); Guilford *et al.*, in, PRACT. HIGH PERFORM. LIQ. CHROMATOGR., Simpson (ed.), 193-206 (1976); Nishikawa, *Proc. Int. Workshop Technol. Protein Sep. Improv. Blood Plasma Fractionation*, Sandberg (ed.), 422-35; (1977) "Affinity chromatography of enzymes," *Affinity Chromatogr., Proc. Int. Symp.* 25-38, (1977) (Pub. 1978); and AFFINITY CHROMATOGRAPHY: A PRACTICAL APPROACH, Dean *et al.* (ed.), IRL Press Limited, Oxford, England (1985). Those of skill in the art have ample guidance in developing particular affinity chromatographic methods utilizing the materials of the invention.

In the present method, affinity chromatographic media of varying chemical structures can be used as supports. For example, agarose gels and cross-linked agarose gels are useful as support materials, because their hydrophilicity makes them relatively free of nonspecific binding. Other useful supports include, for example, controlled-pore glass (CPG) beads, cellulose particles, polyacrylamide gel beads and Sephadex™ gel beads made from dextran and epichlorohydrin.

DRUG CONJUGATE METHODS OF USE

In addition to the compositions and constructs described above, the present invention also provides a number of methods that can be practiced utilizing the compounds and conjugates of the invention. Methods for using the drug-ligand conjugate of the current invention include: killing or inhibiting the growth or replication of a tumor cell or cancer cell, treating cancer, treating a pre-cancerous condition, killing or inhibiting the growth or replication of a cell that expresses an auto-immune antibody, treating an autoimmune disease, treating an infectious disease, preventing the multiplication of a tumor cell or cancer cell,

preventing cancer, preventing the multiplication of a cell that expresses an auto-immune antibody, preventing an autoimmune disease, and preventing an infectious disease. These methods of use comprise administering to an animal such as a mammal or a human in need thereof an effective amount of a drug-ligand conjugate. In some embodiments, an enzyme is separately administered so that it becomes associated with the tumor or target cell.

The drug-ligand conjugate complex of the current invention is useful for treating, for example, cancer, autoimmune disease or infectious disease in an animal. Compositions and methods for treating tumors by providing a subject the composition in a pharmaceutically acceptable manner, with a pharmaceutically effective amount of a composition of the present invention are provided.

The current invention is particularly useful for the treatment of cancer and for the inhibition of the multiplication of a tumor cell or cancer cell in an animal. Cancer, or a precancerous condition, includes, but is not limited to, a tumor, metastasis, or any disease or disorder characterized by uncontrolled cell growth, can be treated or prevented by administration the drug-ligand complexes of the current invention. The complex delivers the drug to a tumor cell or cancer cell.

In one embodiment using a drug-ligand complex, the ligand specifically binds to or associates with a cancer-cell or a tumor-cell-associated antigen. Because of its close proximity to the ligand, the drug can be taken up inside a tumor cell or cancer cell through, for example, receptor-mediated endocytosis. The antigen can be attached to a tumor cell or cancer cell or can be an extracellular matrix protein associated with the tumor cell or cancer cell. Once inside the cell, the linker is cleaved by a tumor-cell or cancer-cell-associated proteases, thereby releasing the drug. The released drug is then free to diffuse and induce cytotoxic activities. In an alternative embodiment, the drug is cleaved from the drug-ligand complex outside the tumor cell or cancer cell, and the drug subsequently penetrates the cell.

The ligand may bind to, for example, a tumor cell or cancer cell, a tumor cell or cancer cell antigen which is on the surface of the tumor cell or cancer cell, or a tumor cell or cancer cell antigen which is an extracellular matrix protein associated with the tumor cell or cancer cell. The ligand can be designed specifically for a particular tumor cell or cancer cell type. Therefore, the type of tumors or cancers that can be effectively treated can be altered by the choice of ligand.

Representative examples of precancerous conditions that may be targeted by the conjugates, include, but are not limited to: metaplasia, hyperplasia, dysplasia, colorectal

polyps, actinic ketatosis, actinic cheilitis, human papillomaviruses, leukoplakia, lychen planus and Bowen's disease.

Representative examples of cancers or tumors that may be targeted by the conjugates include, but are not limited to: lung cancer, colon cancer, prostate cancer, lymphoma, melanoma, breast cancer, ovarian cancer, testicular cancer, CNS cancer, renal cancer, kidney cancer, pancreatic cancer, stomach cancer, oral cancer, nasal cancer, cervical cancer and leukemias. It will be readily apparent to the ordinarily skilled artisan that the particular ligand or cleavable substrate used in the conjugate can be chosen such that it targets the drug to the tumor tissue to be treated with the drug.

In an embodiment, the present invention provides a method of killing a cell. The method includes administering to the cell an amount of a compound of the invention sufficient to kill said cell. In an exemplary embodiment, the compound is administered to a subject bearing the cell. In a further exemplary embodiment, the administration serves to retard or stop the growth of a tumor that includes the cell (*e.g.*, the cell can be a tumor cell). For the administration to retard the growth, the rate of growth of the cell should be at least 10% less than the rate of growth before administration. Preferably, the rate of growth will be retarded at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or completely stopped.

Effective Dosages

Pharmaceutical compositions suitable for use with the present invention include compositions where the active ingredient is contained in a therapeutically effective amount, *i.e.*, in an amount effective to achieve its intended purpose. The actual amount effective for a particular application will depend, *inter alia,* on the condition being treated. Determination of an effective amount is well within the capabilities of those skilled in the art, especially in light of the detailed disclosure herein.

For any compound described herein, the therapeutically effective amount may be initially determined from cell culture assays. Target plasma concentrations will be those concentrations of active compound(s) that are capable of inhibition cell growth or division. In preferred embodiments, the cellular activity is at least 25% inhibited. Target plasma concentrations of active compound(s) that are capable of inducing at least about 50%, 75%, or even 90% or higher inhibition of cellular activity are presently preferred. The percentage of inhibition of cellular activity in the patient can be monitored to assess the appropriateness

of the plasma drug concentration achieved, and the dosage can be adjusted upwards or downwards to achieve the desired percentage of inhibition.

As is well known in the art, therapeutically effective amounts for use in humans can also be determined from animal models. For example, a dose for humans can be formulated to achieve a circulating concentration that has been found to be effective in animals. The dosage in humans can be adjusted by monitoring cellular inhibition and adjusting the dosage upwards or downwards, as described above.

A therapeutically effective dose can also be determined from human data for compounds which are known to exhibit similar pharmacological activities. The applied dose can be adjusted based on the relative bioavailability and potency of the administered compound as compared with the known compound.

Adjusting the dose to achieve maximal efficacy in humans based on the methods described above and other methods as are well-known in the art is well within the capabilities of the ordinarily skilled artisan.

In the case of local administration, the systemic circulating concentration of administered compound will not be of particular importance. In such instances, the compound is administered so as to achieve a concentration at the local area effective to achieve the intended result.

For use in the prophylaxis and/or treatment of diseases related to abnormal cellular proliferation, a circulating concentration of administered compound of about 0.001 μM to 20 μM is preferred, with about 0.01 μM to 5 μM being preferred.

Patient doses for oral administration of the compounds described herein, typically range from about 1 mg/day to about 10,000 mg/day, more typically from about 10 mg/day to about 1,000 mg/day, and most typically from about 50 mg/day to about 500 mg/day. Stated in terms of patient body weight, typical dosages range from about 0.01 to about 150 mg/kg/day, more typically from about 0.1 to about 15 mg/kg/day, and most typically from about 1 to about 10 mg/kg/day, , for example 5 mg/kg/day or 3 mg/kg/day.

In at least some embodiments, patient doses that retard or inhibit tumor growth can be 1 μmol/kg/day or less. For example, the patient doses can be 0.9, 0.6, 0.5, 0.45, 0.3, 0.2, 0.15, 0.1, 0.05, 0.03, 0.01, or 0.005 μmol/kg/day or less (referring to moles of the drug) of the drug or a drug conjugate, such as an antibody-drug conjugate. Preferably, the drug or drug conjugate growth of the tumor when administered in the daily dosage amount over a period of at least five days. In at least some embodiments, the tumor is a human-type tumor in a

SCID mouse. As an example, the SCID mouse can be a CB17.SCID mouse (available from Taconic, Germantown, NY).

For other modes of administration, dosage amount and interval can be adjusted individually to provide plasma levels of the administered compound effective for the particular clinical indication being treated. For example, in one embodiment, a compound according to the invention can be administered in relatively high concentrations multiple times per day. Alternatively, it may be more desirable to administer a compound of the invention at minimal effective concentrations and to use a less frequent administration regimen. This will provide a therapeutic regimen that is commensurate with the severity of the individual's disease.

Utilizing the teachings provided herein, an effective therapeutic treatment regimen can be planned which does not cause substantial toxicity and yet is entirely effective to treat the clinical symptoms demonstrated by the particular patient. This planning should involve the careful choice of active compound by considering factors such as compound potency, relative bioavailability, patient body weight, presence and severity of adverse side effects, preferred mode of administration and the toxicity profile of the selected agent.

The compounds, compositions and methods of the present invention are further illustrated by the examples that follow. These examples are offered to illustrate, but not to limit the claimed invention.

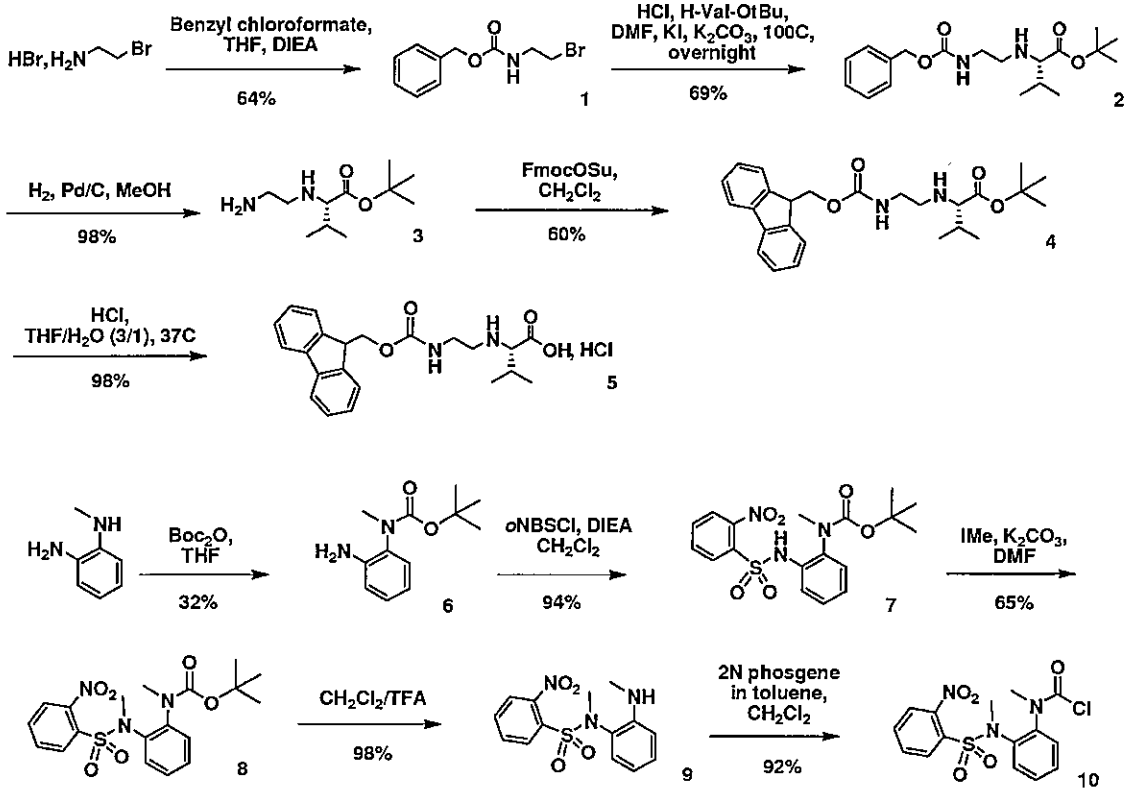
EXAMPLES

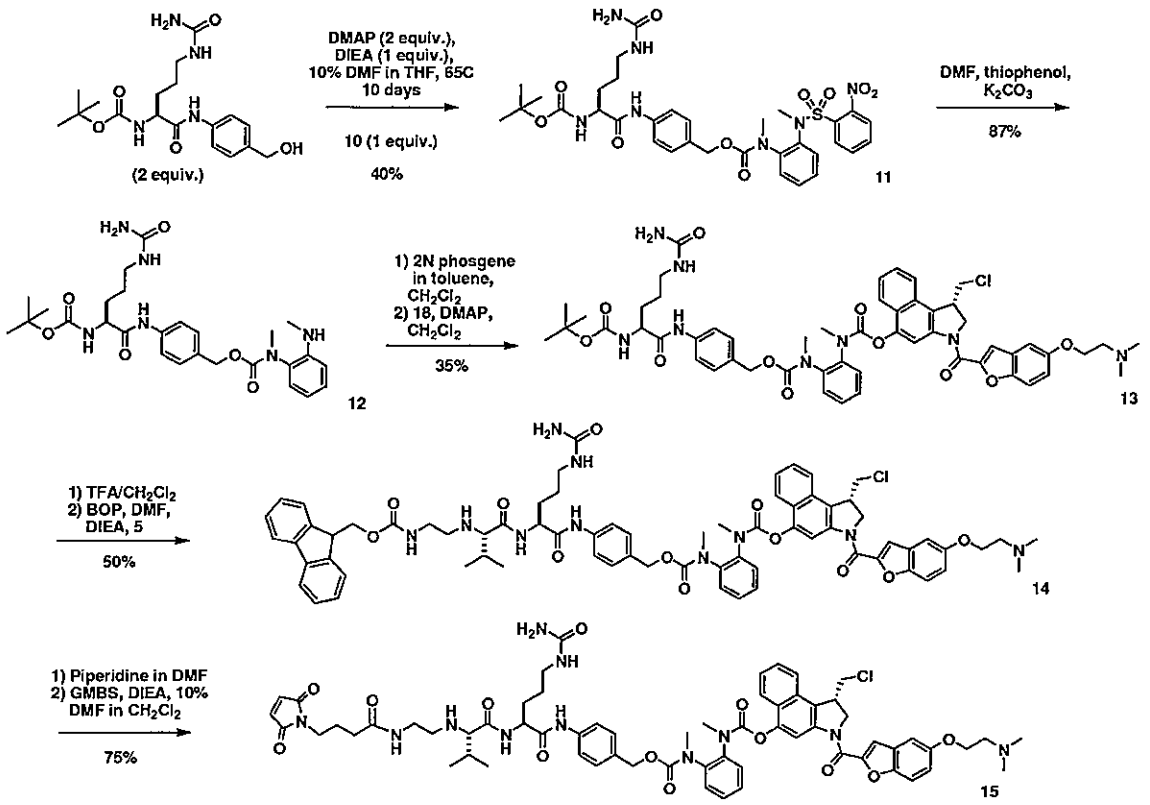
Material and Methods

In the examples below, unless otherwise stated, temperatures are given in degrees Celsius (°C); operations were carried out at room or ambient temperature (typically a range of from about 18-25 °C; evaporation of solvent was carried out using a rotary evaporator under reduced pressure (typically, 4.5-30 mmHg) with a bath temperature of up to 60 °C; the course of reactions was typically followed by TLC and reaction times are provided for illustration only; melting points are uncorrected; products exhibited satisfactory ^{1}H-NMR and/or microanalytical data; yields are provided for illustration only; and the following conventional abbreviations are also used: mp (melting point), L (liter(s)), mL (milliliters), mmol (millimoles), g (grams), mg (milligrams), min (minutes), LC-MS (liquid chromatography-mass spectrometry) and h (hours).

^{1}H-NMR spectra were measured on a Varian Mercury 300 MHz spectrometer and were consistent with the assigned structures. Chemical shifts were reported in parts per million (ppm) downfield from tetramethylsilane. Electrospray mass spectra were recorded on a Perkin Elmer Sciex API 365 mass spectrometer. Elemental analyses were performed by Robertson Microlit Laboratories, Madison, NJ. Silica gel for flash chromatography was E. Merck grade (230-400 mesh). Reverse-Phase analytical HPLC was performed on either a HP 1100 or a Varian ProStar 210 instrument with a Phenomenex Luna 5 μm C-18(2) 150 mm x 4.6 mm column or a Varian Microsorb-MV 0.1 μm C-18 150 mm x 4.6 mm column. A flow rate of 1 mL/min was with either a gradient of 0% to 50% buffer B over 15 minutes or 10% to 100% buffer B over 10 minutes with detection by UV at 254nm. Buffer A, 20 mM ammonium formate + 20% acetonitrile or 0.1% trifluoroacetic acid in acetonitrile; buffer B, 20 mM ammonium formate + 80% acetonitrile or 0.1% aqueous trifluoroacetic acid. Reverse phase preparative HPLC were performed on a Varian ProStar 215 instrument with a Waters Delta Pak 15 μm C-18 300 mm x 7.8 mm column.

EXAMPLE 1





Synthesis of Compound **1**

To a solution of 2-bromoethylamine bromide (5 g, 24.4 mmole) in DMF (50 mL) was added diisopropylethylamine (8.5 mL, 48.8 mmole) and benzyl chlroroformate (3.48 mL, 24.4 mmole). The mixture thus obtained was stirred at room temperature for 2 hours. The reaction mixture was concentrated and the residue was purified by flash chromatography on silica gel with ethyl acetate/hexanes (3/7) as gradient to give Compound **1** as an oil (4 g, 64%). ^{1}H NMR ($CDCl_3$) δ 3.54 (bs, 2H), 3.61 (bs, 2H), 5.12 (s, 2H), 7.36 (m, 5H).

Synthesis of Compound **2**

To a solution of Compound **1** (3.34 g, 12.99 mmole) and valine tert-butyl ester (3.27 g, 15.59 mmole) in DMF (50 mL) was added potassium carbonate (5.39 g, 38.97 mmole) and potassium iodide (2.59 g, 15.59 mmole). The mixture thus obtained was stirred at 100°C overnight. The reaction mixture was concentrated and the residue was purified by flash chromatography on silica gel with ethyl acetate/hexanes (2/8) as gradient to give Compound **2** as an oil (3.12 g, 69%). ^{1}H NMR ($CDCl_3$) δ 0.92 (m, 6H), 1.46 (s, 9H), 1.86 (m, 1H), 2.53 (m, 1H), 2.80 (m, 2H), 3.18 (m, 1H), 3.31 (m, 1H), 5.10 (s, 2H), 5.25 (bs, 1H), 7.36 (m, 5H); LC-MS (ESI) 296 (M+H-*t*butyl$^+$), 352 (M+H$^+$).

Synthesis of Compound 3

A solution of Compound **2** (3.4 g, 9.72 mmole) and palladium on charcoal (200 mg) in methanol (30 mL) was placed under hydrogen atmospheric pressure at room temperature. The mixture thus obtained was stirred at room temperature for 2 hours. The palladium was filtrated and the reaction mixture was concentrated to dryness to give Compound **3** as an oil (2.1 g, 98%). ^{1}H NMR (CD_3OD) δ 0.94 (m, 6H), 1.47 (s, 9H), 1.63 (bs, 2H), 1.90 (m, 1H), 2.47 (m, 1H), 2.73 (m, 2H).

Synthesis of Compound **4**

To a solution of Compound **3** (2.1 g, 9.72 mmole) in dichloromethane (30 mL) was added FmocOSu (N-(9-Fluorenylmethoxycarbonyloxy)succinimide (3.28 g, 9.72 mmole) at 0°C. The mixture thus obtained was stirred for 2 hours at 0°C. The mixture was concentrated to dryness and then the residue was purified by flash chromatography on silica gel with 100% dichloromethane, followed by 0.5% methanol in dichloromethane and finally 1% methanol in dichloromethane as gradient to give Compound **4** as colorless oil (2.55 g, 60%). ^{1}H-NMR ($CDCl_3$) δ 1.03 (d, 3H), 1.14 (d, 3H), 1.52 (s, 9H), 2.28 (m, 1H), 3.14 (m, 2H), 3.46 (m , 2H), 3.89 (d, 1H), 4.24 (m, 1H), 4.44 (m, 2H), 7.29 (m, 2H), 7.40 (m, 2H), 7.64 (m, 2H), 7.80 (d, 2H); LC-MS (ESI) 383 (M+H-*t*butyl$^+$), 440 (M+H$^+$), 462 (M+Na$^+$), 478 (M+K$^+$).

Synthesis of Compound **5**

To a solution of Compound **4** (177 mg, 0.4 mmole) in tetrahydrofurane-water (3/1, 8 mL) was bubbled HCl gas for 5 min. The reaction mixture was stirred at 37°C overnight then the mixture was concentrated to dryness to give Compound **5** as solid (168 mg, 98%) which was used in next step without further purification. ^{1}H-NMR ($CDCl_3$) δ 1.04 (d, 3H), 1.14 (d, 3H), 2.32 (m, 1H), 3.18 (m, 2H), 3.46 (m , 2H), 3.95 (d, 1H), 4.22 (m, 1H), 4.42 (m, 2H), 7.29 (m, 2H), 7.39 (m, 2H), 7.64 (m, 2H), 7.79 (d, 2H); LC-MS (ESI) 383 (M+H$^+$), 405 (M+Na$^+$).

Synthesis of Compound **6**

To a solution of N-methyl-1,2-phenylenediamine (2 ml, 17.6 mmole) in THF (15 mL) was added di-*tert*-butyldicarbonate (3.32 g, 15.2 mmole). The mixture thus obtained was

stirred at room temperature overnight. The solvent was removed and the residue was purified by flash chromatography on silica gel with 30% ethyl acetate in hexanes as gradient to give Compound **6** as colorless oil (1.12 g, 32%). ^{1}H NMR ($CDCl_3$) δ 1.46 (bs, 9H), 3.15 (s, 3H), 3.73 (bs, 2H), 6.75 (d, 2H), 7.06 (m, 2H).

Synthesis of Compound **7**

To a solution of Compound **6** (777 mg, 3.05 mmole) in dichloromethane (30 mL) were added 2-nitrobenzenesulfonyl chloride (811 mg, 3.66 mmole) and diisopropylethylamine (796 μL, 4.57 mmole) at 0°C. The mixture thus obtained was stirred at room temperature overnight. The solvent was removed and the residue was purified by flash chromatography on silica gel with 10% ethyl acetate in hexanes as gradient to give Compound **7** as yellow solid (1.17 g, 94%). ^{1}H NMR ($CDCl_3$) δ 1.49 (bs, 9H), 2.47 and 2.61 (2bs, 3H), 7.05 (bd, 1H), 7.27 (m, 2H), 7.56-7.92 (m, 5H).

Synthesis of Compound **8**

To a solution of Compound **7** (326 mg, 0.8 mmole) in DMF (5 mL) were added carbonate potassium (164 mg, 1.19 mmole) and methyl iodide (148 μL, 2.39 mmole). The mixture thus obtained was stirred at room temperature overnight. The solvent was removed and the residue was purified by flash chromatography on silica gel with 10% ethyl acetate in hexanes as gradient to give Compound **8** as yellow solid (370 mg, 65%). ^{1}H NMR (CD_3OD) δ 1.35 and 1.52 (2s, 9H), 3.16 and 3.21 (2s, 3H), 3.26 and 3.29 (2s, 3H), 6.93 (d, 1H), 7.20 (m, 1H), 7.6 (m, 2H), 7.68-7.80 (m, 4H); LC-MS (ESI) 444 ($M+Na^+$), 460 ($M+K^+$).

Synthesis of Compound **9**

To a solution of Compound **8** (355 mg, 0.85 mmole) in dichloromethane (5 mL) was added trifluoroacetic acid (5 mL) at room temperature. The reaction mixture was stirred for 30 min, then partitioned between ethyl acetate (100 mL) and saturated sodium ammonium bicarbonate (200 mL). The organic layer was washed with brine (100 mL), dried over Na_2SO_4, and concentrated to give the free amine (Compound **9**) as a white solid (270 mg, 98%).^{1}H NMR ($CDCl_3$) δ 2.68 (s, 3H), 3.30 (s, 3H), 6.56 (m, 2H), 6.88 (m, 1H), 7.20 (m, 1H), 7.48 (m, 1H), 7.57 (m, 1H), 7.65 (m, 2H).

Synthesis of Compound **10**

To a solution of Compound **9** (270 mg, 0.84 mmole) in dichloromethane (10 mL) was added 2N phosgene in toluene (440 μL, 2.5 mmole) at 0°C. The mixture was stirred for 30 min and concentrated to dryness to give Compound **10** as white solid (297, 92%) which was used in next step without further purification. ^{1}H NMR ($CDCl_3$) δ 3.25 and 3.30 (2s, 3H), 3.34 and 3.44 (2s, 3H), 6.98 (m, 1H), 7.25-7.40 (m, 2H), 7.45 (m, 1H), 7.55-7.80 (m, 4H); LC-MS (ESI) 384 ($M+H^+$), 407 ($M+Na^+$), 422 ($M+K^+$).

Synthesis of Compound **11**

To a solution of Compound **10** (120 mg, 0.31 mmole) in solution of 10% DMF in THF (3 mL) were added Boc-Cit-PABOH ((S)-*tert*-butyl 1-(4-(hydroxymethyl)phenylamino)-1-oxo-5-ureidopentan-2-ylcarbamate, 238 mg, 0.62 mmole), N,N-dimethylaminopyridine (76 mg, 0.62 mmole) and diisopropylethylamine (55 μL, 0.31 mmole). The mixture thus obtained was stirred at 65°C for 10 days. The solvent was removed and the residue was purified by flash chromatography on silica gel with 5% methanol in dichloromethane as gradient to give Compound **11** as a solid (90 mg, 40%). ^{1}H NMR (CD_3OD) δ 1.43 (bs, 9H), 1.56-1.70 (m, 3H), 1.79 (m, 1H), 3.06-3.27 (m, 8H), 4.17 (2m, 1H), 5.04 and 5.17 (2bs, 2H), 6.89 and 6.95 (2d, 1H), 7.15-7.26 (m, 2H), 7.37-7.72 (m, 8H), 7.80 (m, 1H); LC-MS (ESI) 729 ($M+H^+$), 751 ($M+Na^+$), 767 ($M+K^+$).

Synthesis of Compound **12**

To a solution of Compound **11** (84 mg, 0.11 mmole) in DMF (2 mL) were added potassium carbonate (48 mg, 0.33 mmole) and thiophenol (60 μL, 0.55 mmole). The mixture thus obtained was stirred at room temperature for 2 hours. The solvent was removed and the residue was purified by flash chromatography on silica gel with 5% methanol in dichloromethane as gradient to give Compound **12** as a solid (55 mg, 87%). ^{1}H NMR (CD_3OD) δ 1.44 (bs, 9H), 1.56-1.70 (m, 3H), 1.78 (m, 1H), 2.76 (bs, 3H), 3.05-3.20 (m, 5H), 4.17 (bs, 1H), 4.99 (bs, 2H), 6.62 (m, 2H), 6.93 (m, 1H), 7.15 (m, 2H), 7.40-7.60 (m, 3H); LC-MS (ESI), 566 ($M+Na^+$), 581 ($M+K^+$).

Synthesis of Compound **13**

To a solution of Compound **12** (65 mg, 0.12 mmole) in dichloromethane (3 mL) was added 2N phosgene in toluene (180 μL, 0.36 mmole). The mixture thus obtained was stirred

at 0°C for 1 hour. Then the mixture was concentrated to dryness to give an oil (72 mg, 100%) which was used in next step without further purification.

To a solution of the oil (72 mg, 0.12 mmole) in 5% N-methylpyrrolidone in dichloromethane (1 mL) were added Compound **18** (see Example 2 below) (25 mg, 0.06 mmole) and N,N-dimethylaminopyridine (22 mg, 0.18 mmole). The mixture thus obtained was stirred at room temperature overnight. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give Compound **13** as an oil (15 mg, 35%). ^{1}H NMR (CD_3OD) δ 1.42 (bs, 9H), 1.45-1.80 (m, 4H), 2.97 (bs, 6H), 3.10-3.36 (m, 7H), 3.57 (bs, 3H), 3.98 (bs, 1H), 4.10-4.35 (m, 4H), 4.50-4.75 (m, 3H), 5.15 (bs, 2H), 7.16-7.95 (m, 16H), 8.2 (d, 1H); LC-MS (ESI), 1034 ($M+H^+$), 1056 ($M+Na^+$), 1073 ($M+K^+$).

Synthesis of Compound **14**

To a solution of Compound **13** (13 mg, 0.011 mmole) in dichloromethane (0.5 mL) was added trifluoroacetic acid (0.5 mL) at room temperature. The mixture thus obtained was stirred at room temperature for 30 minutes. Then the mixture was concentrated to dryness to give an oil which was used in next step without further purification.

To a solution of the TFA salted amine in dichloromethane (0.5 mL) were added the Compound **5** (5 mg, 0.012 mmole), Benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphoniumhexafluorophosphate (BOP) (6 mg, 0.013 mmole) and diisopropylethylamine (8 μL, 0.044 mmole). The mixture thus obtained was stirred at room temperature for 1 hour. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give Compound **14** as an oil (9 mg, 50%). ^{1}H NMR (CD_3OD) δ 1.03 and 1.10 (2m, 6H), 1.60 (m, 2H), 1.80 (m, 2H), 2.20 (m, 1H), 2.99 (s, 6H), 3.04-3.50 (m, 11H), 3.60 (bs, 3H), 3.8 (bs, 1H), 4.00 (bs, 1H), 4.20-4.45 (m, 6H), 4.50-4.75 (m, 4H), 5.15 (bs, 2H), 7.20-7.70 (m, 22H), 7.77 (d, 2H), 7.90 (bs, 2H); LC-MS (ESI), 1300 ($M+H^+$).

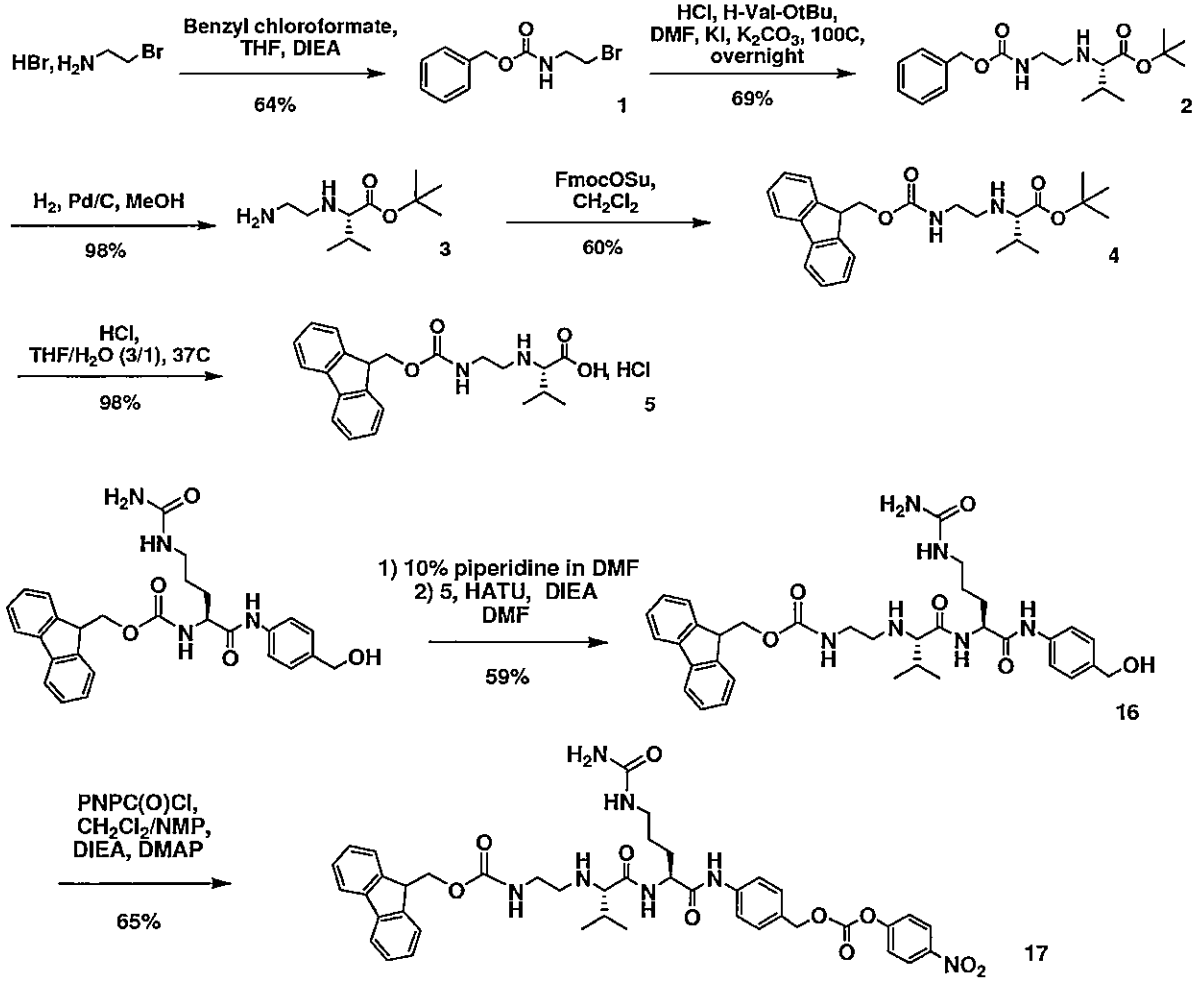
Synthesis of Compound **15**

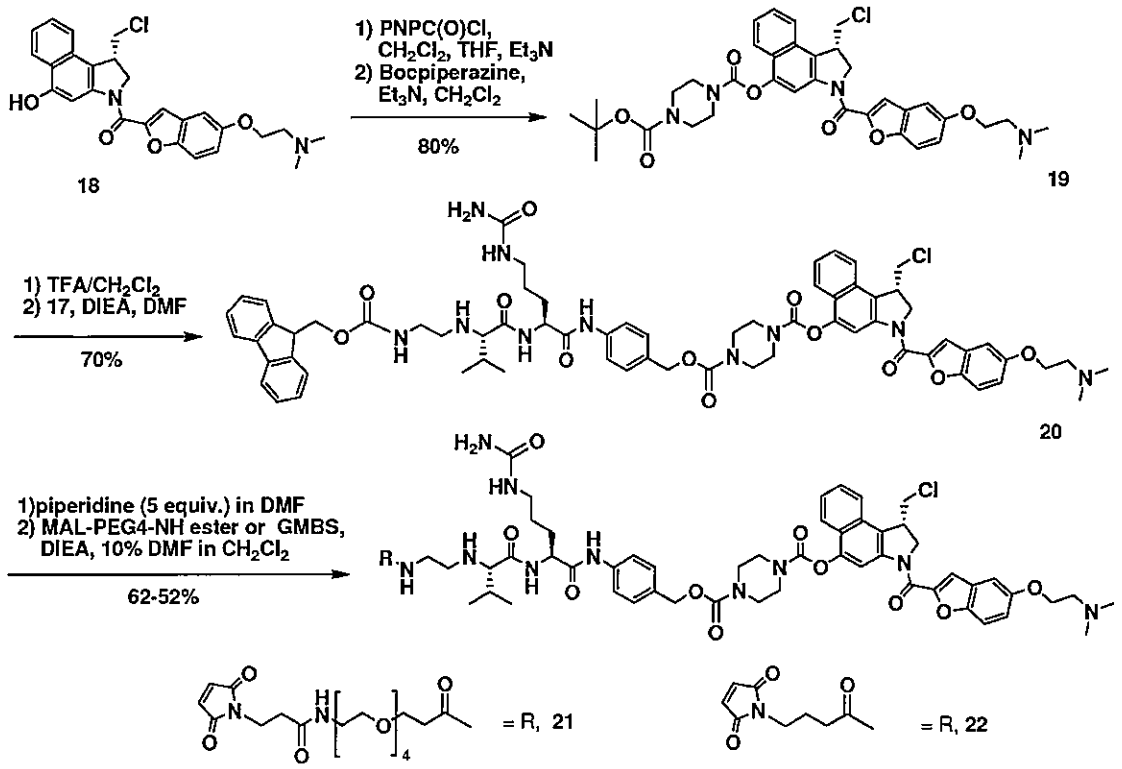
To a solution of Compound **14** (9 mg, 0.006 mmole) in DMF (0.5 mL) was added piperidine (6 μL, 0.06 mmole) at room temperature. The mixture thus obtained was stirred at room temperature for 1 hour. Then the mixture was concentrated to dryness to give an oil which was used in next step without further purification.

To a solution of the oil in a solution of 10% DMF in dichloromethane (1 mL) were added (N-{y-maleimidobutyrloxy}succinimide ester (GMBS) (3.5 mg, 0.012 mmole) and

diisopropylethylamine (2 μL, 0.012 mmole). The mixture thus obtained was stirred at room temperature for 1 hour. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give Compound **15** as an oil (6.5 mg, 75%). ^{1}H NMR (CD_3OD) δ 1.04-1.13 (m, 6H), 1.65 (m, 2H), 1.75 (m, 1H), 1.90 (m, 3H), 2.23 (m, 3H), 2.98 and 3.01 (2s, 6H), 3.05-3.25 (m, 6H), 3.60 (m, 2H), 3.45-3.55 (m, 6H), 3.64 (t, 2H), 3.80 (m, 2H), 4.05 (m, 1H), 4.35 (bs, 1H), 4.41 (m, 2H), 4.55 (m, 2H), 4.80 (m, 1H), 5.15 (bs, 2H), 6.78 (s, 2H), 7.22 (dd, 2H), 7.35-7.65 (m, 11H), 7.90-8.00 (m, 3H), 7.77 (d, 2H), 7.90 (bs, 2H); LC-MS (ESI), 1243 ($M+H^+$).

EXAMPLE 2





Synthesis of Compound **16**

To a solution of Fmoc-Cit-PABOH (2 g, 3.98 mmole) in DMF (45 mL) was added piperidine (5 mL) at room temperature. The mixture thus obtained was stirred at room temperature for 1 hour. The mixture was concentrated to dryness and then the residue was purified by flash chromatography on silica gel with 100% dichloromethane, followed by 10% methanol in dichloromethane and finally 30% methanol in dichloromethane as gradient to give H-Cit-PABOH as colorless oil (1 g, 90%).

To a solution of H-Cit-PABOH (80 mg, 0.28 mmole) in DMF (4 mL) were added the compound **5** (100 mg, 0.24 mmole) (see Example 1 for preparation), HATU (100 mg, 0.26 mmole) and diisopropylethylamine (125 μL, 0.84 mmole). The mixture thus obtained was stirred at room temperature for 1 hour. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give the Compound **16** as an oil (119 mg, 66%). ^{1}H NMR (CD_3OD) δ 1.03-1.11 (2d, 6H), 1.58 (m, 2H), 1.77 (m, 1H), 1.88 (m, 1H), 2.23 (m, 1H), 3.05-3.20 (m, 4H), 3.44 (m, 2H), 3.83 (d, 1H), 4.21 (m, 1H), 4.39 (m, 2H), 4.54 (bs, 2H), 4.60 (m, 1H), 7.30 (m, 4H), 7.37 (m, 2H), 7.51 (m, 2H), 7.62 (m, 2H), 7.77 (m, 2H), 8.80 (d, 1H), 10.05 (s, 1H); LC-MS (ESI), 646 ($M+H^+$), 668 ($M+Na^+$), 684 ($M+K^+$).

Synthesis of Compound **17**

To a solution of Compound **16** (190 mg, 0.25 mmole)) in 10% NMP in dichloromethane (4 mL) were diisopropylethylamine (131 μL, 0.75 mmole), N,N-dimethylaminopyridine (15 mg, 0.12 mmole) and 4-nitrophenyl chloroformate (101 mg, 0.5 mmole). The mixture thus obtained was stirred at room temperature for 1 hour. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give Compound **17** as an oil (151 mg, 65%). (CD_3OD) δ 1.04-1.11 (2d, 6H), 1.59 (m, 2H), 1.79 (m, 1H), 1.89 (m, 1H), 2.24 (m, 1H), 3.05-3.20 (m, 4H), 3.44 (m, 2H), 3.83 (d, 1H), 4.20 (m, 1H), 4.39 (m, 2H), 4.60 (m, 1H), 5.22 (bs, 1H), 7.29 (m, 2H), 7.39 (m, 4H), 7.60 (m, 4H), 7.75 (d, 2H), 8.81 (d, 1H), 10.05 (s, 1H); LC-MS (ESI), 811 ($M+H^+$), 833 ($M+Na^+$), 848 ($M+K^+$).

Synthesis of Compound **19**

To a solution of Compound **18** (50 mg, 0.11 mmole)) in 40% THF in dichloromethane (8 mL) were added 4-Nitrophenyl Chloroformate (87 mg, 0.43 mmole) and triethylamine (88 μL, 0.64 mmole) at 0°C. The mixture thus obtained was let warmed to room temperature and stirred overnight. The solvent was evaporated and the residue was isolated by precipitation in ether and followed by filtration to give PNPC-**18** as a yellow solid (60 mg, 90%).

To a solution of the PNPC-**18** (60 mg, 0.095 mmole) in dichloromethane (5 mL) were added the compound bocpiperazine (71 mg, 0.38 mmole) and triethylamine (53 μL, 0.38 mmole). The mixture thus obtained was stirred at room temperature overnight. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give Compound **18** as an oil (77 mg, 90%). LC-MS (ESI), 678 ($M+H^+$), 700 ($M+Na^+$), 716 ($M+K^+$)

Synthesis of Compound **20**

To a solution of Compound **19** (28 mg, 0.035 mmole) in dichloromethane (0.5 mL) was added trifluoroacetic acid (0.5 mL) at room temperature. The mixture thus obtained was stirred at room temperature for 30 minutes. Then the mixture was concentrated to dryness to give an oil which was used in next step without further purification.

To a solution of the oil in DMF (1 mL) were added the compound **17** (28 mg, 0.035 mmole) and diisopropylethylamine (18 μL, 0.105 mmole). The mixture thus obtained was stirred at room temperature overnight. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give Compound **20** as an oil (36 mg, 70%). ^1H NMR (CD_3OD) δ 1.03-1.10 (2d, 6H), 1.58 (m, 2H), 1.77 (m, 1H), 1.87 (m, 1H), 2.23 (m, 1H), 2.97

(bs, 6H), 3.05-3.20 (m, 4H), 3.30-3.85 (m, 14H), 3.97 (m, 1H), 4.19-4.41 (m, 6H), 4.60 (m, 2H), 4.69 (m, 1H), 5.11 (bs, 2H), 7.16 (dd, 1H), 7.27-7.38 (m, 7H), 7.45 (m, 1H), 7.51-7.63 (m, 7H), 7.76 (d, 2H), 7.85 (d, 2H), 8.24 (bs, 1H), 8.79 (d, 1H), 10.00 (s, 1H); LC-MS (ESI), 1248 ($M+H^+$).

Synthesis of Compound **21**

To a solution of Compound **20** (36 mg, 0.024 mmole) in DMF (1 mL) was added piperidine (12 μL, 0.12 mmole) at room temperature. The mixture thus obtained was stirred at room temperature for 1 hour. Then the mixture was concentrated to dryness to give an oil which was used in next step without further purification.

To a solution of the oil in 10% DMF in dichloromethane (1 mL) were added MAL-PEG_4-NH ester (19 mg, 0.037 mmole) and diisopropylethylamine (8.5 μL, 0.048 mmole). The mixture thus obtained was stirred at room temperature for 1 hour. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give Compound **21** as an oil (25 mg, 62%). ^1H NMR (CD_3OD) δ 1.06-1.12 (2d, 6H), 1.59 (m, 2H), 1.78 (m, 1H), 1.89 (m, 1H), 2.24 (m, 1H), 2.44 (t, 2H), 2.50 (t, 2H), 2.99 (bs, 6H), 3.05-3.20 (m, 4H), 3.46-3.85 (m, 34H), 3.99 (m, 1H), 4.25 (m, 1H), 4.35 (m, 2H), 4.59-4.73 (m, 3H), 5.13 (bs, 2H), 6.79 (s, 2H), 7.18 (dd, 1H), 7.31 (d, 1H), 7.36 (m, 2H), 7.47 (m, 1H), 7.58 (m, 5H), 7.89 (m, 2H), 8.24 (bs, 1H), 8.79 (d, 1H); LC-MS (ESI), 1423 ($M+H^+$).

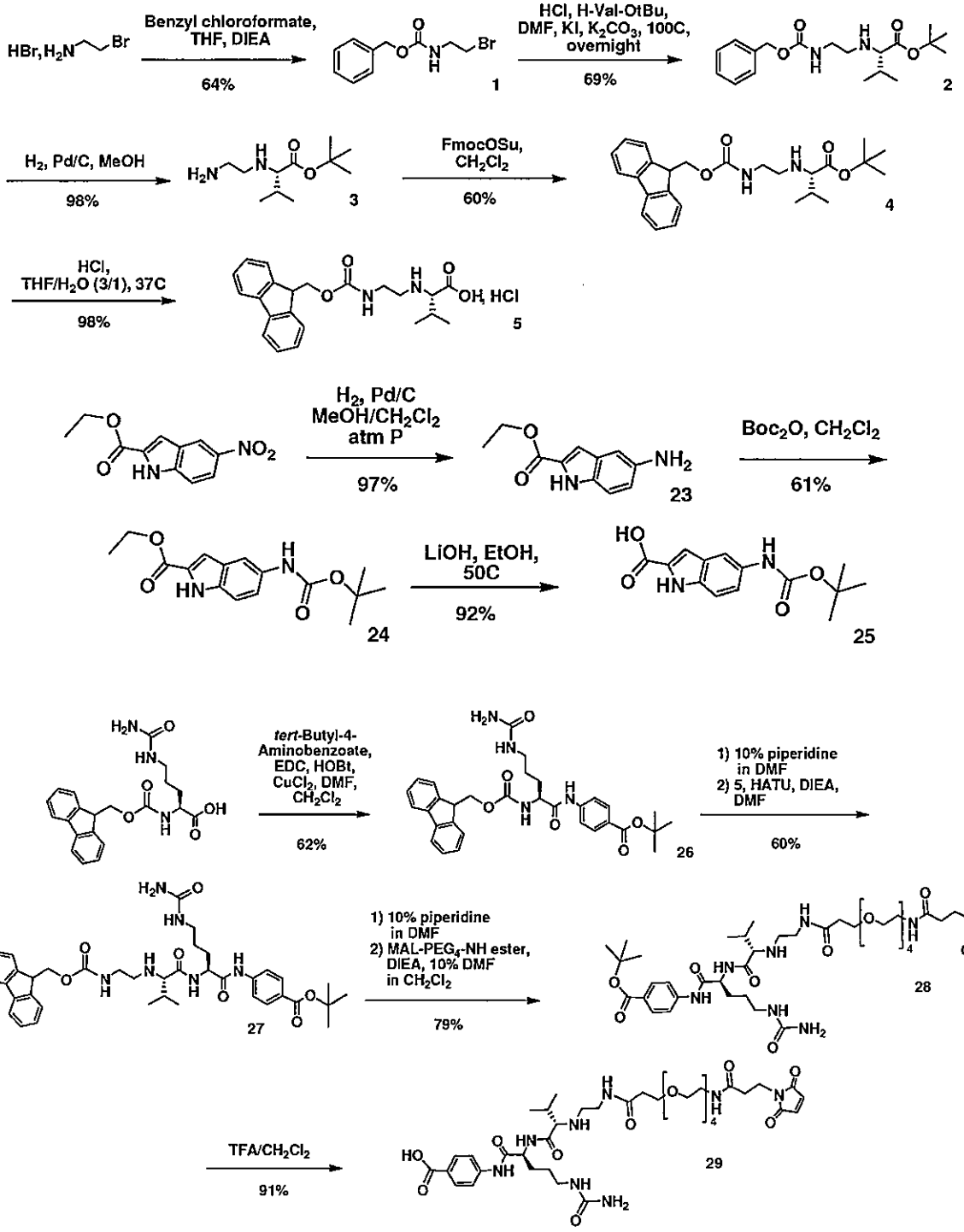
Synthesis of Compound **22**

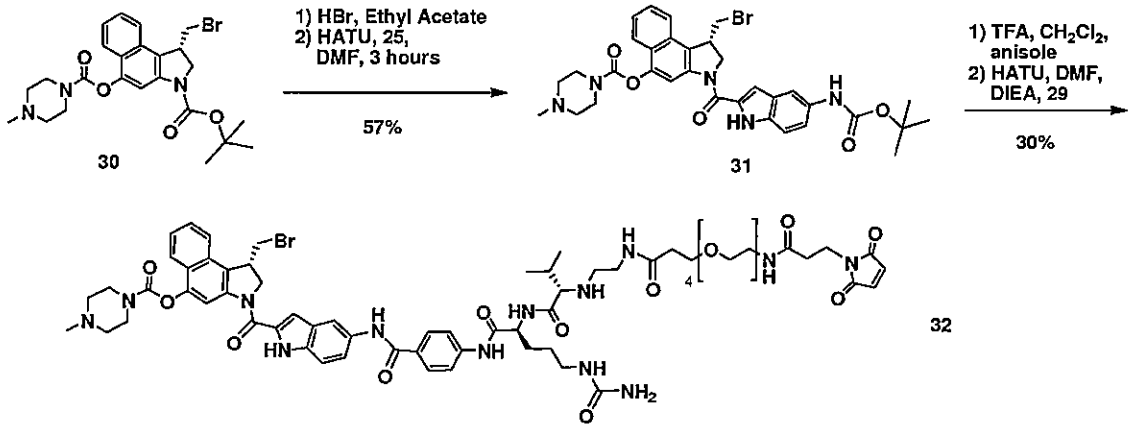
To a solution of Compound **20** (26 mg, 0.017 mmole) in DMF (1 mL) was added piperidine (9 μL, 0.088 mmole) at room temperature. The mixture thus obtained was stirred at room temperature for 1 hour. Then the mixture was concentrated to dryness to give the an oil which was used in next step without further purification.

To a solution of the oil in 10% DMF in dichloromethane (1 mL) were added GMBS (7.5 mg, 0.025 mmole) and diisopropylethylamine (6 μL, 0.034 mmole). The mixture thus obtained was stirred at room temperature for 1 hour. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give Compound **22** as an oil (13 mg, 52%). δ 1.07-1.13 (2d, 6H), 1.60 (m, 2H), 1.78 (m, 1H), 1.90 (m, 3H), 2.24 (m, 3H), 3.00 (bs, 6H), 3.05-3.24 (m, 4H), 3.42-3.74 (m, 16H), 3.78-3.90 (m, 4H), 4.00 (m, 1H), 4.34 (m, 1H), 4.39 (m, 2H), 4.60 (m, 1H), 4.80 (m, 2H), 5.14 (bs, 2H), 6.81 (s, 2H), 7.22 (dd, 1H),

7.37 (m, 3H), 7.50 (m, 1H), 7.60 (m, 5H), 7.92 (m, 2H), 8.24 (bs, 1H), 8.79 (d, 1H), 10.05 (s, 1H); LC-MS (ESI), 1191 ($M+H^+$).

EXAMPLE 3





Synthesis of Compound **23**

A solution of ethyl-5-nitroindole-2 carboxylate (2 g, 8.5 mmole) and palladium on charcoal (200 mg) in 50% methanol in dichloromethane (100 mL) was placed under hydrogen atmospheric pressure at room temperature. The mixture thus obtained was stirred at room temperature for 2 hours. The palladium was filtrated and the reaction mixture was concentrated to dryness to give Compound **23** as colorless oil (1.68 g, 97%). ^{1}H NMR (CD_3OD) δ 1.38 (t, 3H), 4.34 (q, 2H), 6.86 (dd, 1H), 6.95 (d, 1H), 6.98 (d, 1H), 7.25 (d, 1H).

Synthesis of Compound **24**

To a solution of Compound **23** (300 mg, 1.47 mmole) in dichloromethane (5 mL) was added Boc_2O (385 mg, 1.76 mmole). The mixture thus obtained was stirred at room temperature for 2 hours. The reaction mixture was concentrated and the residue was purified by flash chromatography on silica gel with 10% ethyl acetate in hexanes as gradient to give Compound **24** as a white solid (272 mg, 61%). ^{1}H NMR (CD_3OD) δ 1.39 (t, 3H), 1.52 (s, 9H), 4.37 (q, 2H), 7.07 (s, 1H), 7.23 (dd, 1H), 7.34 (d, 1H), 7.68 (bs, 1H).

Synthesis of Compound **25**

A solution of Compound **24** (100 mg, 0.33 mmole) in ethanol (3 mL) was added a solution of LiOH (12 mg, 0.49 mmole) in water (1 mL). The mixture thus obtained was stirred at room temperature for 2 hours at 50°C. The reaction mixture was concentrated to dryness to give an oil. The residue was dissolved in water and acidified to pH 3 with 10% HCl, followed by extraction with EtOAc. The organic solution was dried over Na_2SO_4, filtered and concentrated to dryness to give Compound **25** as colorless oil (85 mg, 92%). ^{1}H NMR (CD_3OD) δ 1.51 (s, 9H), 7.07 (d, 1H), 7.23 (dd, 1H), 7.33 (d, 1H), 7.68 (bs, 1H).

Synthesis of Compound **26**

To a solution of Fmoc-Cit-OH (206 mg, 0.52 mmole) in solution of 30% DMF in dichloromethane (3 mL) were added EDC (120 mg, 0.62 mmole), HOBt (84 mg, 0.62 mmole) and tert-butyl-4-amino benzoate (120 mg, 0.62 mmole) at room temperature. The mixture thus obtained was stirred for 10 minutes then copper chloride (84 mg, 0.62 mmole) was added to the mixture. The mixture was stirred overnight. The mixture was concentrated to dryness and then the residue was purified by flash chromatography on silica gel with 5% methanol in dichloromethane as gradient to give Compound **26** as colorless oil (184 mg, 62%). ^{1}H NMR (CD_3OD) δ 1.53-1.58 (m, 2H), 1.57 (s, 9H), 1.71 (m, 1H), 1.82 (m, 1H), 3.08 (m, 1H), 3.19 (m, 1H), 4.21 (m, 1H), 4.28 (m, 1H), 4.38 (m, 2H), 7.28-7.39 (m, 3H), 7.49 (m, 2H), 7.56-7.86 (m, 5H), 7.89 (m, 2H); LC-MS (ESI), 573 ($M+H^+$), 595 ($M+Na^+$), 611 ($M+K^+$).

Synthesis of Compound **27**

To a solution of Compound **26** (1 g, 1.75 mmole) in DMF (18 mL) was added piperidine (2 mL) at room temperature. The mixture thus obtained was stirred at room temperature for 1 hour. The mixture was concentrated to dryness and then the residue was purified by flash chromatography with 100% dichloromethane, followed by 5% methanol in dichloromethane and finally 20% methanol in dichloromethane as gradient to give a colorless oil (561 mg, 92%).

To a solution of the oil (561 mg, 1.6 mmole) in DMF (10 mL) were added diisopropylethylamine (679 μL, 3.9 mmole), the compound **5** (509 mg, 1.3 mmole) (see Example 1 for preparation) and HATU (494 mg, 1.3 mmole) at room temperature. The mixture thus obtained was stirred at room temperature for 3 hours. The mixture was concentrated to dryness and then the residue was purified by flash chromatography on silica gel with 5% methanol in dichloromethane as gradient to give Compound **27** as colorless oil (691 mg, 65%). ^{1}H NMR (CD_3OD) δ 1.36 (dd, 6H), 1.58-1.62 (m, 2H), 1.6 (s, 9H), 1.71 (m, 1H), 1.82 (m, 1H), 2.00 (m, 1H), 2.65 (m, 2H), 3.2-3.3 (m, 4H), 3.70 (m, 1H), 4.21 (m, 1H), 4.28 (m, 2H), 4.38 (m, 2H), 4.60 (m, 1H), 7.28-7.39 (m, 4H), 7.60-7.70 (m, 4H), 7.8 (d, 2H), 7.89 (d, 2H); LC-MS (ESI), 716 ($M+H^+$), 737 ($M+Na^+$), 753 ($M+K^+$).

Synthesis of Compound **28**

To a solution of Compound **27** (300 mg, 0.45 mmole) in DMF (9 mL) was added piperidine (1 mL) at room temperature. The mixture thus obtained was stirred at room temperature for 1 hour. Then the mixture was concentrated to dryness to give an oil which was crashed out in ether (20 mL). The material was filtered to give a white solid (186 mg, 84%).

To a solution of the free amine (32 mg, 0.065 mmole) in dichloromethane (1 mL) was added MAL-PEG_4-NH ester (50 mg, 0.097 mmole). The mixture thus obtained was stirred at room temperature for 4 hours. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give Compound **28** as an oil (47 mg, 95%). ^{1}H NMR (CD_3OD) δ 1.10 and 1.15 (2d, 6H), 1.58-1.62 (m, 2H), 1.6 (s, 9H), 1.75 (m, 1H), 1.90 (m, 1H), 2.25 (m, 1H), 2.45 (t, 2H), 2.5 (t, 2H), 3.10-3.25 (m, 4H), 3.30 (m, 2H), 3.45-3.65 (m, 16H), 3.75 (m, 4H), 3.85 (d, 1H), 4.65 (m, 1H), 6.80 (s, 2H), 7.67 (d, 2H), 7.90 (d, 2H), 8.80 (d, 1H), 10.20 (s, 1H); LC-MS (ESI), 891 ($M+H^+$), 913 ($M+Na^+$), 929 ($M+K^+$).

Synthesis of Compound **29**

To a solution of Compound **28** (47 mg, 0.062 mmole) in dichloromethane (0.5 mL) was added trifluoroacetic acid (0.5 mL) at room temperature. The mixture thus obtained was stirred at room temperature for 30 minutes. Then the mixture was concentrated to dryness to give Compound **29** as an oil which was used in next step without further purification (40 mg, 92%). ^{1}H NMR (CD_3OD) δ 1.10 and 1.15 (2d, 6H), 1.60 (m, 2H), 1.80 (m, 1H), 1.90 (m, 1H), 2.25 (m, 1H), 2.45 (t, 2H), 2.5 (t, 2H), 3.10-3.25 (m, 4H), 3.30 (m, 2H), 3.45-3.65 (m, 16H), 3.75 (m, 4H), 3.85 (d, 1H), 4.65 (m, 1H), 6.80 (s, 2H), 7.67 (d, 2H), 7.95 (d, 2H), 8.80 (d, 1H); LC-MS (ESI), 836 ($M+H^+$), 858 ($M+Na^+$), 874 ($M+K^+$).

Synthesis of Compound **31**

To a solution of **30** (100 mg, 0.2 mmole) in EtOAc (2 mL) was added a concentrated HBr solution in EtOAc (3 mL) at room temperature. The Boc deprotection was completed after 1 hour. The precipitated material was filtered (quantitative yield). Then the TFA salted amine was dissolved in DMF (3 mL). To this solution were added the compound **25** (55 mg, 0.2 mmole), diisopropylethylamine (173 μL, 1 mmole) and HATU (79 mg, 0.2 mmole). The mixture thus obtained was stirred at room temperature for 3 hours. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give Compound **31** as

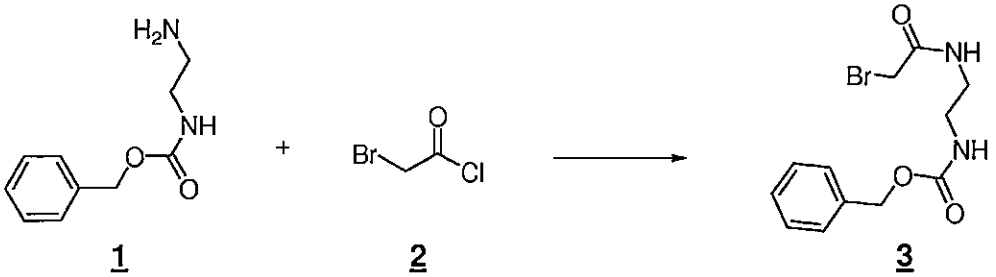
a white solid (86 mg, 57%). ^{1}H NMR (CD_3OD) δ 1.54 (s, 9H), 2.91 (s, 3H), 3.10-3.60 (m, 8H), 3.72 (m, 1H), 3.97 (m, 1H), 4.30-4.60 (m, 3H), 6.94 (bs, 1H), 7.05 (m, 1H), 7.12 (d, 1H), 7.45 (m, 2H), 7.68 (d, 1H), 7.75 (bs, 1H), 7.86 (d, 1H), 8.23 (bs, 1H); LC-MS (ESI), 562 ($M+H-100^+$), 606 ($M+H-56^+$), 662 ($M+H^+$), 685 ($M+Na^+$), 701 ($M+K^+$).

Synthesis of Compound **32**

To a solution of Compound **31** (30 mg, 0.039 mmole) in dichloromethane (0.5 mL) were added anisole (100 μL) and trifluoroacetic acid (0.4 mL) at room temperature. The mixture thus obtained was stirred at room temperature for 30 minutes. Then the mixture was concentrated to dryness to give an oil which was used in next step without further purification.

To a solution of the oil in DMF (1 mL) were added the Compound **29** (36 mg, 0.039 mmole), diisopropylethylamine (40 μL, 0.23 mmole) and HATU (15 mg, 0.039 mmole). The mixture thus obtained was stirred at room temperature for 1 hour. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give Compound **32** as an oil (36 mg, 60%). ^{1}H NMR (CD_3OD) δ 1.09 and 1.15 (2d, 6H), 1.62 (m, 2H), 1.81 (m, 1H), 1.93 (m, 1H), 2.27 (m, 1H), 2.45 (t, 2H), 2.51 (t, 2H), 2.98 (s, .3H), 3.13-3.25 (m, 4H), 3.47-3.62 (m, 24H), 3.76 (m, 4H), 3.82 (m, 1H), 3.85 (d, 1H), 4.20 (m, 1H), 4.55-4.70 (m, 4H), 6.79 (s, 2H), 7.06 (s, 1H), 7.36 (bs, 1H), 7.43-7.54 (m, 2H), 7.72-7.81 (m, 3H), 7.91 (m, 3H), 8.05 (s, 1H), 8.25 (bs, 1H), 8.82 (d, 1H), 10.25 (s, 1H); LC-MS (ESI), 691 ($M+2H^+$)/2, 1381 ($M+H^+$), 1419 ($M+K^+$).

EXAMPLE 4

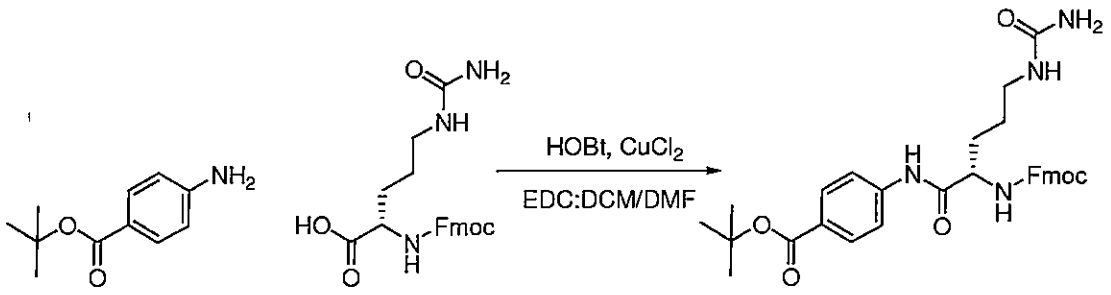


1 **2** **3**

Synthesis of Compound **3**

Bromo acetyl chloride **2** (240uL, 2.86 mMoles) was added dropwise to a solution of benzyl 2-aminoethylcarbamate **1** (500 mg, 2.6 mMoles) and TEA (800 uL, 5.7 mMoles) in 10

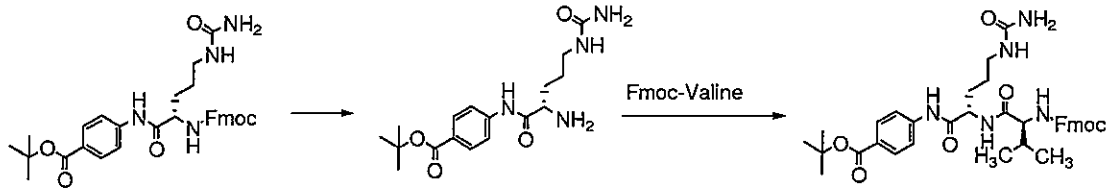
mL dichloromethane at 0° C. The reaction mixture was allowed to stir for 2 hours and the the temperature gradually rose to room temperature.. The solvents were evaporated followed by aqueous work up and extraction with ethyl acetate. The organic layer was washed with 10 % citric acid, water and saturated sodium bicarbonate and brine and dried over anhydrous sodium sulfate, giving Compound **3** (260 mg, 32 % yield) MS: $M^{+1} = 315.3$. H^1 NMR (CDCL3): 7.37 ppm (5H), 5.11ppm (2H), 3.83 ppm (2H), 3.40 ppm (4H).



4 **5** **6**

Synthesis of Compound **6**

1.5 grams of Fmoc-Citruline **5** (0.0038 Moles), 0.88 grams of t-butyl-4-amino benzoate **4** (0.0045 Moles), 0.6 g of HOBt 0.0045 Moles), 0.68 g of EDC (0.0043 Moles) and a catalytic amount of copper chloride were allowed to stir overnight in a mixture of DCM/DMF (2:1) 9mL. The solvents were removed and the product purified by silica gel flash column chromatography using 5-10 % MeOH in DCM to give Compound **6** (1.54 g, 71 % yield). MS: $M^{+1} = 573.9$.

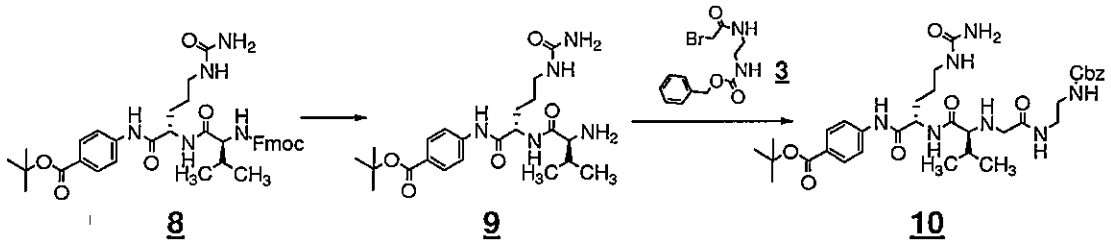


6 **7** **8**

Synthesis of Compound **8**

Deprotection of the Fmoc protecting group in Compound **6**, 300 mg, 0.66 mMoles was done using 5 % piperidine in DMF in 20 minutes. The solvents were evaporated and the crude solid rinsed with diethyl ether to give 230 mg of Compound **7** (99% yield). MS: M^{+1} = 352

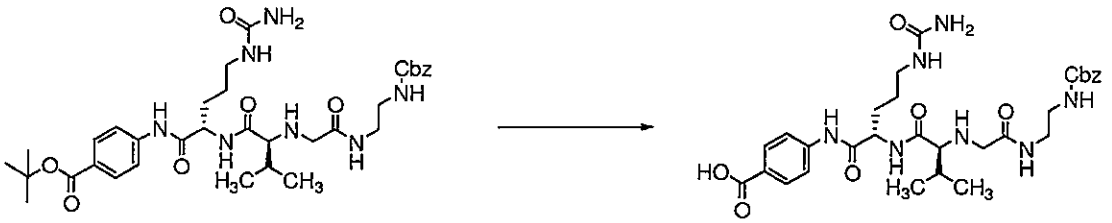
230 mg of Compound **7** (0.65 mMoles) was reacted with Fmoc-Valine 333 mg (0.98 mMoles) and 188 mg of EDC (0.98 mmoles) in DMF and DCM to give 240 mg of Compound **8** (55 % yield) after purification over silica gel 5-10 % MeOH in DCM. MS: M^{+1} = 673.



8 **9** **10**

Synthesis of Compound **10**

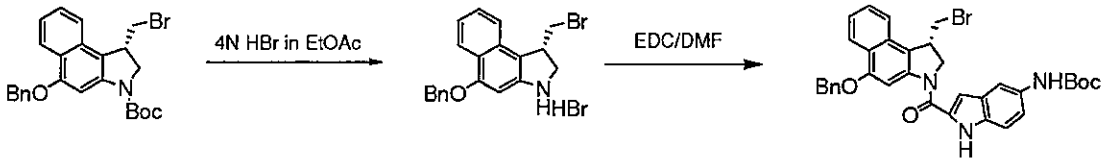
500 mg of Compound **8** (0.75 mMoles) was deprotected using piperidine in DMF to give Compound **9** which was rinsed with Diethyl ether after removing solvents. Compound **9** without further purification was reacted with 235 mg of **3** (0.75 mmoles) in the presence of 125 mg of KI (0.75 mMoles) and 313 uL triethyl amine at 40° C for 2 hours. The crude reaction mixture was concentrated and purified by reverse phase HPLC to give 300mg of Compound **10** in 55.8 % yield. MS: $M^{[+1]}$ = 685.



10 **11**

Synthesis of Compound **11**

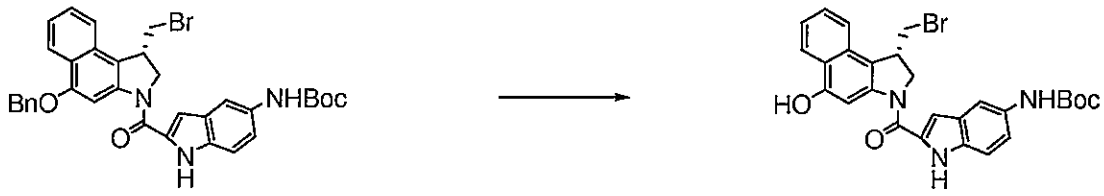
Compound **10** (300 mg) was deprotected with HCL-EA for 3 hours. The solvents were evaporated and the product dried under high vacuum to give Compound **11**. MS: $M^{[+1]}$ = 628. . H^1 NMR (DMSO): 10.45 ppm (1H), 8.8 ppm (1H), 8.5 (1H), 7.88 ppm (2H), 7.75 ppm (2H), 7.3 ppm (5H), 6.1 (1H), 5.5 (2H), 4.99 (2H), 4.5 ppm (1H), 3.7-3.4 (3H), 3.2-2.9 (5H), 2.16 (1H), 1.45 (2H). 1.1 (3H), 0.92 ppm (3H)



12 **13** **14**

Synthesis of Compound **14**

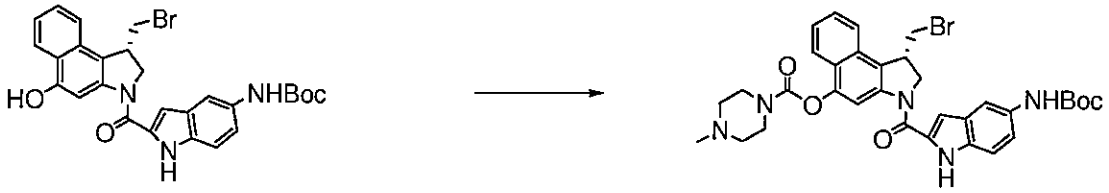
A solution of Compound **12** (42 mg, 0.09 mmol) in 4 N HBr in EtOAc (5 ml) was stirred at 25 ^{0}C for 45 min. The solvent was removed and was further dried in high vacuo for 4 h. To the residue in DMF (2mL) was added 5-(amino-*tert*-butoxycarbonyl)-indone-2-carboxylic acid (39.2 mg, 0.14 mmol) and BOP (71mg, 0.16 mmol) followed by adding DIPEA (83uL, 0.48 mmol). The reaction mixture was stirred at 25 ^{0}C for 20 min and passed through a short column of silica gel. The solvent was removed, and the crude product was chromatographed on silica gel eluted with 20% EtOAc in hexane to give Compound **14** (49 mg, 88%). ^{1}HNMR DMSO-d_6) δ11.63 (s, 1H), 9.18 (br s, 1H), 8.19 (d, 1H, J=8.4 Hz), 8.09 (br s, 1H), 7.90 (d, 1H, J=8.4 Hz), 7.79 (br s, 1H), 7.53-7.58 (m, 3H), 7.39-7.43 (m, 3H), 7.28-7.35 (m, 3H), 7.11 (s, 1H), 5.29 (s, 2H), 4.80 (t, 1H, 11.2 Hz), 4.54 (dd, 1H, 8.8 Hz), 4.31 (m, 1H), 3.92 (dd, 1H, J=10.2 Hz), 3.82 (dd, 1H, J=10.7 Hz), 1.47 (s, 9H).



14 **15**

Synthesis of compound **15**

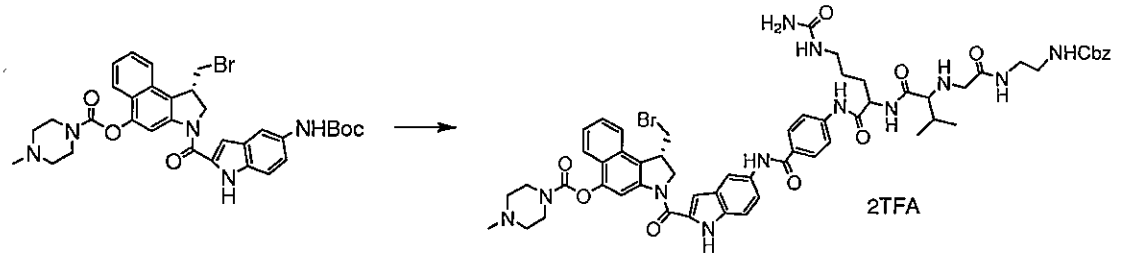
A mixture of Compound **14** (49 mg, 0.08 mmol) and 10% Pd-C (35 mg) in MeOH/CH_2Cl_2 (1/2, 10 ml) was degassed in vacuo for 40 s. The resulting mixture was placed under an atmosphere of hydrogen and stirred at 25 ^{0}C for 7 h. The reaction mixture was filtered through Celite (MeOH-CH_2Cl_2 wash). The solvent was removed in vacuo. Chromatography on silica gel eluted with 2% MeOH in DCM to afford Compound **15** (40.6 mg, 97%). ^{1}NMR DMSO-d_6) δ 11.59(s, 1H), 10.43 (s, 1H), 9.18 (br s, 1H), 8.09 (d, 1H, J=8.2 Hz), 7.93 (br s, 1H), 7.81 (d, 1H, J=8.2 Hz), 7.78 (br s, 1H), 7.49 (t, 1H, J=8.4 Hz), 7.27-7.35 (m, 3H), 7.08 (s, 1H), 4.80 (t, 1H, 11.2 Hz), 4.54 (dd, 1H, 8.8 Hz), 4.31 (m, 1H), 3.92 (dd, 1H, J=10.2 Hz), 3.82 (dd, 1H, J=10.7 Hz), 1.47 (s, 9H).



15 **16**

Synthesis of Compound **16**

A solution mixture of Compound **15** (36 mg, 0.07 mmol), 4-methyl-1-piperazinecarbonyl chloride hydrochloride (20 mg, 0.10 mmol), allyl alcohol (0.1 mL) and anhydrous pyridine (63 μml) in CH_2Cl_2 (7mL) was stirred for 16 h at room temperature. Without removing solvent, the crude product was chromatographed on silica gel, eluted with 2%-10% MeOH in CH_2Cl_2 to obtain Compound **16** (32.4 mg, 73%). ^{1}NMR DMSO-d_6) δ 11.59(s, 1H), 9.18 (br s, 1H), 8.09 (d, 1H, J=8.2 Hz), 7.93 (br s, 1H), 7.81 (d, 1H, J=8.2 Hz), 7.78 (br s, 1H), 7.49 (t, 1H, J=8.4 Hz), 7.27-7.35 (m, 3H), 7.08 (s, 1H), 4.80 (t, 1H, 11.2 Hz), 4.54 (dd, 1H, 8.8 Hz), 4.31 (m, 1H), 3.92 (dd, 1H, J=10.2 Hz), 3.82 (dd, 1H, J=10.7 Hz), 3.77 (br s, 2H), 3.47 (br s, 2H), 3.37 (br s, 2H), 2.63 (s, 3H), 2.38 (br s, 2H), 1.47 (s, 9H).

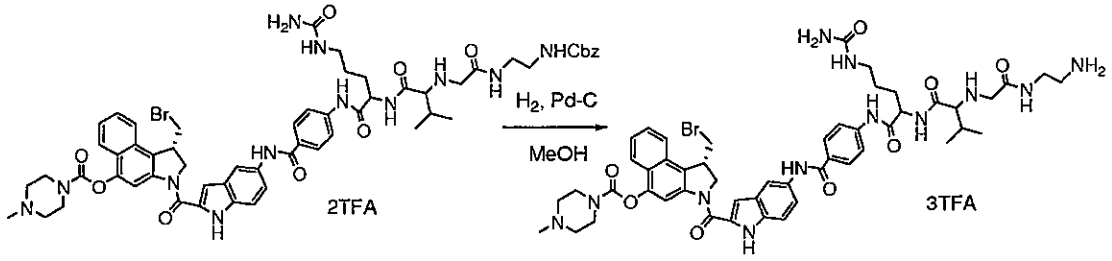


16 **17**

Synthesis of Compound **17**

A solution of Compound **16** (32 mg, 0.05 mmol) in 4 N HBr in EtOAc (4 ml) was stirred at 25 ^{0}C for 45 min. The solvent was removed and was further dried in high vacuo for 4 h. To the residue in DMF (2 mL) was added Compound **11** (48.2 mg, 0.07 mmol) and Benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphoniumhexafluorophosphate (BOP) (34.5 mg, 0.08 mmol) followed by adding DIPEA (68 uL) and the reaction mixture was stirred for 25 min at 25^{0}C. The solvent was removed under vacuo and the product was purified by Prep

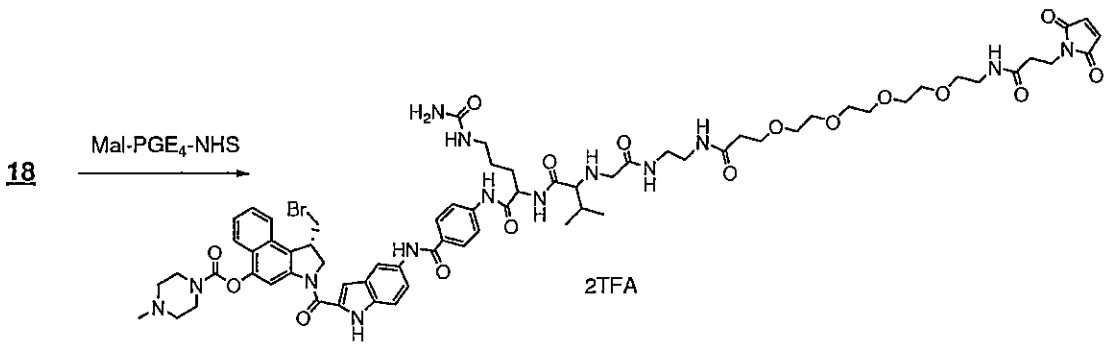
HPLC (SymmetrPrep C18, 7μm, 19 x 150 mm column), eluted at 10 ml/min (0.01% TFA in water/acetonitrile) with a gradient: 10% acetonitrile in 5 min, 10% to 50% acetonitrile in 5 min, maintaining 50% acetonitrile in 5 min, 50% to 100% acetonitrile in 5 min, to give Compound **17** (42.1 mg, 64%). MS: calcd for $C_{58}H_{67}BrN_{12}O_{10}$ (M+H) m/z 1171.43 found 1172.40.



17 **18**

Synthesis of compound **18**

To a solution of Compound **17** (33.6 mg, 0.025 mmol) in MeOH (5mL) was added 10% Pd-C (28 mg), and the mixture was degassed with N_2. The reaction mixture was flushed with H_2 and then stirred under a H_2 atmosphere. Upon complete reaction (40 min), the reaction mixture was filtered and concentrated to afford Compound **18** (31.5 mg, 96%). MS: calcd for $C_{50}H_{61}BrN_{12}O_8$ (M+H) m/z 1037.39 found 1038.20.



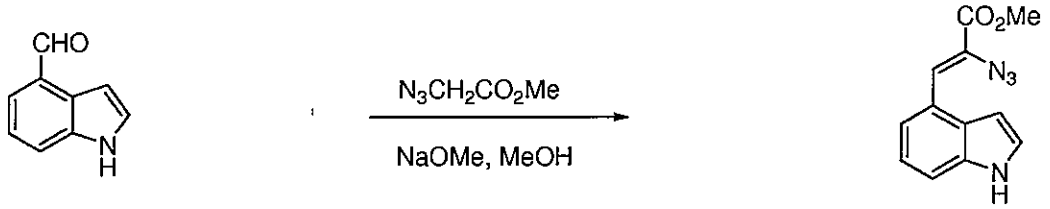
19

Synthesis of compound **19**

A solution of Compound **18** (31.5 mg, 0.024 mmol) in DMF (2mL) was added Mal-PEG4-NHS ester (42 mg, 0.08 mmol) in DCM (0.5mL). The reaction mixture was stirred for

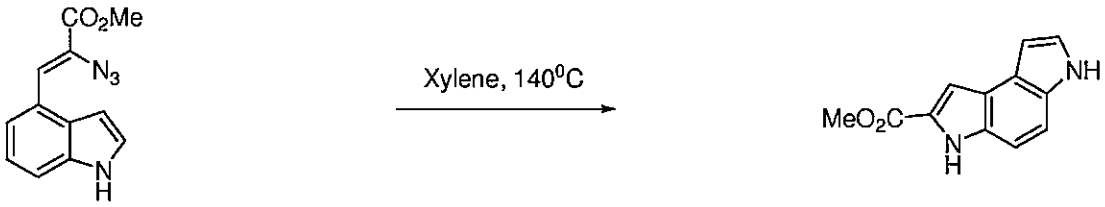
1 hr at 25°C. The final product was purified by Prep HPLC (SymmetrPrep C18, 7µm, 19 x 150 mm column), eluted at 10 ml/min (0.01% TFA in water/acetonitrile) with a gradient: 10% acetonitrile in 5 min, 10% to 50% acetonitrile in 15 min, maintaining 50% acetonitrile in 5 min, 50% to 100% acetonitrile in 5 min, to obtain Compound **19** (29.7 mg, 77%). MS: calcd for $C_{68}H_{87}BrN_{14}O_{16}$ (M+H) m/z 1435.56 found 1437.00.

EXAMPLE 5



1 **2**

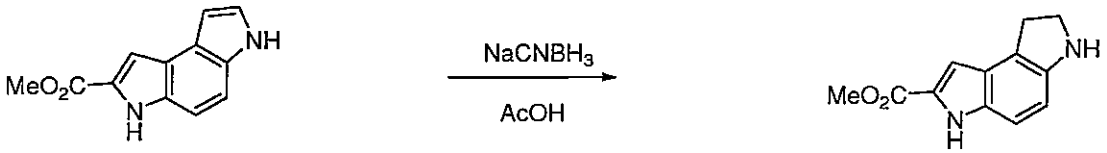
Synthesis of compound 2: To a solution of indole-4-carboxaldehyde (**1**, 583 mg, 4 mmol) and methyl azidoacetate (460 mg, 40 mmol) in dry methanol (20 mL) was added sodium methoxide in methanol dropwise (6.9 mL of 25% NaOMe, 32 mmol) at -25°C (dry ice/CCl_4) under N_2. The reaction mixture was warmed to 0°C and was stirred 3.5 h. The reaction mixture was poured into water (120 mL) and was extracted with EtOAc (2 x 60 mL). The combined extracts were washed with saturated aqueous NaCl (60 mL) and were dried ($MgSO_4$). The solvent was removed in vacuo to give **2** (890 mg, 91%). ^{1}HNMR ($CDCl_3$) δ 8.28 (br s, 1H), 8.06 (d, 1H, J=7.6 Hz), 7.45 (s, 1H), 7.42 (d, 1H, J=7.6 Hz), 7.30 (t, 1H, J=3.2 Hz), 7.26 (s, 1H), 6.73 (br s, 1H), 3.96 (s, 3H).



2 **3**

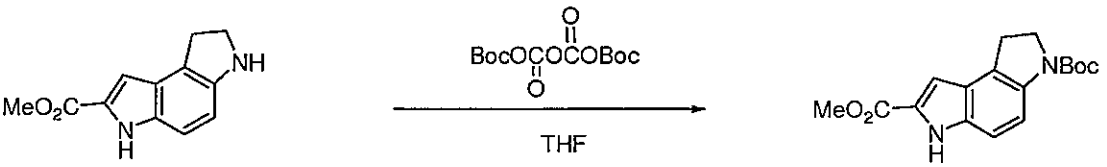
Synthesis of compound 3: A suspension of methyl 2-azido-3-(1*H*-indol-4-yl)acrylate (**2**, 890 mg, 3.68 mmol) in dry xylenes (100 mL) was refluxed under N_2 for 1h. The solvent was removed in vacuo and the residue passed through a column of silica gel (30% EtOAc-hexane)

to afford **3** (663 mg, 85%). ^{1}HNMR ($CDCl_3$) δ 8.97 (br s, 1H), 8.36 (br s, 1H), 7.47 (d, 1H, J=2 Hz), 7.40 (d, 1H, J=9.2 Hz), 7.26 (t, 1H, J=2.8 Hz), 7.22 (d, 1H, J=9.2 Hz), 6.82 (t, 1H, J=2.4 Hz), 3.95 (s, 3H).



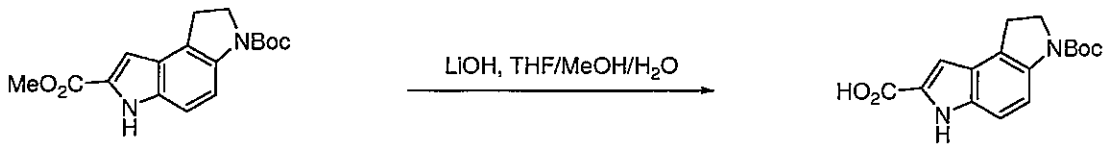
3 **4**

Synthesis of compound 4: A solution of methyl pyrrolo[3,2-*e*]indole-2-carboxylate (**3**, 663 mg, 3.1 mmol) in glacial acetic acid (9 mL) under N_2 at 15^{0}C was added sodium cyanoborohydride (600 mg, 9.5 mmol), and the reaction mixture was stirred for 2.5 h (13-18^{0}C). The reaction mixture was poured into water (60 mL) and was made pH 8-9 by the careful addition of solid sodium carbonate. The aqueous mixture was extracted with EtOAc (3 x 60 mL), and the combined extracts were dried ($MgSO_4$). The solvent was removed in vacuo. Flash chromatography (silica gel, 20% EtOAc-hexane) afforded **4** (562 mg, 84%). ^{1}HNMR ($CDCl_3$) δ 8.85 (br s, 1H), 7.15 (d, 1H, J=8.4 Hz), 7.04 (s, 1H), 6.89 (d, 1H, J=8.4 Hz), 3.94 (s, 3H), 3.68 (t, 2H, J=8.4 Hz), 3.24 (t, 2H, J=8.4 Hz).



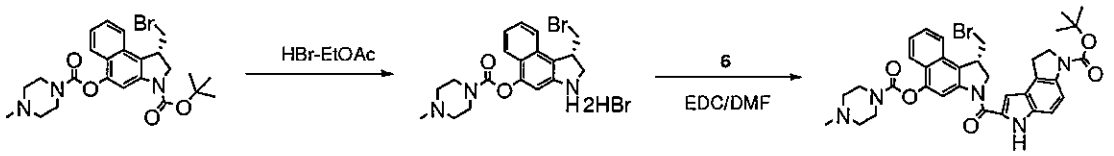
4 **5**

Synthesis of compound 5: A solution of methyl 1,2-dihydro-3*H*-pyrrolo[3,2-*e*]indole-7-carboxylate (**4**, 450 mg, 1.42 mmol) in dry THF (8 mL) was treated with di-*tert*-butyl dicarbonate (621 g, 2.8 mmol) at 25^{0}C under N_2. The reaction mixture was stirred 16 h, then the solvent was removed in vacuo. Flash chromatography (silica gel, 30% EtOAc-hexane) gave **5** (551 mg, 84%).^{1}HNMR ($CDCl_3$) δ 8.78 (br s, 1H), 7.26 (s, 1H), 7.25 (d, 1H, J=8.5 Hz), 7.07 (s, 1H), 4.12 (br s, 2H), 3.94 (s, 3H), 3.27 (t, 2H, J=8.8 Hz), 1.57 (s, 9H).



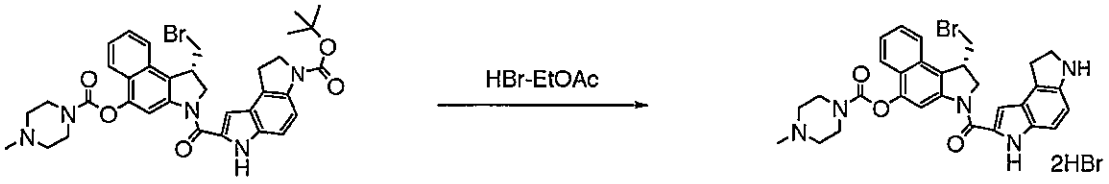
5 6

Synthesis of compound 6: An aqueous solution of LiOH (2.2 mL of 4.0 M solution, 8.7 mmol) was added to a slurry of methyl 3-(*tert*-Butyloxycarbonyl)-1,2-dihydro-3*H*-pyrrolo[3,2-*e*]indole-7-carboxylate (**5**, 551 mg, 1.74 mmol) in 60 mL of THF/MeOH/H_2O (3:2:1), and the reaction mixture was stirred at 25^{0}C for 14 h. The reaction mixture was diluted with water (30mL) and was then adjusted pH to 4 with 2N HCl, producing a white precipitate. The solid was collected by filtering and was washed with water (10 mL). Drying the solid in high vacuo afforded **6** (524 mg, 99%). ^{1}HNMR (DMSO-d_6) δ 12.93 (br s, 1H), 11.69 (s, 1H), 7.80 (br s, 1H), 7.20 (d, 1H, J=8.8 Hz), 6.91 (s, 1H), 3.96 (t, 2H, J=8.8 Hz), 3.18 (t, 2H, J=8.8 Hz), 1.48 (s, 9H).



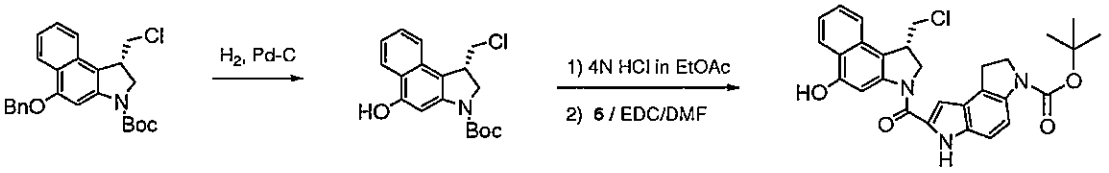
7 8 9

Synthesis of compound 9: A solution of 7 (48 mg, 0.1 mmol) in 4 N HBr in EtOAc (5 ml) was stirred at 25 ^{0}C under nitrogen for 45 min. The solvent was removed and was further dried in high vacuo for 4 h. To the residue was added 3-(tert-butyloxycarbonyl)-1,2-dihydro-3*H*-pyrrolo[3,2-*e*]indole-7-carboxylic acid (**6**, 36.24 mg, 0.12 mmol). A solution of EDC (22.9 mg, 0.12 mmol) in DMF (3 ml) was added and the reaction mixture was stirred at 25 ^{0}C for 6 h. The solvent was removed. The crude product was chromatographed on silica gel eluted with 3-10% MeOH in CH_2Cl_2 to give **9** (26 mg, 40%). ^{1}HNMR (DMSO-d_6) δ11.69 (s, 1H), 8.17 (s, 1H), 8.01 (d, 1H, J=8.4 Hz), 7.81 (d, 2H, J=8.8 Hz), 7.59 (t, 1H, J=7.6 Hz), 7.49 (t, 1H, J=7.6 Hz), 7.29 (d, 1H, J=9.2 Hz), 7.07 (s, 1H), 4.87 (t, 1H, 10 Hz), 4.54 (d, 1H, 8.8 Hz), 4.43 (br s, 1H), 3.89-4.03 (m, 4H), 3.76 (br s, 2H), 3.46 (br s, 2H). 3.26 (m, 2H), 2.46 (br s, 2H), 2.38 (br s, 2H), 2.24 (s, 3H), 1.49 (s, 9H).



9 **10**

Synthesis of compound 10: A solution of **9** (26 mg, 0.04 mmol) in 4 N HBr in EtOAc (5 ml) was stirred at 25 ^{0}C under nitrogen for 45 min. The solvent was removed and was further dried in vacuo for 14 h to afford **10** (27.7 mg, 98%). ^{1}HNMR (DMSO-d_6) δ12.09 (s, 1H), 8.25 (s, 1H), 8.03 (d, 1H, J=8.4 Hz), 7.92 (d, 1H, J=8.8 Hz), 7.63 (t, 1H, J=7.6 Hz), 7.49-7.55 (m, 2H), 7.34 (d, 1H, J=8.8 Hz), 7.32 (s, 1H), 4.91 (t, 1H, 10.8 Hz), 4.54 (d, 1H, 10.8 Hz), 4.47 (br s, 1H), 3.84-3.99 (m, 4H), 3.27- 3.50 (m, 10H), 2.88 (s, 3H).

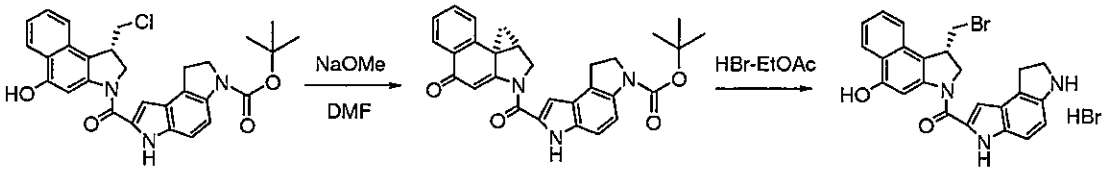


11 **12** **13**

Synthesis of compound 12: A solution of **11** (20 mg, 0.05 mmol) and 10% Pd-C (15 mg) in MeOH/CH_2Cl_2 (1/2, 10 ml) was degassed in vacuo for 40 s. The resulting mixture was placed under an atmosphere of hydrogen and stirred at 25 ^{0}C for 7 h. The reaction mixture was filtered through Celite (CH_2Cl_2 wash). The solvent was removed in vacuo. Chromatography on silica gel eluted with EtOAc/Hex (2/8) afforded **12** (15.4 mg, 98%). ^{1}NMR (DMSO-d_6) δ 10.36 (s, 1H), 8.04 (d, 1H, J=8.2 Hz), 7.72 (d, 1H, J=8.2 Hz), 7.61 (br s, 1H), 7.45 (t, 1H, J=8.4 Hz), 7.261 (t, 1H, J=8.4 Hz), 4.06 (m, 4H), 3.73 (br s, 1H), 1.52 (s, 9H).

Synthesis of compound 13: A solution of **12** (14 mg, 0.04 mmol) in 4 N HCl in EtOAc (3 ml) was stirred at 25 ^{0}C under nitrogen for 30-45 min. The solvent was removed and was further dried in high vacuo for 14 h. To the residue was added 3-(tert-butyloxycarbonyl)-1,2-dihydro-3*H*-pyrrolo[3,2-*e*]indole-7-carboxylic acid (**6**, 15.1 mg, 0.05 mmol). A solution of EDC (9.6 mg, 0.05 mmol) in DMF (2 ml) was added and the reaction mixture was stirred at

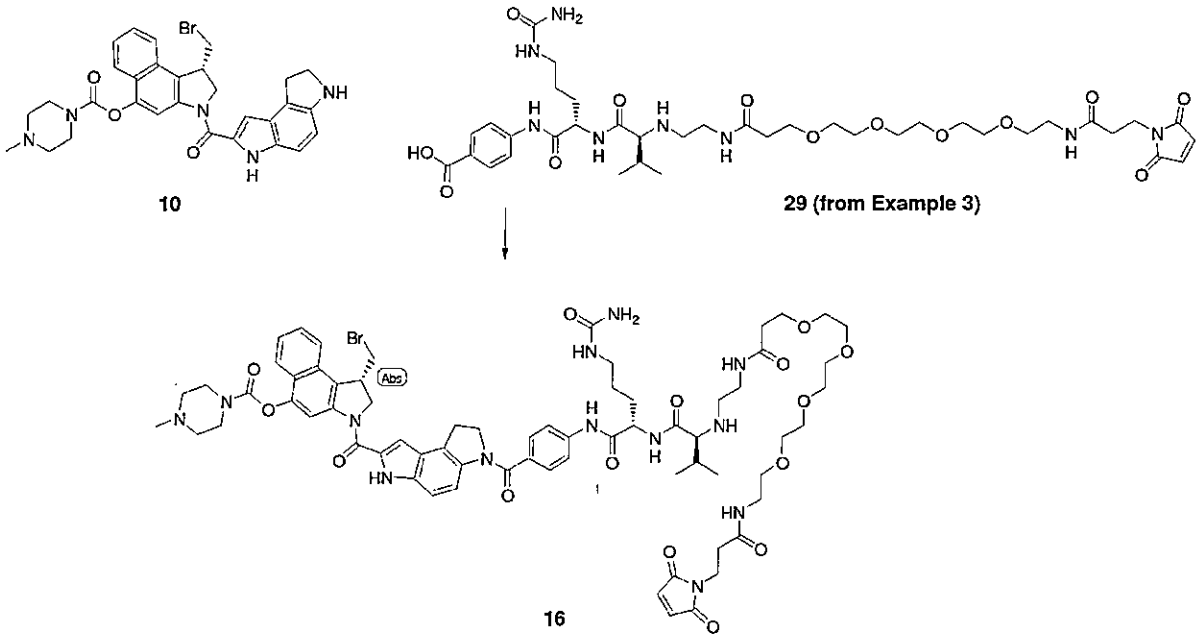
25 ^{0}C for 15 h. The solvent was removed. The crude product was chromatographed on silica gel eluted with 3-10% MeOH in CH_2Cl_2 to give **13** (18.6 mg, 86%). ^{1}HNMR ($CDCl_3$) δ 9.45 (s, 1H), 8.31 (s, 1H), 8.29 (d, 1H, J=8.4 Hz), 8.02 (s, 2H), 7.63 (d, 1H, J=8.4 Hz), 7.54 (t, 1H, J=6.8 Hz), 7.43 (t, 1H, J=6.8 Hz), 7.29 (d, 1H, J=8.0 Hz), 6.89 (s, 1H), 4.75 (d, 1H, 10.8 Hz), 4.64 (t, 1H, 8.8 Hz), 4.12 (br s, 2H), 4.03 (t, 1H, J=8.8 Hz), 3.93 (dd, 1H, J=11.2 Hz), 3.42 (t, 1H, J=10.8 Hz), 3.22-3.31 (m, 2H), 1.61 (s, 9H).



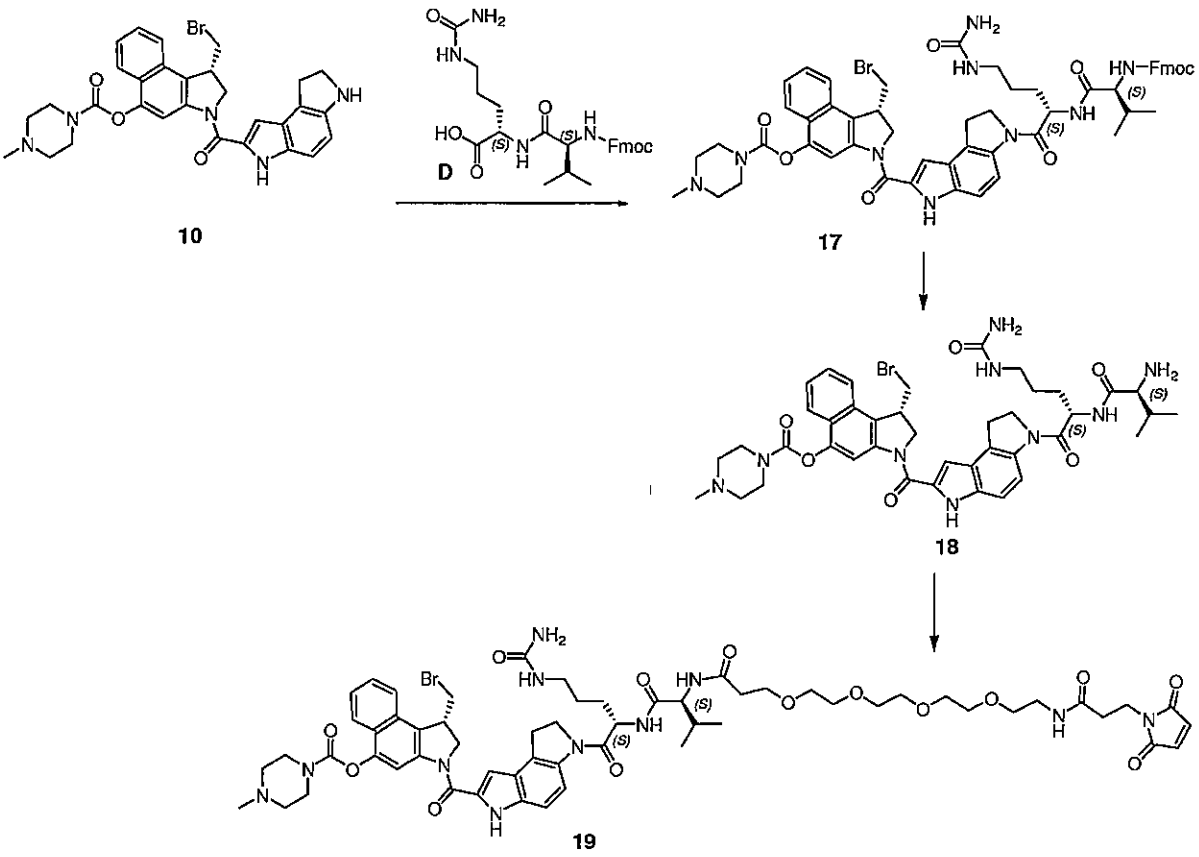
13 **14** **15**

Synthesis of compound 14: To a solution of **13** (19 mg, 0.04 mmol) in DMF (3 ml) was added sodium methoxide (0.5 M in MeOH, 79 uL, 0.04 mmol) at 0-5 ^{0}C and was stirred for 5 min. To the reaction mixture was added water (20 mL), and the aqueous mixture was extracted with EtOAc (2 x 10 mL). The combined extracts were dried ($MgSO_4$). The solvent was removed in vacuo, and the crude product was chromatographed on silica gel eluted with 10% MeOH in CH_2Cl_2 to give **14** (15.3 mg, 87%). ^{1}NMR ($CDCl_3$) δ 9.24 (br s, 1H), 8.25 (dd, 1H, J=9.2, 1.6 Hz), 8.02 (s, 1H), 7.54 (tt, 1H, J=7.6, 1.6 Hz), 7.43 (tt, 1H, J=7.6, 1.6 Hz), 7.28 (d, 1H, J=9.2 Hz), 7.20 (s, 1H), 6.94 (d, 1H, J=7.6 Hz), 6.88 (s, 1H), 4.49 (m, 2H), 4.12 (br s, 2H), 3.26 (m, 2H), 2.92 (m, 1H), 1.76 (dd, 2H, J=7.6 Hz), 1.61 (s, 9H).

Synthesis of compound 15: A solution of **14** (6.4 mg, 0.013 mmol) in 4 N HBr in EtOAc (3 ml) was stirred at 25 ^{0}C under nitrogen for 30 min. The solvent was removed in vacuo and was further dried in high vacuo for 14 h to give **15** (7.1 mg, 99%). ^{1}HNMR (DMSO-d_6) δ12.16 (s, 1H), 10.45 (s, 1H), 8.11 (d, 1H, J=8.4 Hz), 7.94 (br s, 1H), 7.83 (d, 1H, J=8.0 Hz), 7.51 (m, 2H), 7.34 (m, 2H), 7.27 (s, 1H), 4.81 (t, 1H, 10.8 Hz), 4.48 (d, 1H, 10.8 Hz), 4.28 (br s, 1H), 3.77-3.91 (m, 4H), 3.41- 3.48 (m, 2H).

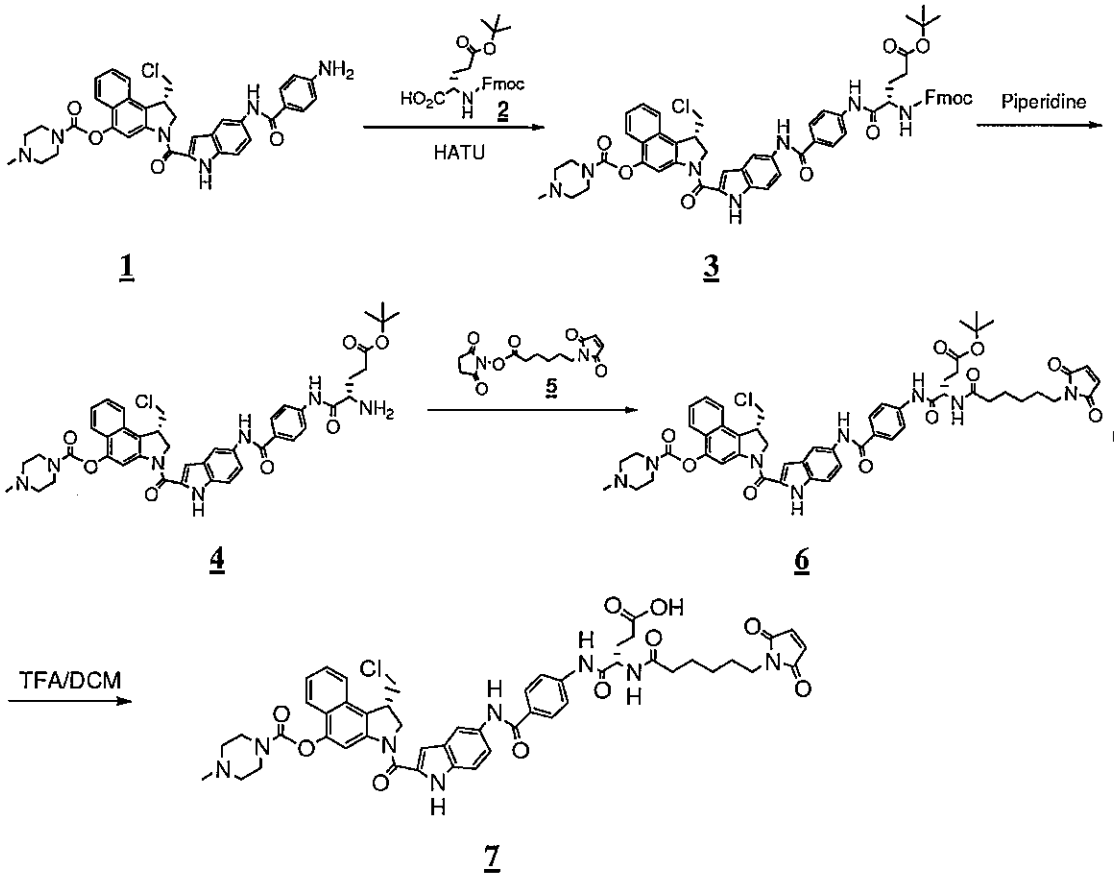


Synthesis of compound 16: Compound **10** (0.033 mMoles, 2HBr salt) was reacted with Compound **29** of Example 3 (45 mg, 0.054 mMoles) in 2.5 mL DMF in the presence of HATU (20 mg, 0.054 mMoles) and TEA (15-20 µL) for 50 minutes. The solvents were evaporated and the Crude was purified by Reverse phase HPLC to give 10 mg of Compound **16** (21 % yield). MS: 1405.6, 1427.8 and 1444.6.



Synthesis of compound 19: Compound **10** (25mg, 0.033 mMoles, 2HBr Salt)) was reacted with compound **D** of Example 5 (21.5 mg, 0.043 mMoles) in 2.5 mL DMF in the presence of HATU (16.5 mg, 0.0433 mMoles) and TEA (10-15 μL) for 45 minutes. The solvent was evaporated and crude purified by reverse phase HPLC to give 25 mg of Compound **17**. (71 % yield). MS: 1067.0. Compound **17** (0.0187 mMoles) was deprotected with 5 % piperidine in DMF (3 mL) in 45 minutes. The solvent was evaporated and the residue washed with diethyl ether to give **18** (MS: 845.2). To a Solution of 0.00935 mMoles of **18** in 3 mL DMF was added Mal-dPEG4-NHS ester (10 mg, 0.019 mMoles) in DCM 1 mL, followed by TEA (5 μL). After 30 minutes the solvents were evaporated and the crude purified by Reverse Phase HPLC to give 5.2 mg of pure Compound **19** (MS: 1243.2, 1266.8 and 1281.2)

EXAMPLE 6



1 3 4 6 7

Synthesis of compound 3: To compound **1** (18.2 mg, 0.03 mmol) in DMF (2 mL) was added **2** (16 mg, 0.036 mmol) and HATU (14 mg, 0.036 mmol) followed by adding DIPEA (19 uL) and the reaction mixture was stirred for 7 hrs at 25^{0}C. The solvent was removed under vacuo and the product was purified by Prep HPLC (SymmetrPrep C18, 7μm, 19 x 150 mm column), eluted at 10 ml/min (0.01% TFA in water/ acetonitrile) with a gradient: 10% acetonitrile in 5 min, 10% to 50% acetonitrile in 15 min, maintaining 50% acetonitrile in 5 min, 50% to 100% acetonitrile in 5 min, to give **3** (15.8 mg, 53%). MS: calcd for $C_{59}H_{58}ClN_7O_9$ (M+H) m/z 1044.4 found 1045.

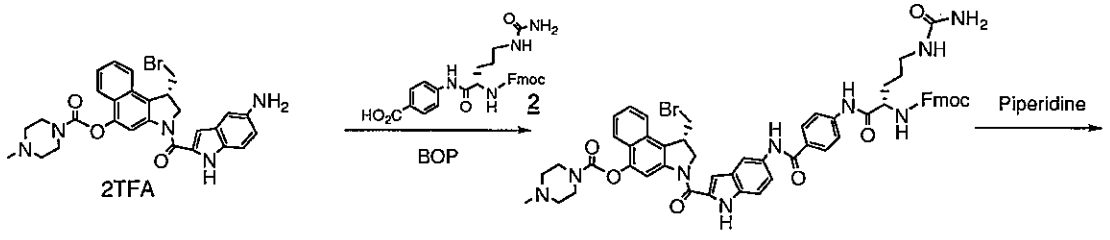
Synthesis of compound 4: A solution of **3** (15 mg, 0.014 mmol) in DMF (2mL) was treated with 5% piperidine in DMF (2 mL). The reaction mixture was stirred for 5min at 25^{0}C. The final product was purified by Prep HPLC (SymmetrPrep C18, 7μm, 19 x 150 mm column), eluted at 10 ml/min (0.01% TFA in water/acetonitrile) with a gradient: 10% acetonitrile in 5 min, 10% to 50% acetonitrile in 15 min, maintaining 50% acetonitrile in 5 min, 50% to 100%

acetonitrile in 5 min, to obtain **4** (11.2 mg, 95%). MS: calcd for $C_{44}H_{48}ClN_7O_7$ (M+H) m/z 822.33 found 822.6.

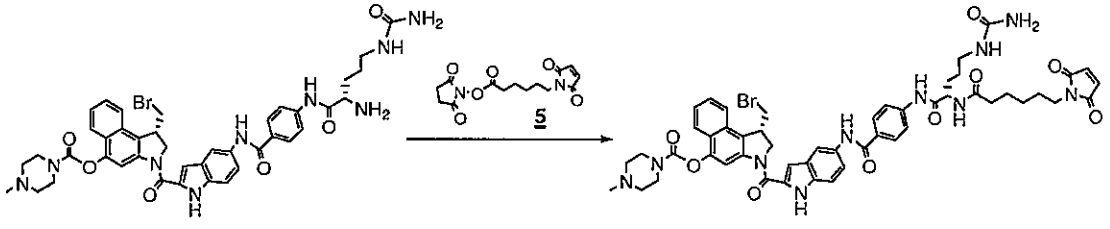
Synthesis of compound 6: A solution of **4** (11.2 mg, 0.014 mmol) in DMF (2mL) was added to **5** (5.5 mg, 0.018 mmol) in DCM (0.5mL). The reaction mixture was stirred for 1 hr at 25^{0}C. The final product was purified by Prep HPLC (SymmetrPrep C18, 7μm, 19 x 150 mm column), eluted at 10 ml/min (0.01% TFA in water/acetonitrile) with a gradient: 10% acetonitrile in 5 min, 10% to 50% acetonitrile in 15 min, maintaining 50% acetonitrile in 5 min, 50% to 100% acetonitrile in 5 min, to obtain **19** (13 mg, 94%). MS: calcd for $C_{54}H_{59}ClN_8O_{10}$ (M+H) m/z 1015.4 found 1016.0.

Synthesis of compound 7: A solution of **6** (13 mg, 0.013 mmol) in TFA/DCM (1/1, 2mL) was stired for 15 min. The solvent was removed under vacuo and the product was purified by Prep HPLC (SymmetrPrep C18, 7μm, 19 x 150 mm column), eluted at 10 ml/min (0.01% TFA in water/ acetonitrile) with a gradient: 10% acetonitrile in 5 min, 10% to 50% acetonitrile in 15 min, maintaining 50% acetonitrile in 5 min, 50% to 100% acetonitrile in 5 min, to give **7** (12 mg, 97%). MS: calcd for $C_{50}H_{51}ClN_8O_{10}$ (M+H) m/z 959.34 found 960.

EXAMPLE 7



1 **3**



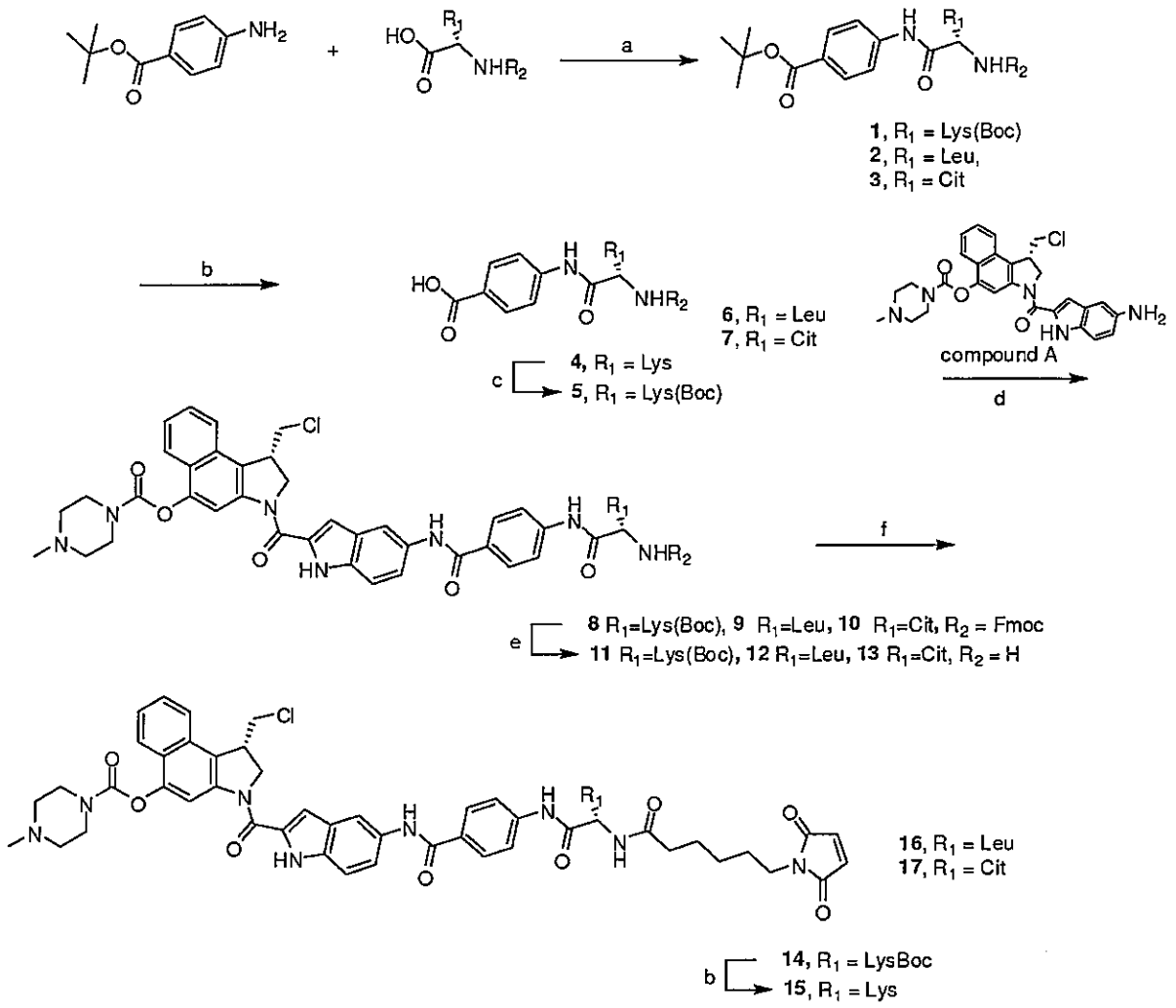
4 **6**

Synthesis of compound 3: A solution of **1** (47 mg, 0.06 mmol), **2** (40mg, 0.078 mmol) and BOP (34.5 mg, 0.078 mmol) in DMF (3 ml) was treated with DIPEA (68 uL). The reaction mixture was stirred at 25 ^{0}C for 30 min. The solvent was removed and was further dried in high vacuo for 4 h. The solvent was removed under vacuo and the crude product was purified by Prep HPLC (SymmetrPrep C18, 7µm, 19 x 150 mm column), eluted at 10 ml/min (0.01% TFA in water/acetonitrile) with a gradient: 10% acetonitrile in 5 min, 10% to 50% acetonitrile in 15 min, maintaining 50% acetonitrile in 5 min, 50% to 100% acetonitrile in 5 min, to give **3** (56 mg, 85%). MS: calcd for $C_{56}H_{54}BrN_9O_8$ (M+H) m/z 1060.33 found 1060.44.

Synthesis of compound 4: A solution of **3** (22 mg, 0.02 mmol) in DMF (2mL) was treated with 5% piperidine in DMF (2 mL). The reaction mixture was stirred for 5min at 25^{0}C. The final product was purified by Prep HPLC (SymmetrPrep C18, 7µm, 19 x 150 mm column), eluted at 10 ml/min (0.01% TFA in water/acetonitrile) with a gradient: 10% acetonitrile in 5 min, 10% to 50% acetonitrile in 15 min, maintaining 50% acetonitrile in 5 min, 50% to 100% acetonitrile in 5 min, to obtain **4** (16.2 mg, 93%). MS: calcd for $C_{41}H_{44}BrN_9O_6$ (M+H) m/z 838.26 found 839.2.

Synthesis of compound 6: A solution of **4** (16 mg, 0.015 mmol) and **5** (6.2 mg, 0.02 mmol) in DMF (2mL) was treated with DIPEA (10 uL). The reaction mixture was stirred for 1 hr at 25^{0}C. The final product was purified by Prep HPLC (SymmetrPrep C18, 7µm, 19 x 150 mm column), eluted at 10 ml/min (0.01% TFA in water/acetonitrile) with a gradient: 10% acetonitrile in 5 min, 10% to 50% acetonitrile in 15 min, maintaining 50% acetonitrile in 5 min, 50% to 100% acetonitrile in 5 min, to obtain **6** (13 mg, 75%). MS: calcd for $C_{51}H_{55}BrN_{10}O_9$ (M+H) m/z 1031.33 found 1031.6.

EXAMPLE 8



a EDC, HOBt, $CuCl_2$, 5% DMF in CH_2Cl_2 40-60% **b** TFA, CH_2Cl_2 98% **c** Boc_2O, DMF 70% **d** HATU, compound **A**, DMF, DIEA 57-66% **e** piperidine, DMF 98% **f** 20% DMF in CH_2Cl_2, DIEA, N-succinimidyl-6-maleimidohexanoate 60-79%

Synthesis of compound 1: To a solution of Fmoc-Lys(Boc)-OH (500 mg, 1.07 mmol) in solution of 5% DMF in THF (21 mL) were added EDC (245 mg, 1.28 mmol), HOBt (173 mg, 1.28 mmol) and tert-butyl-4-amino benzoate (247 mg, 1.28 mmol) at room temperature. The mixture thus obtained was stirred for 10 minutes and then copper chloride (172 mg, 1.28 mmol) was added to the mixture. The mixture was stirred overnight. The mixture was concentrated to dryness and then the residue was purified by flash chromatography on silica and eluted with 5% methanol in dichloromethane to give compound **1** as a colorless oil (286 mg, 42%). ^{1}H NMR (CD_3OD) δ 1.38 (s, 9H), 1.45-1.54 (m, 4H), 1.58 (s, 9H), 1.80 (m, 2H),

3.05 (t, 2H), 4.22 (m, 2H), 4.38 (d, 2H), 7.30 (m, 2H), 7.38 (m, 2H), 7.68 (m, 4H), 7.78 (d, 2H), 7.90 (d, 2H); LC-MS (ES^+), 544 $(M+H\text{-}Boc)^+$, 667 $(M+Na)^+$.

Synthesis of compound 2: See the preparation of **1** for general EDC coupling procedure. Coupling of Fmoc-Leu-OH (500 mg, 1.42 mmol) with tert-Butyl-4-amino benzoate (328 mg, 1.70 mmol) gave 335 mg of **2** (44%). ^1H NMR (CD_3OD) δ 0.95 (t, 6H), 1.58 (s, 9H), 1.55-1.80 (m, 3H), 4.22 (m, 1H), 4.28 (m, 1H), 4.40 (m, 2H), 7.35 (m, 4H), 7.65 (m, 4H), 7.78 (d, 2H), 7.90 (d, 2H).

Synthesis of compound 3: See the preparation of **1** for general EDC coupling procedure. Coupling of Fmoc-Cit-OH (206 mg, 0.52 mmol) with tert-Butyl-4-amino benzoate (120 mg, 0.62 mmol) gave 184 mg of **3** (62%). ^1H NMR (CD_3OD) δ 1.53-1.58 (m, 2H), 1.57 (s, 9H), 1.71 (m, 1H), 1.82 (m, 1H), 3.08 (m, 1H), 3.19 (m, 1H), 4.21 (m, 1H), 4.28 (m, 1H), 4.38 (m, 2H), 7.28-7.39 (m, 3H), 7.49 (m, 2H), 7.56-7.86 (m, 5H), 7.89 (m, 2H); LC-MS (ES^+), 573 $(M+H)^+$, 595 $(M+Na)^+$, 611 $(M+K)^+$.

Synthesis of compound 4: To a solution of **1** (280 mg, 0.44 mmol) in dichloromethane (4 mL) was added TFA (2 mL). The resulting solution was stirred for 20 minutes. The solvent was evaporated *in vacuo*. The residue (260 mg, 98%) was used without further purification for the next step. ^1H NMR (CD_3OD) δ 1.45-1.80 (m, 5H), 1.88 (m, 1H), 2.09 (t, 2H), 4.25 (m, 2H), 4.45 (m, 2H), 7.30 (m, 2H), 7.38 (m, 2H), 7.68 (m, 4H), 7.80 (d, 2H), 7.98 (d, 2H).

Synthesis of compound 5: To a solution of **4** (214 mg, 0.44 mmol) in DMF (5 mL) were added diisopropylethylamine (153 μL, 0.88 mmol), Boc_2O (144 mg, 0.66mmol) at room temperature. The mixture was stirred overnight. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give compound **5** as a white solid (181 mg, 70%). ^1H NMR (CD_3OD) δ 1.40 (s, 9H), 1.45-1.50 (m, 4H), 1.80 (m, 2H), 3.03 (t, 2H), 4.21 (m, 2H), 4.40 (d, 2H), 7.35 (m, 4H), 7.65 (m, 4H), 7.68 (d, 2H), 7.95 (d, 2H); LC-MS (ES^+) 489 $(M+H\text{-}Boc)^+$, 610 $(M+Na)^+$.

Synthesis of compound 6: See the preparation of **4** for *tert*-butyl ester deprotection procedure. Deprotection of **2** (15 mg, 0.028 mmol) gave 13 mg of **6** (98%). The residue was used without further purification for the next step. LC-MS (ES^+) 495 $(M+H)^+$.

Synthesis of compound 7: See the preparation of **4** for *tert*-butyl ester deprotection procedure. Deprotection of **3** (20 mg, 0.035 mmol) gave 17 mg of **7** (98%). The residue was used without further purification for the next step.

Synthesis of compound 8: To a solution of compound **A** (compound A was synthesized in a similar way as described for compound 16 in example 4) (24 mg, 0.032 mmol) in DMF (1 mL) were added **5** (19 mg, 0.032 mmol), diisopropylethylamine (28 μL, 0.16 mmol) and HATU (12 mg, 0.032 mmol) at room temperature. The mixture thus obtained was stirred for 2 hours. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give the title compound as a white solid (25 mg, 66%). ^{1}H NMR (CD_3OD) δ 1.40 (s, 9H), 1.45-1.50 (m, 4H), 1.80 (m, 2H), 2.93 (s, 3H), 3.03 (t, 2H), 3.10-3.60 (m, 8H), 3.85 (d, 1H), 4.05 (m, 1H), 4.21 (m, 2H), 4.40 (m, 4H), 4.52 (m, 1H), 6.95 (s, 1H), 7.27-7.50 (m, 8H), 7.63-7.77 (m, 7H), 7.84 (m, 3H), 7.99 (s, 1H), 8.25 (bs, 1H); LC-MS (ES^+) 1088 $(M+H)^+$.

Synthesis of compound 9: See the preparation of **8** for HATU coupling procedure. Coupling of **6** (13 mg, 0.027 mmol) with **25** (20 mg, 0.027mmol) gave 17 mg of **9** (57%).^{1}H NMR (CD_3OD) δ 0.86 (m, 6H), 1.45-1.70 (m, 3H), 2.78 (s, 3H), , 3.05-3.50 (m, 8H), 3.70 (d, 1H), 3.80 (m, 1H), 4.07 (m, 1H), 4.20-4.40 (m, 6H), 6.76 (s, 1H), 7.13-7.30 (m, 8H), 7.51-7.72 (m, 10H), 7.86 (s, 1H), 8.15 (bs, 1H); LC-MS (ES^+) 973 $(M+H)^+$.

Synthesis of compound 10: See the preparation of **8** for HATU coupling procedure. Coupling of **7** (27 mg, 0.053 mmol) with **25** (39 mg, 0.053 mmol) gave 36 mg of **10** (60%).

Synthesis of compound 11: To a solution of **8** (25 mg, 0.02 mmol) in DMF (1 mL) was added piperidine (10 μL, 0.1 mmol). The resulting solution was stirred 1 hour. The solvent was evaporated *in vacuo*. The residue was crashed out in ether and filtered (18 mg, 98%). The compound was used without further purification. LC-MS (ES^+) 866 $(M+H)^+$, 888 $(M+Na)^+$.

Synthesis of compound 12: See the preparation of **11** for general Fmoc deprotection procedure. Deprotection of **9** (17 mg, 0.016 mmol) gave 14 mg of **12** (98%). The residue was used without further purification for the next step.

Synthesis of compound 13: See the preparation of **11** for general Fmoc deprotection procedure. Deprotection of **10** (36 mg, 0.036 mmol) gave 28 mg of **13** (98%). The residue was used without further purification for the next step.

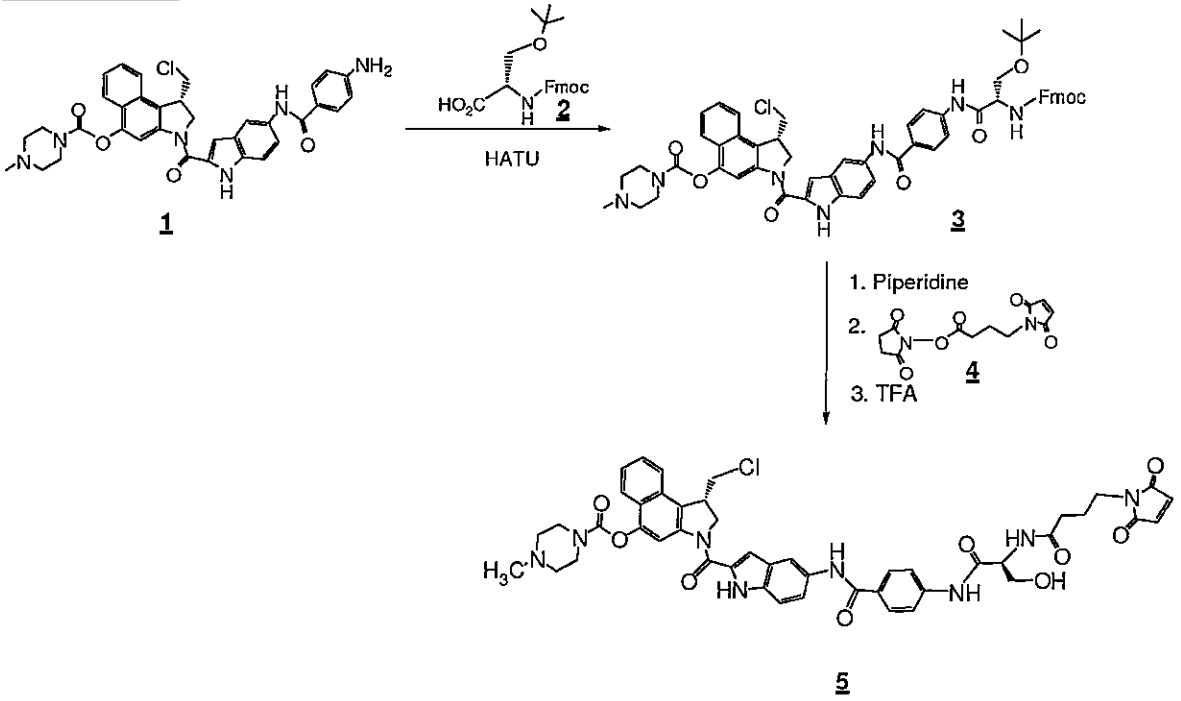
Synthesis of compound 14: To a solution of **11** (18 mg, 0.021 mmol) in 20% DMF in dichloromethane (1 mL) were added diisopropylethylamine (11 μL, 0.63 mmol) and N-succinimidyl-6-maleimidohexanoate (10 mg, 0.031 mmol) at room temperature. The mixture thus obtained was stirred for 2 hours. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give compound **14** as a white solid (19 mg, 79%). ^{1}H NMR (CD_3OD) δ 1.32 (m, 2H), 1.42 (s, 9H), 1.45-1.80 (m, 8H), 1.78 (m, 1H), 1.85 (m, 1H), 2.28 (t, 2H), 2.96 (s, 3H), 3.05 (t, 3H), 3.35-3.70 (bs, 8H), 3.47 (t, 2H), 3.60 (m, 1H), 3.90 (m, 1H), 4.05 (m, 1H), 4.44 (m, 2H), 4.55 (m, 1H), 6.76 (s, 2H), 6.96 (s, 1H), 7.31 (bs, 2H), 7.42 (m, 1H), 7.46 (m, 1H), 7.71 (m, 3H), 7.86 (m, 3H), 7.99 (s, 1H), 8.25 (bs, 1H); LC-MS (ES^+) 1059 $(M+H)^+$, 1082 $(M+Na)^+$.

Synthesis of compound 15: To a solution of **14** (19 mg, 0.08 mmol) in dichloromethane (0.8 mL) was added TFA (0.2 mL). The resulting solution was stirred for 20 minutes. The solvent was evaporated and lyophilized to give compound **15** as a white solid (17 mg, 90%). ^{1}H NMR (CD_3OD) δ 1.34 (m, 2H), 1.45-1.80 (m, 9H), 1.95 (m, 1H), 2.29 (t, 2H), 2.96 (t, 3H), 3.00 (s, 3H), 3.35-3.70 (bs, 8H), 3.48 (t, 2H), 3.75 (m, 1H), 3.98 (dd, 1H), 4.20 (m, 1H), 4.50 (m, 1H), 4.69 (m, 2H), 6.78 (s, 2H), 7.12 (s, 1H), 7.42 (bs, 2H), 7.48 (m, 1H), 7.57 (m, 1H), 7.73 (m, 2H), 7.87-7.95 (m, 4H), 8.05 (s, 1H), 8.25 (bs, 1H); LC-MS (ES^+) 959 $(M+H)^+$, 982 $(M+Na)^+$.

Synthesis of compound 16: See the preparation of **14** for N-succinimidyl-6-maleimidohexanoate coupling procedure. Coupling of **12** (11 mg, 0.015 mmol) with N-succinimidyl-6-maleimidohexanoate (7 mg, 0.022 mmol) gave 10 mg of **16** (66%).^{1}H NMR (CD_3OD) δ 0.97 (d, 3H), 1.01 (d, 3H), 1.31 (m, 2H), 1.56-1.75 (m, 7H), 2.28 (t, 2H), 2.97 (s, 3H), 3.35-3.55 (m, 8H), 3.47 (t, 2H), 3.62 (m, 1H), 3.90 (m, 1H), 4.06 (m, 1H), 4.51 (m, 1H), 4.55 (m, 2H), 6.76 (s, 2H), 6.99 (s, 1H), 7.33 (bs, 2H), 7.42 (m, 1H), 7.49 (m, 1H), 7.69 (d, 2H), 7.75 (m, 1H), 7.86 (m, 3H), 8.00 (s, 1H), 8.25 (bs, 1H); LC-MS (ES^+) 944 $(M+H)^+$, 966 $(M+Na)^+$.

Synthesis of compound 17: See the preparation of **14** for N-succinimidyl-6-maleimidohexanoate coupling procedure. Coupling of **13** (28 mg, 0.035 mmol) with N-succinimidyl-6-maleimidohexanoate (16 mg, 0.053 mmol) gave 24 mg of **17** (60%).^{1}H NMR (CD_3OD) δ 1.34 (m, 2H), 1.55-1.68 (m, 6H), 1.68-190 (m, 2H), 2.29 (t, 2H), 3.00 (s, 3H), 3.12-3.30 (m, 2H), 3.35-3.70 (bs, 8H), 3.48 (t, 2H), 3.70 (m, 1H), 3.96 (dd, 1H), 4.20 (m, 1H), 4.50 (m, 1H), 4.65 (m, 2H), 6.78 (s, 2H), 7.08 (s, 1H), 7.41 (bs, 2H), 7.47 (m, 1H), 7.56 (m, 1H), 7.73 (d, 2H), 7.86 (d, 4H), 7.90 (d, 2H), 8.03 (s, 1H), 8.25 (bs, 1H); LC-MS (ES^+) 988 $(M+H)^+$, 1010 $(M+Na)^+$.

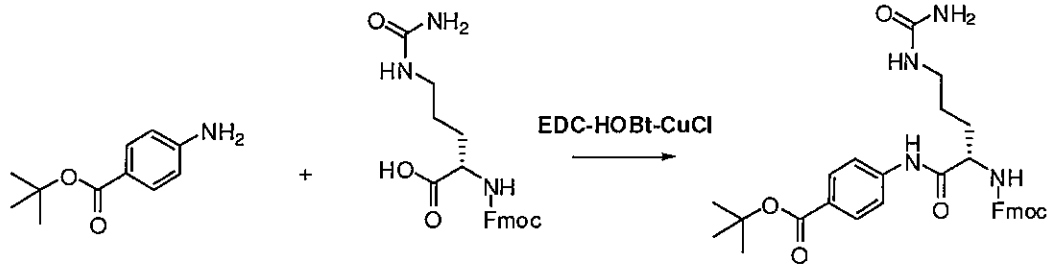
EXAMPLE 9



Synthesis of compound 3: To compound **1** (50 mg, 0.079 mmol) in DMF (2 mL) was added **2** (49 mg, 0.085 mmol) and HATU (33 mg, 0.086 mmol) followed by adding DIPEA (45 uL) and the reaction mixture was stirred for 4 hrs at room temperature. The solvent was removed under vacuo and the product was purified by Prep HPLC (SymmetrPrep C18, 7μm, 19 x 150 mm column), eluted at 10 ml/min (0.01% TFA in water/ acetonitrile) with a gradient: 10% acetonitrile in 5 min, 10% to 50% acetonitrile in 15 min, maintaining 50% acetonitrile in 5 min, 50% to 100% acetonitrile in 5 min, to give **3** (47 mg, 60%). MS: $(M+H)^+$ 1003.

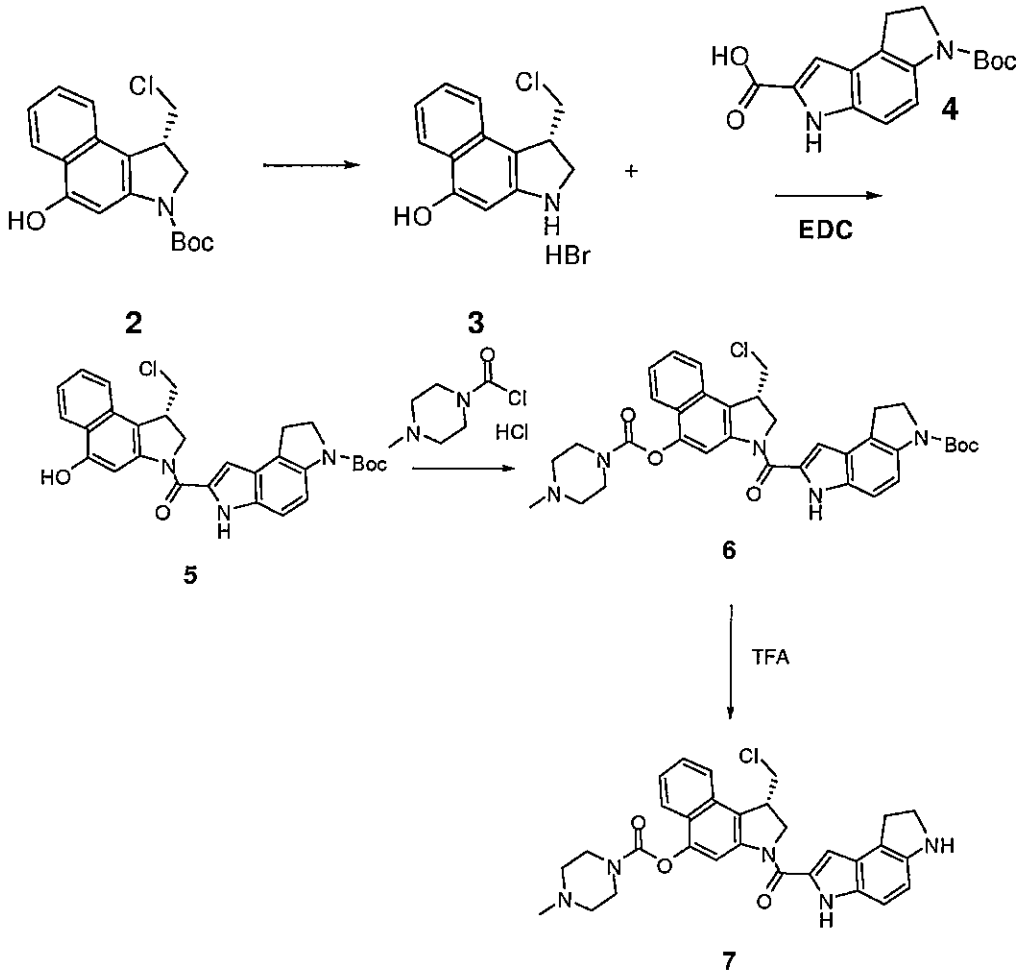
Synthesis of compound 5: A solution of **3** (15 mg, 0.014 mmol) in DMF (2mL) was treated with 5% piperidine in DMF (2 mL) and the reaction mixture was stirred for 10 min at 25^{0}C. The crude product was precipitated with diethyl ether and used for the next step without further purification. The crude product was dissolved in DMF (3 mL) followed by the addition of **4** (25 mg, 0.025 mmol) in DMF (2 mL) and DIEA (20 uL). The reaction mixture was stirred for 2 hrs at room temperature. TFA was added to the reaction mixture and the reaction mixture was stirred at room temperature for 45 min to yield compound **5** as indicated by analytical HPLC. Crude product was purified by Prep HPLC (SymmetrPrep C18, 7µm, 19 x 150 mm column), eluted at 10 ml/min (0.01% TFA in water/acetonitrile) with a gradient: 10% acetonitrile in 5 min, 10% to 50% acetonitrile in 15 min, maintaining 50% acetonitrile in 5 min, 50% to 100% acetonitrile in 5 min, to obtain **5** (3 mg, 94%). MS: $(MH)^+$ 889.

EXAMPLE 10



tert-butyl 4-aminobenzoate Fmoc Citruline **1**

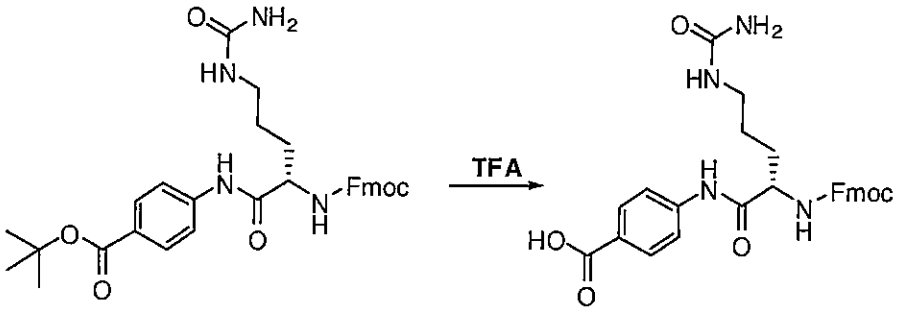
Synthesis of compound 1: 1.5 grams (3.77 mMol) of Fmoc-citruline were dissolved in 3 mL of DMF in a round bottom flask to which was added 0.87 grams (4.5 mMol) of EDC, 0.61 grams (4.5 mMol) of HOBt. 6 mL of DCM were then added followed by the addition 0.88 grams (4.5 mMol) of *t*-butyl-4-aminobenzoate and a catalytic amount of copper chloride. The reaction mixture was allowed to stir overnight The solvents were evaporated and the crude product purified over silica gel with 5 to 10 % MeOH in DCM to give 2 grams of **1** in 92 % yield. $M^{+1} = 574$



Synthesis of compound 7: 210 mg (0.63 mMol) of **2** was treated with an HBr-ethyl acetate solution for 30 minutes. The solvent was evaporated and the resulting salt **3** was dried under high vacuum. The 0.63 mmol of **3** prepared above was reacted with 210 mg (0.69 mMol) of **4** in 10 mL of DMF in the presence of 133 mg (0.69 mMol) of EDC for 2 hours. The solvent was evaporated and the crude purified over silica. To give 160 mg of **5** (47.6% yield). M^{+1} = 518

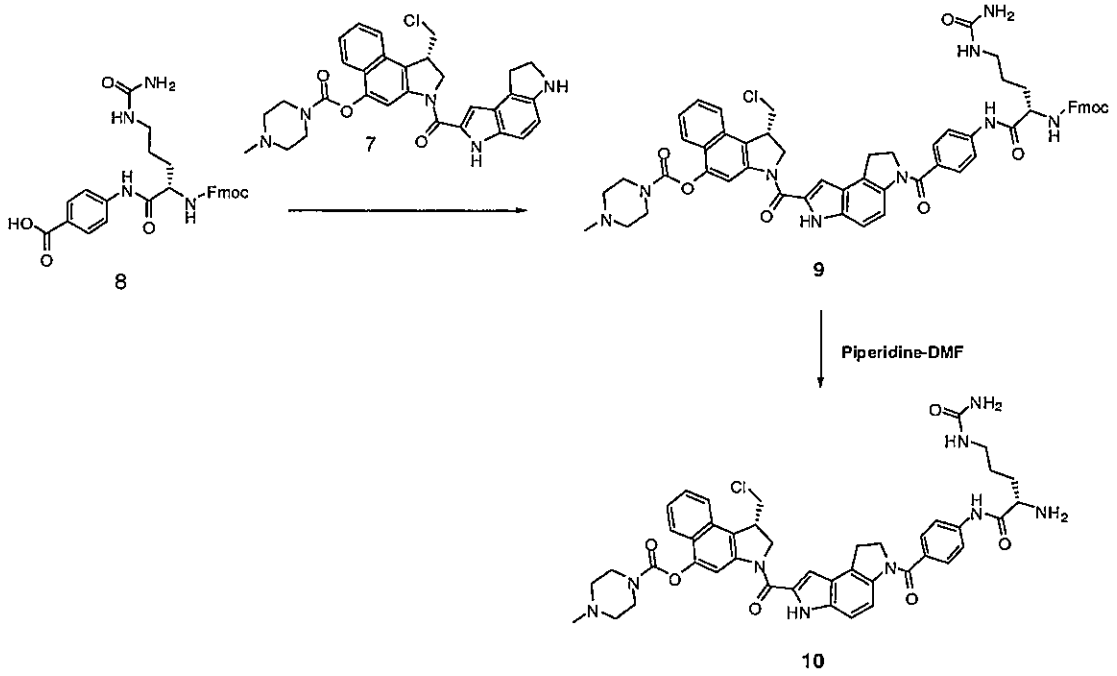
40 mg (0.27 mMol) of **5** was reacted overnight with 160 mg (0.81 mMol) of commercially available 4-methylpiperazine carbonylchloride hydrochloride in 17 mL of DCM with 1.7 mL of allylalcohol and 214 μL pyridine. The aolvent was evaporated and the crude compound was purified over silica with 5% MeOH/DCM as eluent to give 70 mg (40 % yield) of **6**. M^{+1} = 645.

35 mg (0.054 mMol) of **6** was treated with 5mL of 1:2 TFA-DCM for 5 minutes. The solvent was evaporated and the resulting salt **7** was dried overnight under high vacuum. M^{+1} = 544 and used in the next step.



1 8

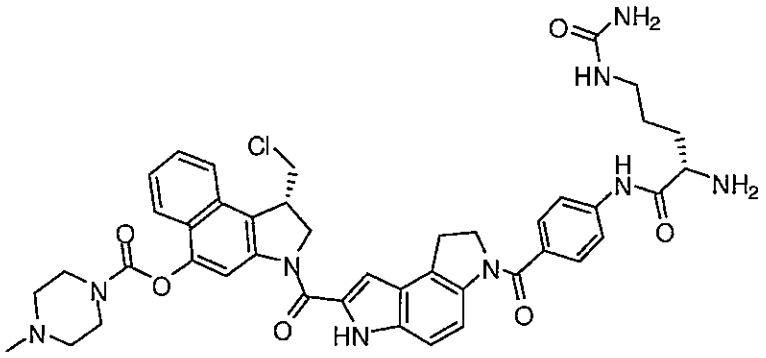
Synthesis of compound 8: 66 mg (0.11 mMol) of the **1** was converted to **8** by stirring with 3 mL of 1:2 TFA:DCM for 20 minutes. The solvents were evaporated and the acid dried under high vacuum. M^{+1} = 517.8, M^{+Na} = 540



8 9 10

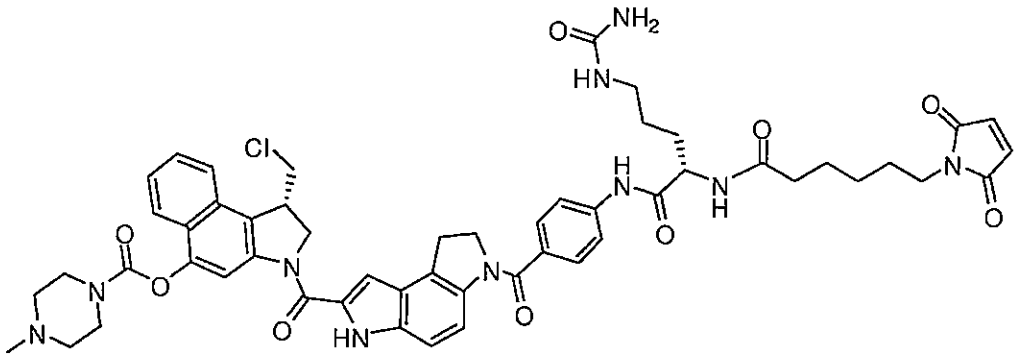
Synthesis of compound 10: 0.065 mmol of **8** and 44.7 mg (0.065 mMol) of BOP was added as a solution in 2 mL of anhydrous DMF to flask containing 0.054 mMol of **7** (MED-2477). DIPEA (93 uL, 0.54 mMol) was then added and stirred for 1 hour. The solvent was evaporated and the crude mixture purified by reverse phase prep HPLC. To give 48 mg of **9** (as its TFA salt 77 % yield) after purification. M^{+1} = 1043. 16 mg (0.015 mMol) of **9** was

treated with 1 mL of a 5% solution of piperidine in DMF for 10 minutes. The solvent was evaporated and the solid residue washed with hexanes and diethyl ether. The product **10** was dried under high vacuum. $M^{+1} = 821$



10

N-Succinimidyl 6-Maleimidohexanoate



11

Synthesis of compound 11: The 0.015 mMol of **10** prepared above was reacted with 11 mg (0.035 mMol) of commercially available *N*-succinimidyl 6-maleimidohexanoate in 1.5 mL of anhydrous DMF and 10 µL of DIPEA for 1 hour. The solvents was evaporated the crude purified by Reverse phase HPLC to give 10.7 mg of **11** (as its TFA salt, 63 % yield). $M^{+1} =$ 1014

EXAMPLE 11: Conjugation of Drug-Linker Molecules to Antibodies

For amino acid and peptide linkers: This example describes reaction conditions and methodologies for conjugating a drug-linker molecule of the invention (optionally including other groups, such as spacers, reactive functional groups and the like) to an antibody as a

targeting agent, X^4. The conditions and methodologies are intended to be exemplary only and non-limiting. Other approaches for conjugating drug-linker molecules to antibodies are known in the art.

The conjugation method described herein is based on introduction of free thiol groups to the antibody through reaction of lysines of the antibody with 2-iminothiolane, followed by reaction of the drug-linker molecule with an active maleimide group. Initially the antibody to be conjugated was buffer exchanged into 0.1M phosphate buffer pH 8.0 containing 50mM NaCl, 2mM DTPA, pH 8.0 and concentrated to 5-10 mg/ml. Thiolation was achieved through addition of 2-iminothiolane to the antibody. The amount of 2-iminothiolane to be added was determined in preliminary experiments and varies from antibody to antibody. In the preliminary experiments, a titration of increasing amounts of 2-iminothiolane was added to the antibody, and following incubation with the antibody for one hour at room temperature, the antibody was desalted into 50mM HEPES buffer pH 6.0 using a Sephadex G-25 column and the number of thiol groups introduced determined rapidly by reaction with dithiodipyridine (DTDP). Reaction of thiol groups with DTDP resulted in liberation of thiopyridine which was monitored at 324nm. Samples at a protein concentration of 0.5-1.0 mg/ml were used. The absorbance at 280nm was used to accurately determine the concentration of protein in the samples, and then an aliquot of each sample (0.9ml) was incubated with 0.1 ml DTDP (5mM stock solution in ethanol) for 10 minutes at room temperature. Blank samples of buffer alone plus DTDP were also incubated alongside. After 10 minutes, absorbance at 324nm was measured and the number of thiols present quantitated using an extinction coefficient for thiopyridine of $19800M^{-1}$.

Typically a thiolation level of three thiol groups per antibody is desired. For example, with one particular antibody this was achieved through adding a 15 fold molar excess of 2-iminothiolane followed by incubation at room temperature for 1 hour. Antibody to be conjugated was therefore incubated with 2-iminothiolane at the desired molar ratio and then desalted into conjugation buffer (50mM HEPES buffer pH 6.0 containing 5mM glycine, 0.5% povidone (10k) and 2mM DTPA). The thiolated material was maintained on ice whilst the number of thiols introduced was quantitated as described above.

After verification of the number of thiols introduced, the drug-linker molecule containing an active maleimide group was added at a 3-fold molar excess per thiol. The conjugation reaction was carried out in conjugation buffer also containing a final concentration of 5% DMSO (or a suitable alternative solvent). Commonly, the drug-linker

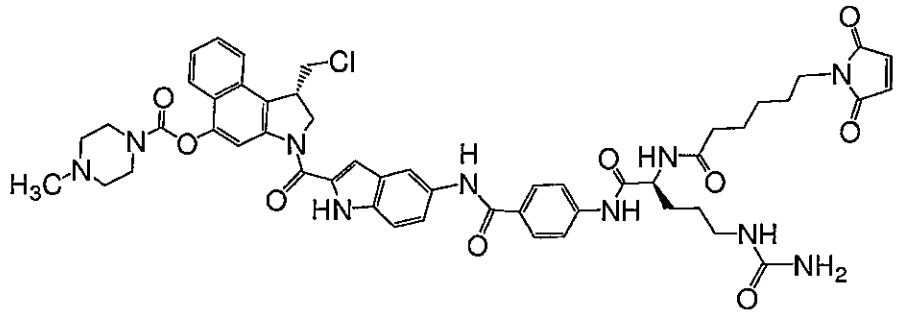
stock solution was dissolved in 100% dimethyl sulfoxide. For addition to antibody, the stock solution was added directly to the thiolated antibody, which has enough DMSO added to bring the final concentration to 10%, or pre-diluted in conjugation buffer containing a final concentration of 10% DMSO, followed by addition to an equal volume of thiolated antibody.

The conjugation reaction was incubated at room temperature for 2 hours with mixing. Following incubation the reaction mix was centrifuged and filtered through 0.2 micrometer filter. Purification of conjugate was achieved through chromatography using a number of methods. Conjugate can be purified using size-exclusion chromatography on a Sephacryl S200 column pre-equilibrated with 50mM HEPES buffer pH 7.2 containing 5mM glycine, 50mM NaCl and 0.5% povidone (10k). Chromatography was carried out at a linear flow rate of 28 cm/h. Fractions containing conjugate were collected, pooled and concentrated. Alternatively purification can be achieved through ion-exchange chromatography. Conditions vary from antibody to antibody and need to be optimized in each case. For example, antibody-drug conjugate reaction mix was applied to an SP-Sepharose column pre-equilibrated in 50mM HEPES, 5mM glycine, 0.5% povidone (10k), pH 5.5. The antibody conjugate was eluted using a gradient of 0-1M NaCl in equilibration buffer at pH 5.5. Relevant fractions containing the conjugate were pooled and dialyzed against formulation buffer (with 50mM HEPES buffer pH 7.2 containing 5mM glycine, 100 mM NaCl and 0.5% povidone (10k).

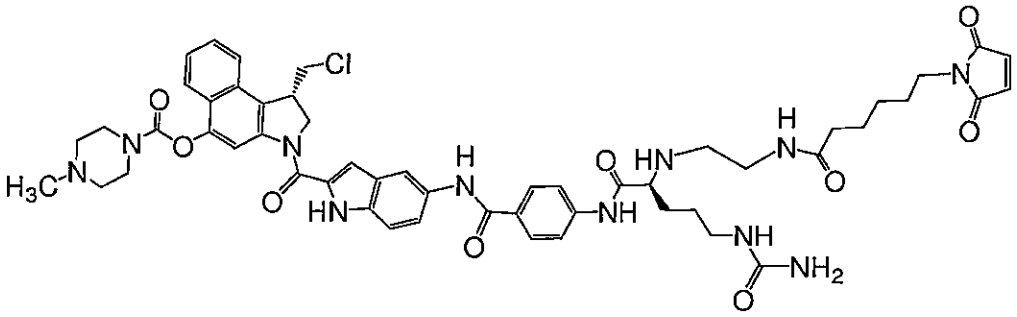
EXAMPLE 12: In Vivo Study

786-O (ATCC Accession No. CRL-1932) cells were expanded *in vitro* using standard laboratory procedures. Male CB17.SCID mice (Taconic, Hudson, NY) between 6-8 weeks of age were implanted subcutaneously in the right flank with 2.5 million 786-O in 0.2 ml of PBS/Matrigel (1:1) per mouse. Mice were weighed and measured for tumors three dimensionally using an electronic caliper twice weekly after implantation. Tumor volumes were calculated as height x width x length. Mice with tumors averaging 200 mm^3 were randomized into treatment groups. The mice were dosed intraperitoneally with PBS vehicle, toxin-conjugated isotype control antibody or toxin-conjugated anti-CD70 HuMAb 2H5 on Day 0. Each group contained 8 mice.

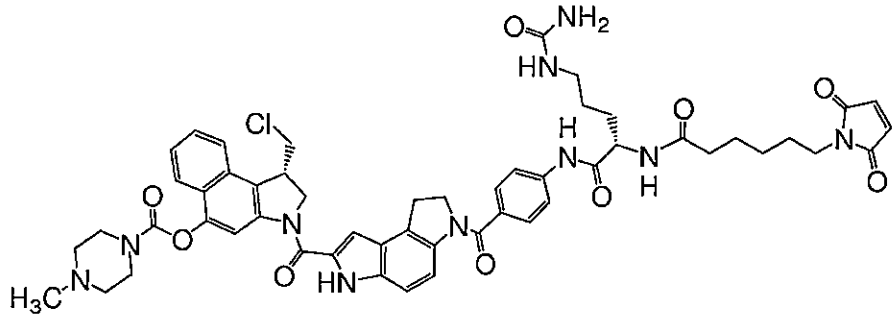
The following toxins were studied:



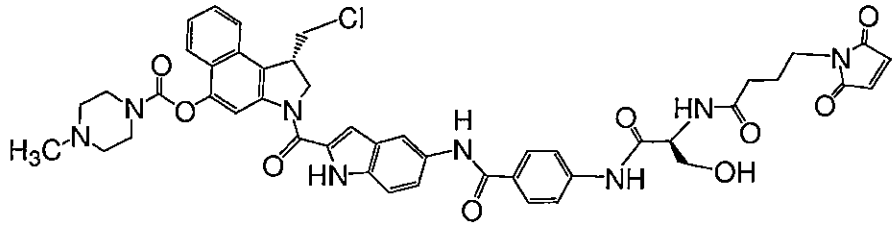
Compound A



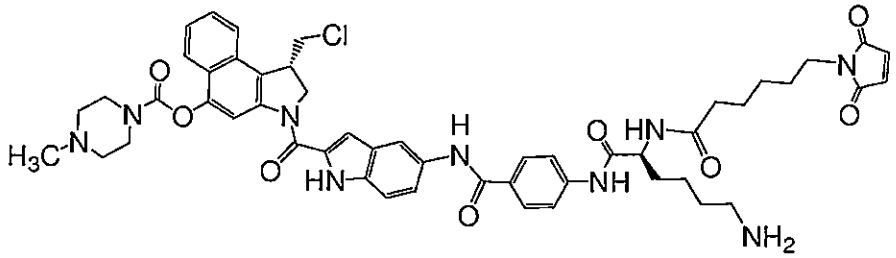
Compound B



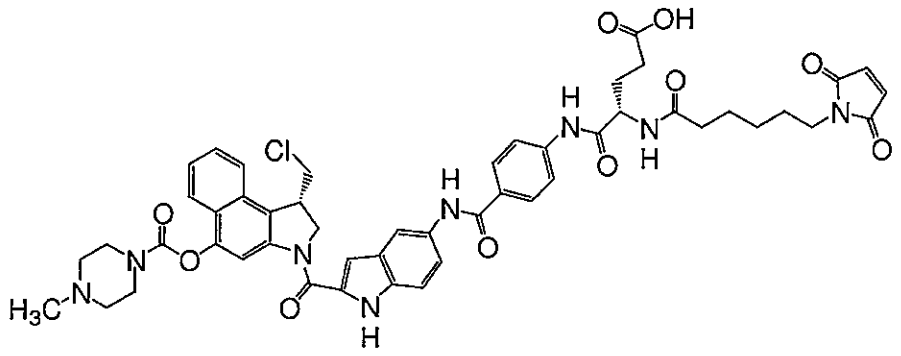
Compound C



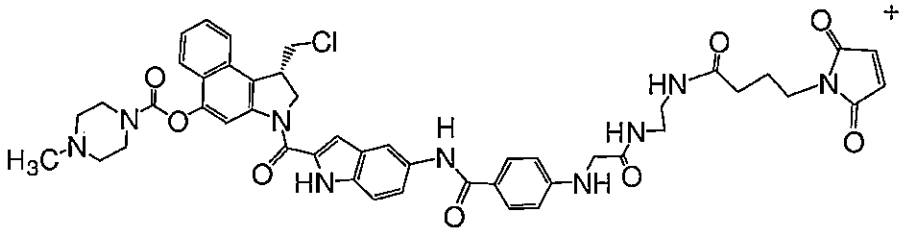
Compound D



Compound E



Compound F



Compound G

Table 1 is a summary of the dosing groups based on μmole of Toxin (Compounds A-G).

Table 1. Study Summary

Group		Dose (Toxin μmole, Ab mg/kg)
1	Vehicle IP SD	Matching 0.03 vol
2	CD70.1 0.1 IP SD	0.03
3-4	CD70.1- Cmpd A IP SD	0.03, 0.005
5-6	CD70.1- Cmpd B IP SD	0.03, 0.005
7-8	CD70.1- Cmpd C IP SD	0.03, 0.005
9-10	CD70.1- Cmpd D IP SD	0.03, 0.005
11-12	CD70.1- Cmpd E IP SD	0.03, 0.005
13-14	CD70.1- Cmpd F IP SD	0.03, 0.005
15-16	CD70.1- Cmpd G IP SD	0.03, 0.005

Figures 1, 2, 5, and 6 show the mean tumor volume versus days past dosing and Figures 3, 4, 7, and 8 show the median tumor volume versus days past dosing (0.03 μmol - Figures 1, 3, 5, and 7; 0.005 μmol - Figures 2, 4, 6, and 8). Figure 9 shows the median percent body weigh change versus days post dosing for both 0.03 and 0.0005 μmol doses.

Efficacy, based on the 0.03 μmol dosage experiments, appears to be in the following descending order Compound E > Compound F > Compound A> Compound C, Compound D > Compound B > Compound G.

EXAMPLE 13: Cathepsin B Mediated Cleavage of Single Amino acid Linkers

A panel of single amino acid compounds, each differing with respect to amino acid composition but of otherwise similar structure, was assayed for cathepsin B activity. The following compounds were tested: compounds A,B,C,D,E and F. The appearance of cathepsin B-mediated products was monitored by application to RP-HPLC with quantitation of drug products by their absorbance at 340 nm. Compounds were incubated with cathepsin B for various periods of time and their approximate half lives ($t_{1/2}$) are reported in Table 2 below. Of the compounds tested, only compound A and compound D were cleaved by cathepsin B.

Table 2: Cathepsin B mediated cleavage of compounds with single amino acid cleavable linkers.

Compound	Cathepsin B cleavage	Approx. substrate half-life
Compound A	Yes	24 hrs.
Compound B	No	
Compound C	No	
Compound D	No	
Compound E	Yes	4 hrs.
Compound F	No	

Cathepsin B Enzyme Assay: A stock solution of bovine spleen cathepsin B (Sigma product code C-6286) was prepared by dissolving lyophilized solid (6 mg at 56% protein) in 25 mM sodium acetate/1 mM EDTA pH 5.0 buffer (10 mL). Enzyme activation was accomplished by mixing the stock solution (50 uL) with activation buffer (100 ul) comprised of 30 mM DTT/ 15 mM EDTA, followed by incubation at room temperature for 15 minutes. The activated cathepsin B was diluted 1:1 with a solution of 25 mM sodium acetate/ 1 mM EDTA, pH 5.0 prior to use. To assay for drug release, cysteine modified compound (4 uL of 2.5 mM solution in DMSO) was added to 25 mM sodium acetate/ 1 mM EDTA pH 5.0 buffer (86 uL) and activated cathepsin B (10 uL at 3.6 U/mL). Samples were incubated at 37°C for appropriate time points and stopped with methanol (100 uL).

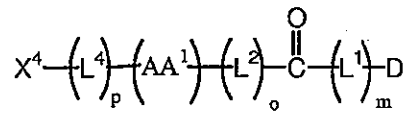
Compounds samples (40 uL) were applied to a Waters 2795 HPLC system fitted with a reverse phase column (Waters Nova-pak C18, 3.9 x 150 mm column, product code WAT086344). Sample chromatography was accomplished using a dual mobile phase system comprised of water/0.1 % TFA (A buffer) and acetonitrile/0.1% TFA (B buffer) with a gradient elution of 10-100% B buffer over 20 min at 1.0 mL/min. compounds were detected by its absorbance at 340 nm (A_{340}).

Each of the patent applications, patents, publications, and other published documents mentioned or referred to in this specification is herein incorporated by reference in its entirety, to the same extent as if each individual patent application, patent, publication, and other published document was specifically and individually indicated to be incorporated by reference.

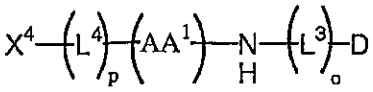
While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention and the appended claims. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

WHAT IS CLAIMED IS:

1. A single amino acid compound of the formula



or



wherein

L^1 is substituted or unsubstituted alkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl or substituted or unsubstituted heteroalkyl group;

m is an integer 0, 1, 2, 3, 4, 5, or 6;

AA^1 is an amino acid selected from the group consisting of natural amino acids and unnatural α- amino acids;

L^2 is substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, unsubstituted heterocycloalkyl, substituted heterocycloalkyl, substituted or unsubstituted aryl, or substituted or unsubstituted heteroaryl;

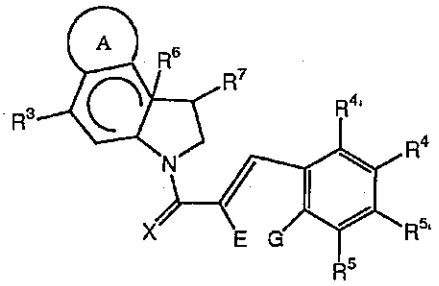
L^3 is substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, or substituted or unsubstituted heterocycloalkyl;

o is 0 or 1 ;

L^4 is a linker member selected from the group consisting of substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroalkyl and substituted heteroalkyl; p is 0 or 1;

X^4 is a member selected from the group consisting of a reactive functional group, that is an N-hydroxysuccinimide ester or a maleimide, a detectable label that is a radioactive or fluorescent label, and a targeting agent that is an antibody or antibody fragment; and

D comprises a structure:



wherein the ring system A is substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl or substituted or unsubstituted heterocycloalkyl;

E and G are members independently selected from H, substituted and unsubstituted alkyl, substituted and unsubstituted heteroalkyl, a heteroatom, and a single bond, or E and G are joined to form a ring system selected from substituted and unsubstituted aryl, substituted and unsubstituted heteroaryl and substituted and unsubstituted heterocycloalkyl;

X is a member selected from O, S and NR^{23};

R^{23} is H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, or acyl;

R^3 is OR^{11},

wherein R^{11} is H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, diphosphate, triphosphate, acyl, $C(O)R^{12}R^{13}$, $C(O)OR^{12}$, $C(O)NR^{12}R^{13}$, $P(O)(OR^{12})_2$, $C(O)CHR^{12}R^{13}$, SR^{12}, or $SiR^{12}R^{13}R^{14}$,

in which R^{12}, R^{13}, and R^{14} are members independently selected from H, substituted and unsubstituted alkyl, substituted and unsubstituted heteroalkyl and substituted and unsubstituted aryl, wherein R^{12} and R^{13} together with the nitrogen or carbon atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

R^4, $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, SO_3, SO_2R^{15}, $NR^{15}R^{16}$, $NR^{16}C(O)R^{15}$,

$OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, $CR^{15}=NR^{16}$, and $O(CH_2)_nN(CH_3)_2$, or any adjacent pair of R^4, $R^{4'}$, R^5 and $R^{5'}$, together with the carbon atoms to which they are attached, are joined to form a substituted or unsubstituted cycloalkyl or heterocycloalkyl ring system having from 4 to 6 members;

wherein

n is an integer from 1 to 20;

R^{15} and R^{16} are independently selected from H, substituted and unsubstituted alkyl, substituted and unsubstituted heteroalkyl, substituted and unsubstituted aryl, substituted and unsubstituted heteroaryl, substituted and unsubstituted heterocycloalkyl, and substituted and unsubstituted peptidyl, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

R^6 is a single bond which is either present or absent and when present R^6 and R^7 are joined to form a cyclopropyl ring; and

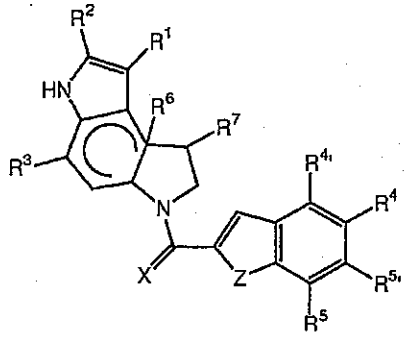
R^7 is CH_2-X^1 or -CH_2- joined in said cyclopropyl ring with R_6, wherein

X^1 is a leaving group selected from the group consisting of halogen, azides, sulfonic esters, oxonium ions, alkyl perchlorates, ammonioalkanesulfonate esters, alkylfluorosulfonates, triflates, nonaflates, and tresylates,

wherein at least one of R^4, $R^{4'}$, R^5, $R^{5'}$, R^{11}[[,]]$_z$ R^{12}, R^{13}, R^{15} or R^{16} links D to the remainder of the compound;

or a pharmaceutically acceptable salt thereof.

2. The compound of claim 1, wherein D has the structure:



wherein

Z is a member selected from O, S and NR^{23}

wherein

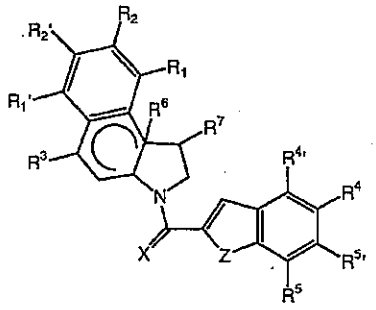
R^{23} is H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, or acyl;

R^1 is H, substituted or unsubstituted lower alkyl, $C(O)R^8$, or CO_2R^8, wherein R^8 is substituted alkyl, unsubstituted alkyl, NR^9R^{10}, NR^9NHR^{10}, or OR^9

in which R^9 and R^{10} are members independently selected from H, substituted and unsubstituted alkyl and substituted and unsubstituted heteroalkyl; and

R^2 is H, substituted alkyl or unsubstituted lower alkyl.

3. The compound of claim 1, wherein D has the structure:



wherein

Z is a member selected from O, S and NR^{23},

wherein

R^{23} is H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, or acyl;

R^1 is H, substituted or unsubstituted lower alkyl, $C(O)R^8$, or CO_2R^8, wherein R^8 is a member selected from NR^9R^{10} and OR^9,

in which

R^9 and R^{10} are members independently selected from H, substituted and unsubstituted alkyl and substituted and unsubstituted heteroalkyl;

$R^{1'}$ is H, substituted or unsubstituted lower alkyl, or $C(O)R^8$, wherein R^8 is a member selected from NR^9R^{10} and OR^9,

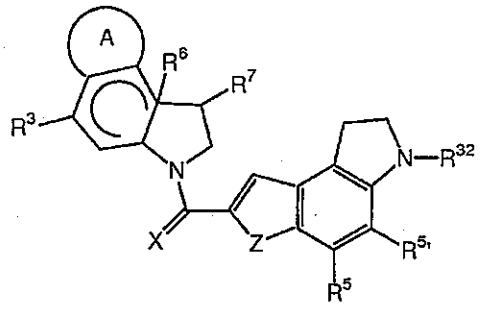
in which

R^9 and R^{10} are members independently selected from H, substituted and unsubstituted alkyl and substituted and unsubstituted heteroalkyl;

R^2 is H, substituted or unsubstituted lower alkyl, unsubstituted heteroalkyl, cyano or alkoxy; and

$R^{2'}$ is H, substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl.

4. The compound of claim 1 wherein D is



[[(13)]]

wherein

Z is a member selected from O, S and NR^{23}

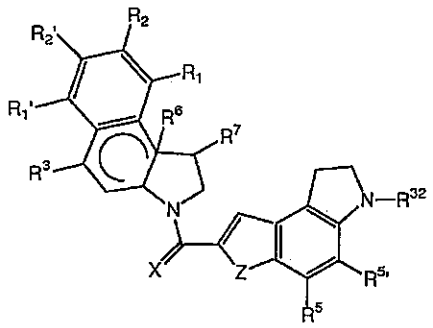
wherein

R^{23} is H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, or acyl;

R^{32} is selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NR^{16}C(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, $CR^{15}{=}NR^{16}$, or $O(CH_2)_nN(CH_3)_2$, where n is an integer from 1 to 20, and

at least one of R^5, $R^{5'}$, R^{11}[[,]]$_z$ R^{12}, R^{13}, R^{15}, R^{16}, or R^{32} links D to the remainder of the compound.

5. The compound of claim 4, wherein D is:



wherein

R^1 is H, substituted or unsubstituted lower alkyl, $C(O)R^8$, or CO_2R^8, wherein R^8 is a member selected from NR^9R^{10} and OR^9,

in which

R^9 and R^{10} are members independently selected from H, substituted and unsubstituted alkyl and substituted and unsubstituted heteroalkyl;

$R^{1'}$ is H, substituted or unsubstituted lower alkyl, or $C(O)R^8$, wherein R^8 is a member selected from NR^9R^{10} and OR^9,

in which

R^9 and R^{10} are members independently selected from H, substituted and unsubstituted alkyl and substituted and unsubstituted heteroalkyl;

R^2 is H, substituted or unsubstituted lower alkyl, unsubstituted heteroalkyl, cyano or alkoxy; and

$R^{2'}$ is H, substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl.

6. The compound of any one of claims 1-5, wherein AA^1 is selected from the group consisting of: Ala, Arg, Asn, Asp, Cit, Cys, Gln, Glu, Gly, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val.

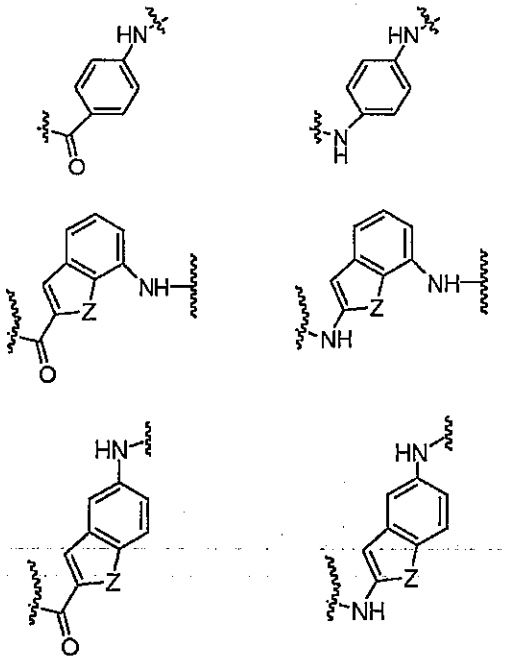
7. The compound of claim 6, wherein AA^1 is selected from the group consisting of Cit, Glu, Lys, and Ser.

8. The compound of claim any one of claims 1-7, wherein at least one of R^4, $R^{4'}$, R^5, $R^{5'}$, R^{15} or R^{16} links D to the remainder of the compound.

9. The compound of any one of claims 1-8, wherein m is not 0 and L^1 is a self- immolative linker.

10. The compound of any one of claims 1-9, wherein L^2 is present and is a self- immolative linker.

11. The compound of any one of claims 1-8, wherein L^3 is present and $-L^3-NH-$ is selected from



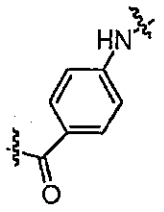
wherein

Z is a member selected from O, S and NR^{23}

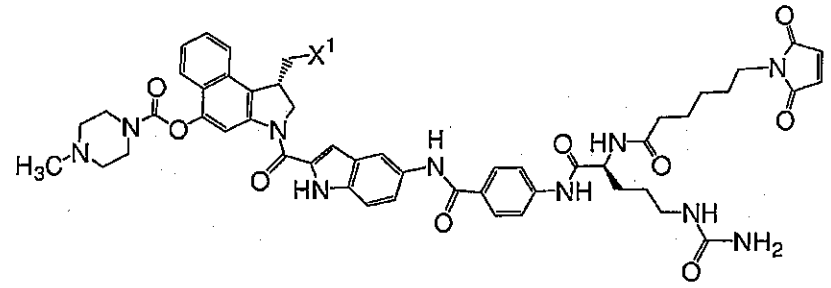
wherein

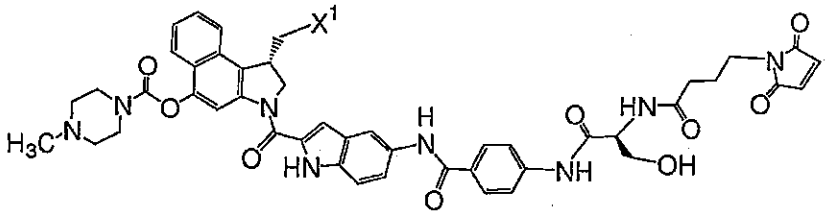
R^{23} is H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, or acyl.

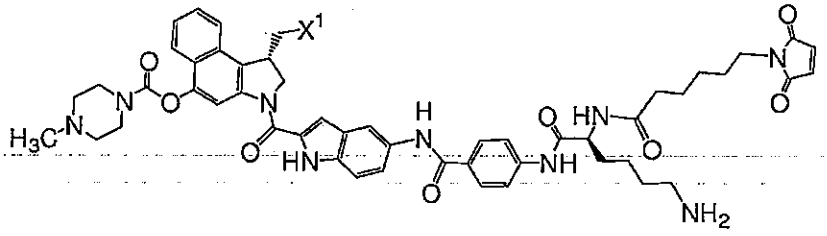
12. The compound of claim 11, wherein $-L^3-NH-$ is

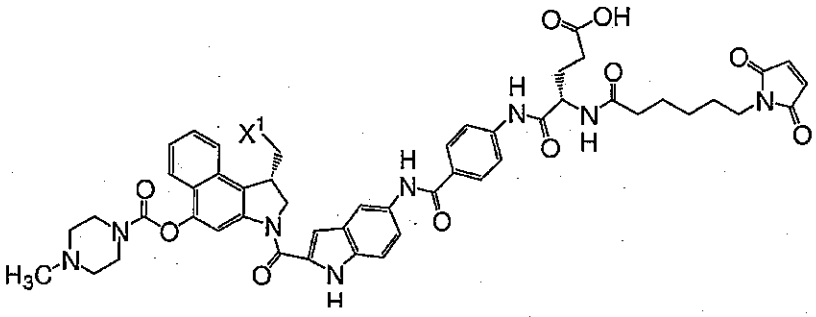


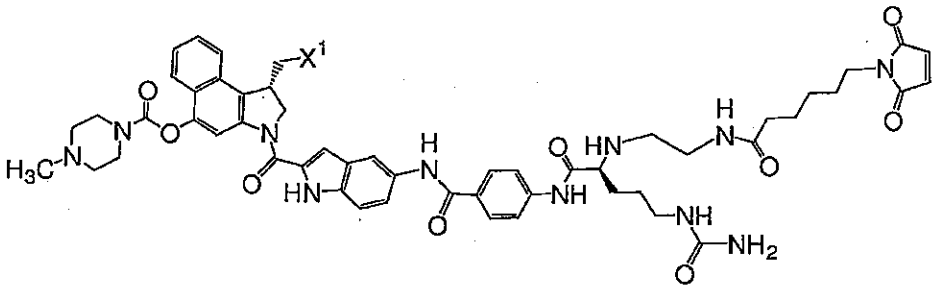
13. The compound of claim 1, wherein the compound is selected from



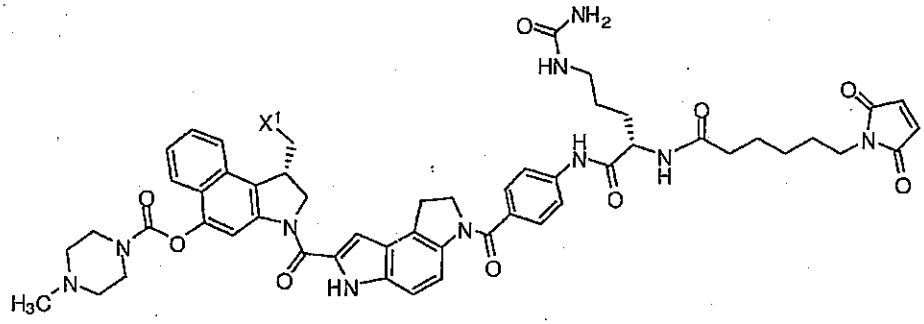








and



wherein X^1 is Cl or Br.

14. The compound of claim 13, wherein X^1 is Cl.

15. The compound of any one of claims 1-12, wherein X^4 is an antibody or antibody fragment.

16. A pharmaceutical formulation comprising a compound according to any one of claims 1-15 and a pharmaceutically acceptable carrier.

17. A compound according to any of claims 1-15 for use in killing a cell.

18. The compound of claim 17, wherein the cell is a tumor cell.

19. A compound according to any of claims 1-15 for use in retarding or stopping the growth of a tumor in a mammalian subject.

SEQUENCE LISTING

<110> Medarex, Inc.
Chen, Liang
Gangwar, Sanjeev
Guerlavais, Vincent
Lonberg, Nils
Zhang, Qian

<120> CHEMICAL LINKERS WITH SINGLE AMINO ACIDS AND CONJUGATES THEREOF

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