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(54) Benævnelse: **SAMMENSÆTNINGER SOM INDEHOLDER, FREMGANGSMÅDER SOM INVOLVERER, OG ANVENDELSER AF IKKE-NATURLIGE AMINOSYREBUNDNE DOLASTATINDERIVATER**

(56) Fremdragne publikationer:
F. TIAN ET AL: "A general approach to site-specific antibody drug conjugates", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 111, no. 5, 17 January 2014 (2014-01-17), pages 1766-1771, XP055101730, ISSN: 0027-8424, DOI: 10.1073/pnas.1321237111
None

Description

CROSS REFERENCE

[0001] This application claims priority to U.S. Provisional Application No. 61/491,076, entitled, "Compositions Containing, Methods Involving, and Uses of Non-Natural Amino Acid Linked Dolastatin Derivatives," filed May 27, 2011.

BACKGROUND OF THE INVENTION

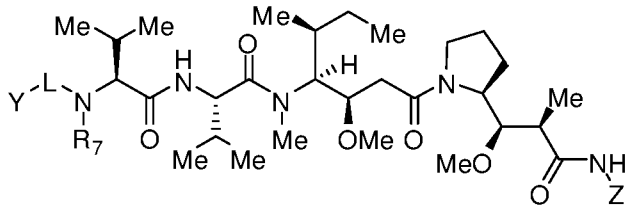
[0002] The ability to incorporate non-genetically encoded amino acids (i.e., "non-natural amino acids") into proteins permits the introduction of chemical functional groups that could provide valuable alternatives to the naturally-occurring functional groups, such as the epsilon $-NH_2$ of lysine, the sulfhydryl -SH of cysteine, the imino group of histidine, etc. Certain chemical functional groups are known to be inert to the functional groups found in the 20 common, genetically-encoded amino acids but react cleanly and efficiently to form stable linkages with functional groups that can be incorporated onto non-natural amino acids.

[0003] Methods are now available to selectively introduce chemical functional groups that are not found in proteins, that are chemically inert to all of the functional groups found in the 20 common, genetically-encoded amino acids and that may be used to react efficiently and selectively with reagents comprising certain functional groups to form stable covalent linkages. US 2009/047296 A1 discloses auristatin-ligand conjugates.

SUMMARY OF THE INVENTION

[0004] Disclosed herein are toxic moieties with one or more linker(s), toxic groups linked to non-natural amino acids, and methods for making such non-natural amino acids and polypeptides.

[0005] Some embodiments of the present invention describe a compound, or salt thereof, comprising Formula (I):

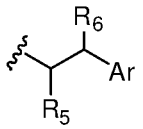


(I)

;

wherein:

Z has the structure of:



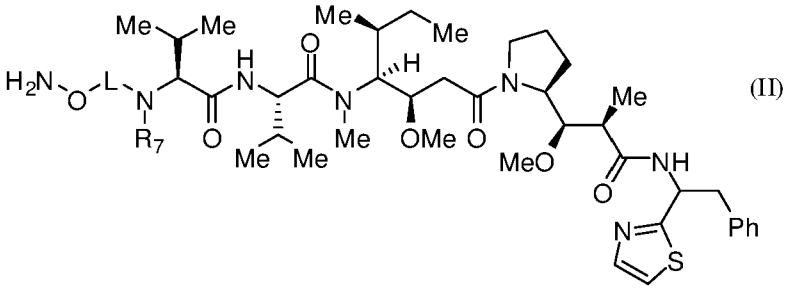
;

R_5 is H, COR_8, C_1-C_6 alkyl, or thiazole;
R_8 is OH;
R_6 is OH or H;
Ar is phenyl or pyridine;

R_7 is C_1-C_6 alkyl or hydrogen;
Y is selected from the group consisting of an hydroxylamine, azide, alkyne and cycloalkyne;
L is-alkylene- or -(alkylene-O)$_n$-alkylene-; and
n is an integer greater than or equal to one;
wherein alkyl means 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 and also includes heteroalkyl and haloalkyl;
wherein alkylene includes heteroalkylene.

[0006] In some embodiments, R_5 is thiazole. In other embodiments, R_6 is H. In certain embodiments, Ar is phenyl. In further or additional embodiments, R_7 is methyl. In some embodiments, n is an integer from 0 to 20, 0 to 10 or 0 to 5.

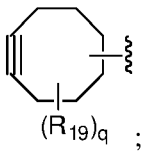
[0007] In some embodiments, a compound is described comprising Formula (II):



(II)

In certain embodiments, L is -(alkylene-O)$_n$-alkylene-. In specific embodiments, each alkylene is - CH_2CH_2-, n is equal to 3, and R_7 is methyl. In other embodiments, L is -alkylene-. In specific embodiments, each alkylene is -CH_2CH_2- and R_7 is methyl or hydrogen.

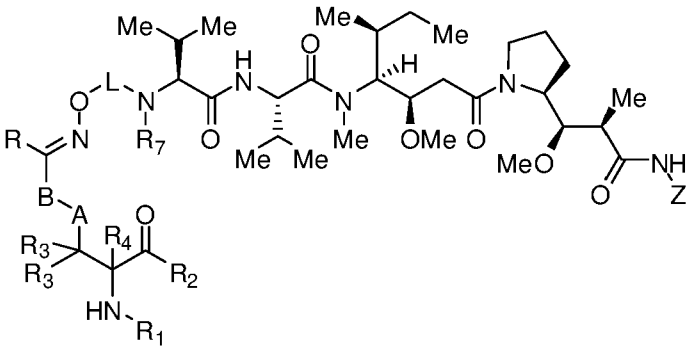
[0008] In some embodiments, Y is azide. In other embodiments, Y is cyclooctyne. In specific embodiments, the cyclooctyne has a structure of:



;

each R_{19} is independently selected from the group consisting of C_1-C_6 alkyl, C_1-C_6 alkoxy, ester, ether, thioether, aminoalkyl, halogen, alkyl ester, aryl ester, amide, aryl amide, alkyl halide, alkyl amine, alkyl sulfonic acid, alkyl nitro, thioester, sulfonyl ester, halosulfonyl, nitrile, alkyl nitrile, and nitro; and
q is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11.

[0009] Certain embodiments of the present invention describe a compound comprising Formula (VIII):



(VIII)

;

wherein:

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;
B is optional, and when present is a linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene, -O-, -O-(alkylene or substituted alkylene)-, - S-, -S-(alkylene or substituted alkylene)-, -$S(O)_k$- where k is 1, 2, or 3, -$S(O)_k$(alkylene or substituted alkylene)-, -C(O)-, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -C(S)-(alkylene or substituted alkylene)-, -N(R')-, -NR'-(alkylene or substituted alkylene)-, -C(O)N(R')-, -CON(R')-(alkylene or sub-

stituted alkylene)-, -CSN(R')-, -CSN(R')-(alkylene or substituted alkylene)-, -N(R')CO-(alkylene or substituted alkylene)-, -N(R')C(O)O-, -S(O)$_k$N(R')-, -N(R')C(O)N(R')-, -N(R')C(S)N(R')-, -N(R')S(O)$_k$N(R')-, -N(R')-N=, -C(R')=N-, -C(R')=N-N(R')-, -C(R')=N-N=, -C(R')$_2$-N=N-, and -C(R')$_2$-N(R')-N(R')-, where each R' is independently H, alkyl, or substituted alkyl;

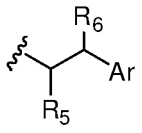
R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;

R_1 is H, an amino protecting group, resin, at least one amino acid, polypeptide, or polynucleotide;

R_2 is OH, an ester protecting group, resin, at least one amino acid, polypeptide, or polynucleotide;

R_3 and R_4 are each independently H;

Z has the structure of:



;

R_5 is H, CO_2H, C_1-C_6 alkyl, or thiazole;

R_6 is OH or H;

Ar is phenyl or pyridine;

R_7 is C_1-C_6 alkyl or hydrogen; and

L is-alkylene- or -(alkylene-O)$_n$-alkylene-; and

n is an integer greater than or equal to one;

wherein alkyl means 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 and also includes heteroalkyl and haloalkyl;

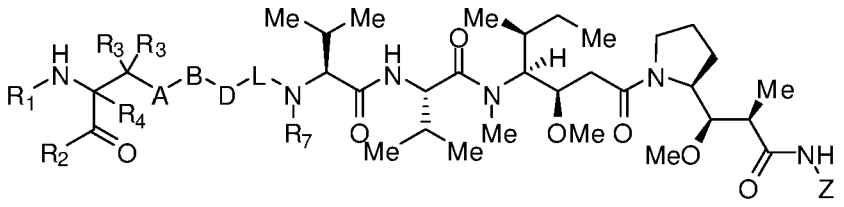
wherein alkylene includes heteroalkylene;

wherein arylene means a divalent aryl radical, wherein aryl includes heteroaryl;

or solvate thereof.

[0010] In some embodiments, R_1 is a polypeptide. In specific embodiments, the polypeptide is an antibody. In certain specific embodiments, the antibody is trastuzumab. In other embodiments, R_2 is a polypeptide. In specific embodiments, the polypeptide is an antibody. In certain specific embodiments, the antibody is trastuzumab.

[0011] Some embodiments of the present invention describe a compound comprising Formula (XXXI):

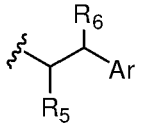


(XXXI)

;

wherein:

Z has the structure of:



;

R_5 is H, CO_2H, C_1-C_6 alkyl, or thiazole;

R_6 is OH or H;

Ar is phenyl or pyridine;

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substi-

tuted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;

B is optional, and when present is a linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene, -O-(alkylene or substituted alkylene)-, -S-(alkylene or substituted alkylene)-, -C(O)R"-, -$S(O)_k$(alkylene or substituted alkylene)-, where k is 1, 2, or 3, -C(O)-(alkylene or substituted alkylene)-, -C(S)-(alkylene or substituted alkylene)-, -NR"-(alkylene or substituted alkylene)-, -CON(R")-(alkylene or substituted alkylene)-, -CSN(R")-(alkylene or substituted alkylene)-, and -N(R")CO-(alkylene or substituted alkylene)-, where each R" is independently H, alkyl, or substituted alkyl ;

R_1 is H, an amino protecting group, resin, at least one amino acid, polypeptide, or polynucleotide;

R_2 is OH, an ester protecting group, resin, at least one amino acid, polypeptide, or polynucleotide;

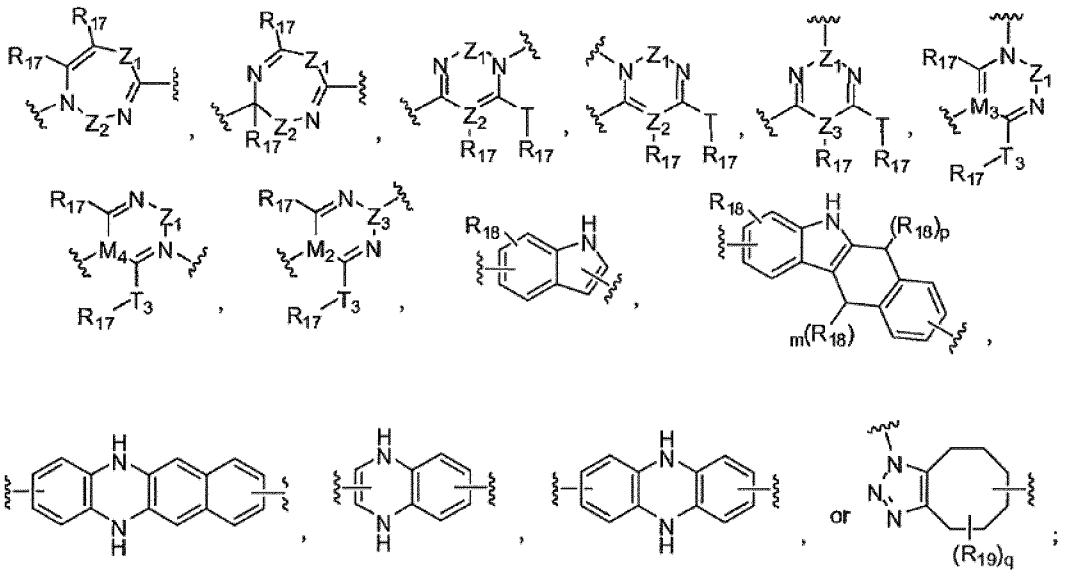
R_3 and R_4 are each independently H, halogen, lower alkyl, or substituted lower alkyl;

R_7 is C_1-C_6 alkyl or hydrogen;

L is-alkylene- or -(alkylene-O)n-alkylene-; and

n is an integer greater than or equal to one;

D has the structure of:



each R_{17} is independently selected from the group consisting of H, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, alkoxy, substituted alkoxy, alkylalkoxy, substituted alkylalkoxy, polyalkylene oxide, substituted polyalkylene oxide, aryl, substituted aryl, heteroaryl, substituted heteroaryl, alkaryl, substituted alkaryl, aralkyl, substituted aralkyl, - (alkylene or substituted alkylene)-$ON(R")_2$, -(alkylene or substituted alkylene)-C(O)SR", - (alkylene or substituted alkylene)-S-S-(aryl or substituted aryl), -C(O)R", -$C(O)_2$R", or -$C(O)N(R")_2$, wherein each R" is independently hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkoxy, substituted alkoxy, aryl, substituted aryl, heteroaryl, alkaryl, substituted alkaryl, aralkyl, or substituted aralkyl;

each Z_1 is a bond, $CR_{17}R_{17}$, O, S, NR', $CR_{17}R_{17}$-$CR_{17}R_{17}$, $CR_{17}R_{17}$-O, O-$CR_{17}R_{17}$, $CR_{17}R_{17}$-S, S-$CR_{17}R_{17}$, $CR_{17}R_{17}$-NR', or NR'-$CR_{17}R_{17}$;

each R' is H, alkyl, or substituted alkyl;

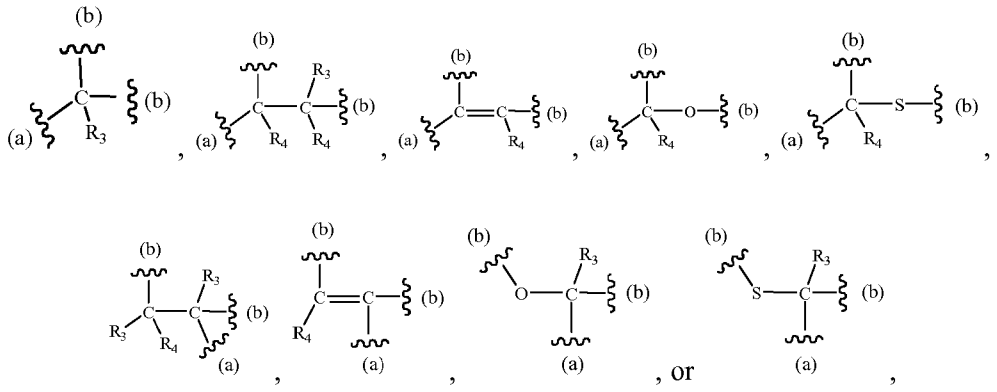
each Z_2 is selected from the group consisting of a bond, -C(O)-, -C(S)-, optionally substituted C_1-C_3 alkylene, optionally substituted C_1-C_3 alkenylene, and optionally substituted heteroalkyl;

each Z3 are independently selected from the group consisting of a bond, optionally substituted C_1-C_4 alkylene, optionally substituted C_1-C_4 alkenylene, optionally substituted heteroalkyl, -O-, -S-, -C(O)-, -C(S)-, and -N(R')-;

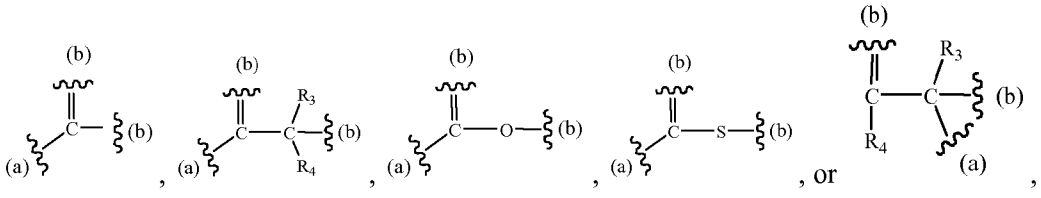
each T_3 is a bond, C(R")(R"), O, or S; with the proviso that when T_3 is O or S, R" cannot be halogen;

each R" is H, halogen, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl; m and p are 0, 1, 2, or 3, provided that at least one of m or p is not 0;

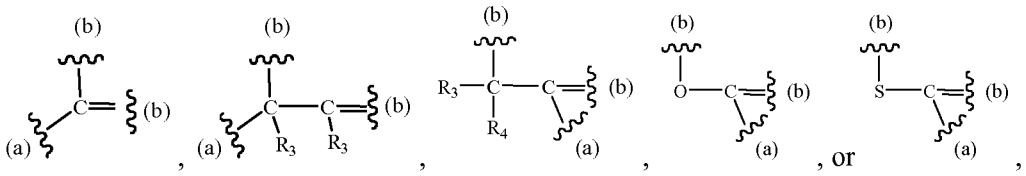
M_2 is



where (a) indicates bonding to the B group and (b) indicates bonding to respective positions within the heterocycle group;
M_3 is



where (a) indicates bonding to the B group and (b) indicates bonding to respective positions within the heterocycle group;
M_4 is



where
(a) indicates bonding to the B group and (b) indicates bonding to respective positions within the heterocycle group;
each R_{19} is independently selected from the group consisting of C_1-C_6 alkyl, C_1-C_6 alkoxy, ester, ether, thioether, aminoalkyl, halogen, alkyl ester, aryl ester, amide, aryl amide, alkyl halide, alkyl amine, alkyl sulfonic acid, alkyl nitro, thioester, sulfonyl ester, halosulfonyl, nitrile, alkyl nitrile, and nitro;
q is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11; and
each R_{16} is independently selected from the group consisting of hydrogen, halogen, alkyl, NO_2, CN, and substituted alkyl;

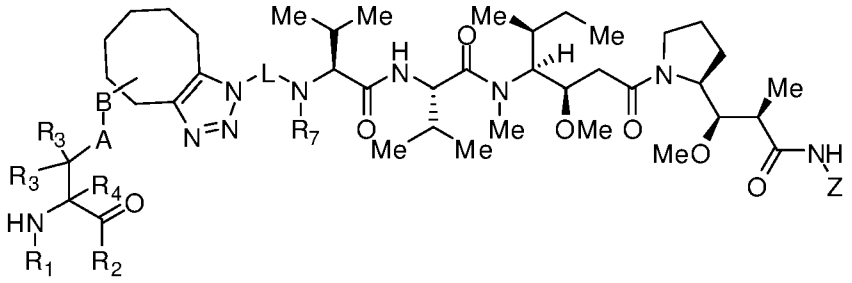
wherein alkyl means a straight or branched chain, or cyclic hydrocarbon radical, or combination thereof, which may be fully saturated, mono- or polyunsaturated and can include di- and multivalent radicals and also includes heteroalkyl and haloalkyl;

wherein alkylene includes heteroalkylene;

wherein arylene means a divalent aryl radical, wherein aryl includes heteroaryl.

[0012] In some embodiments, R_1 is a polypeptide. In specific embodiments, the polypeptide is an antibody. In certain specific embodiments, the antibody is trastuzumab. In other embodiments, R_2 is a polypeptide. In specific embodiments, the polypeptide is an antibody. In certain specific embodiments, the antibody is trastuzumab.

[0013] In some embodiments, a compound is described comprising Formula (XXXI-A):



(XXXI-A)

[0014] In certain embodiments, a pharmaceutical composition is provided comprising any of the compounds described and a pharmaceutically acceptable carrier, excipient, or binder.

[0015] Also described are methods for detecting the presence of a polypeptide in a patient, the method comprising administering a polypeptide comprising at least one heterocycle-containing non-natural amino acid and the resulting heterocycle-containing non-natural amino acid polypeptide modulates the immunogenicity of the polypeptide relative to the homologous naturally-occurring amino acid polypeptide.

[0016] It is to be understood that the methods and compositions described herein are not limited to the particular methodology, protocols, cell lines, constructs, and reagents described herein and as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the methods and compositions described herein, which will be limited only by the appended claims.

[0017] As used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly indicates otherwise.

[0018] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which the inventions described herein belong. Although any methods, devices, and materials similar or equivalent to those described herein can be used in the practice or testing of the inventions described herein, the preferred methods, devices and materials are now described.

[0019] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors described herein are not entitled to antedate such disclosure by virtue of prior invention or for any other reason.

[0020] The terms "aldol-based linkage" or "mixed aldol-based linkage" refers to the acid- or base-catalyzed condensation of one carbonyl compound with the enolate/enol of another carbonyl compound, which may or may not be the same, to generate a β-hydroxy carbonyl compound-an aldol.

[0021] The term "affinity label," as used herein, refers to a label which reversibly or irreversibly binds another molecule, either to modify it, destroy it, or form a compound with it. By way of example, affinity labels include enzymes and their substrates, or antibodies and their antigens.

[0022] The terms "alkoxy," "alkylamino" and "alkylthio" (or thioalkoxy) are used in their conventional sense, and refer to those alkyl groups linked to molecules via an oxygen atom, an amino group, or a sulfur atom, respectively.

[0023] The term "alkyl," by itself or as part of another molecule 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 herein, such as "heteroalkyl", "haloalkyl" and "homoalkyl".

[0024] The term "alkylene" by itself or as part of another molecule means a divalent radical derived from an alkane, as exemplified, by $(-CH_2-)_n$, wherein n may be 1 to about 24. By way of example only, such groups include, but are not limited to, groups having 10 or fewer carbon atoms such as the structures $-CH_2CH_2-$ and $-CH_2CH_2CH_2CH_2-$. A "lower alkyl" or "lower alkylene" is a shorter chain alkyl or alkylene group, generally having eight or fewer carbon atoms. The term "alkylene," unless otherwise noted, is also meant to include those groups described herein as "heteroalkylene."

[0025] The term "amino acid" refers to naturally occurring and non-natural 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 encoded amino acids are the 20 common amino acids (alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan,

tyrosine, and valine) and pyrolysine and selenocysteine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, by way of example only, an α-carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group. Such analogs may have modified R groups (by way of example, norleucine) or may have modified peptide backbones while still retaining the same basic chemical structure as a naturally occurring amino acid. Non-limiting examples of amino acid analogs include homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium.

[0026] Amino acids may be referred to herein by either their name, their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Additionally, nucleotides, may be referred to by their commonly accepted single-letter codes.

[0027] An "amino terminus modification group" refers to any molecule that can be attached to a terminal amine group. By way of example, such terminal amine groups may be at the end of polymeric molecules, wherein such polymeric molecules include, but are not limited to, polypeptides, polynucleotides, and polysaccharides. Terminus modification groups include but are not limited to, various water soluble polymers, peptides or proteins. By way of example only, terminus modification groups include polyethylene glycol or serum albumin. Terminus modification groups may be used to modify therapeutic characteristics of the polymeric molecule, including but not limited to increasing the serum half-life of peptides.

[0028] By "antibody fragment" is meant any form of an antibody other than the full-length form. Antibody fragments herein include antibodies that are smaller components that exist within full-length antibodies, and antibodies that have been engineered. Antibody fragments include but are not limited to Fv, Fc, Fab, and (Fab')2, single chain Fv (scFv), diabodies, triabodies, tetrabodies, bifunctional hybrid antibodies, CDR1, CDR2, CDR3, combinations of CDR's, variable regions, framework regions, constant regions, heavy chains, light chains, and variable regions, and alternative scaffold non-antibody molecules, bispecific antibodies, and the like (Maynard & Georgiou, 2000, Annu. Rev. Biomed. Eng. 2:339-76; Hudson, 1998, Curr. Opin. Biotechnol. 9:395-402). Another functional substructure is a single chain Fv (scFv), comprised of the variable regions of the immunoglobulin heavy and light chain, covalently connected by a peptide linker (S-z Hu et al., 1996, Cancer Research, 56, 3055-3061). These small (Mr 25,000) proteins generally retain specificity and affinity for antigen in a single polypeptide and can provide a convenient building block for larger, antigen-specific molecules. Unless specifically noted otherwise, statements and claims that use the term "antibody" or "antibodies" specifically includes "antibody fragment" and "antibody fragments."

[0029] By "antibody-drug conjugate, or "ADC", as used herein, refers to an antibody molecule, or fragment thereof, that is covalently bonded to one or more biologically active molecule(s). The biologically active molecule may be conjugated to the antibody through a linker, polymer, or other covalent bond.

[0030] The term "aromatic" or "aryl", as used herein, refers to a closed ring structure which has at least one ring having a conjugated pi electron system and includes both carbocyclic aryl and heterocyclic aryl (or "heteroaryl" or "heteroaromatic") groups. The carbocyclic or heterocyclic aromatic group may contain from 5 to 20 ring atoms. The term includes monocyclic rings linked covalently or fused-ring polycyclic (i.e., rings which share adjacent pairs of carbon atoms) groups. An aromatic group can be unsubstituted or substituted. Non-limiting examples of "aromatic" or "aryl", groups include phenyl, 1-naphthyl, 2-naphthyl, 4-biphenyl, anthracenyl, and phenanthracenyl. Substituents for each of the above noted aryl and heteroaryl ring systems are selected from the group of acceptable substituents described herein.

[0031] For brevity, the term "aromatic" or "aryl" when used in combination with other terms (including but not limited to, aryloxy, arylthioxy, aralkyl) includes both aryl and heteroaryl rings as defined above. Thus, the term "aralkyl" or "alkaryl" is meant to include those radicals in which an aryl group is attached to an alkyl group (including but not limited to, benzyl, phenethyl, pyridylmethyl and the like) including those alkyl groups in which a carbon atom (including but not limited to, a methylene group) has been replaced by a heteroatom, by way of example only, by an oxygen atom. Examples of such aryl groups include, but are not limited to, phenoxymethyl, 2-pyridyloxymethyl, 3-(1-naphthyloxy)propyl, and the like.

[0032] The term "arylene", as used herein, refers to a divalent aryl radical. Non-limiting examples of "arylene" include phenylene, pyridinylene, pyrimidinylene and thiophenylene. Substituents for arylene groups are selected from the group of acceptable substituents described herein.

[0033] A "bifunctional polymer", also referred to as a "bifunctional linker", refers to a polymer comprising two functional groups that are capable of reacting specifically with other moieties to form covalent or non-covalent linkages. Such moieties may include, but are not limited to, the side groups on natural or non-natural amino acids or peptides which contain such natural or non-natural amino acids. The other moieties that may be linked to the bifunctional linker or bifunctional polymer may be the same or different moieties. By way of example only, a bifunctional linker may have a functional group reactive with a group on a first peptide, and another functional group which is reactive with a group on a second peptide, whereby forming a conjugate that includes the first peptide, the bifunctional linker and the second peptide. Many procedures and linker molecules for attachment of various compounds to peptides are known. See, e.g., European Patent Application No. 188,256; U.S. Patent Nos. 4,671,958, 4,659,839, 4,414,148, 4,699,784; 4,680,338; and 4,569,789. A "multi-functional polymer" also referred to as a "multi-functional linker", refers to a polymer comprising

two or more functional groups that are capable of reacting with other moieties. Such moieties may include, but are not limited to, the side groups on natural or non-natural amino acids or peptides which contain such natural or non-natural amino acids. (including but not limited to, amino acid side groups) to form covalent or non-covalent linkages. A bi-functional polymer or multi-functional polymer may be any desired length or molecular weight, and may be selected to provide a particular desired spacing or conformation between one or more molecules linked to a compound and molecules it binds to or the compound.

[0034] The term "bioavailability," as used herein, refers to the rate and extent to which a substance or its active moiety is delivered from a pharmaceutical dosage form and becomes available at the site of action or in the general circulation. Increases in bioavailability refers to increasing the rate and extent a substance or its active moiety is delivered from a pharmaceutical dosage form and becomes available at the site of action or in the general circulation. By way of example, an increase in bioavailability may be indicated as an increase in concentration of the substance or its active moiety in the blood when compared to other substances or active moieties. A non-limiting example of a method to evaluate increases in bioavailability is given in examples 21-25. This method may be used for evaluating the bioavailability of any polypeptide.

[0035] The term "biologically active molecule", "biologically active moiety" or "biologically active agent" when used herein means any substance which can affect any physical or biochemical properties of a biological system, pathway, molecule, or interaction relating to an organism, including but not limited to, viruses, bacteria, bacteriophage, transposon, prion, insects, fungi, plants, animals, and humans. In particular, as used herein, biologically active molecules include but are not limited to any substance intended for diagnosis, cure, mitigation, treatment, or prevention of disease in humans or other animals, or to otherwise enhance physical or mental well-being of humans or animals. Examples of biologically active molecules include, but are not limited to, peptides, proteins, enzymes, small molecule drugs, hard drugs, soft drugs, prodrugs, carbohydrates, inorganic atoms or molecules, dyes, lipids, nucleosides, radionuclides, oligonucleotides, toxins, cells, viruses, liposomes, microparticles and micelles. Classes of biologically active agents that are suitable for use with the methods and compositions described herein include, but are not limited to, drugs, prodrugs, radionuclides, imaging agents, polymers, antibiotics, fungicides, anti-viral agents, anti-inflammatory agents, anti-tumor agents, cardiovascular agents, anti-anxiety agents, hormones, growth factors, steroidal agents, microbially derived toxins, and the like.

[0036] By "modulating biological activity" is meant increasing or decreasing the reactivity of a polypeptide, altering the selectivity of the polypeptide, enhancing or decreasing the substrate selectivity of the polypeptide. Analysis of modified biological activity can be performed by comparing the biological activity of the non-natural polypeptide to that of the natural polypeptide.

[0037] The term "biomaterial," as used herein, refers to a biologically-derived material, including but not limited to material obtained from bioreactors and/or from recombinant methods and techniques.

[0038] The term "biophysical probe," as used herein, refers to probes which can detect or monitor structural changes in molecules. Such molecules include, but are not limited to, proteins and the "biophysical probe" may be used to detect or monitor interaction of proteins with other macromolecules. Examples of biophysical probes include, but are not limited to, spin-labels, a fluorophores, and photoactivatible groups.

[0039] The term "biosynthetically," as used herein, refers to any method utilizing a translation system (cellular or non-cellular), including use of at least one of the following components: a polynucleotide, a codon, a tRNA, and a ribosome. By way of example, non-natural amino acids may be "biosynthetically incorporated" into non-natural amino acid polypeptides using the methods and techniques described herein, "In vivo generation of polypeptides comprising non-natural amino acids", and in the non-limiting example 20. Additionally, the methods for the selection of useful non-natural amino acids which may be "biosynthetically incorporated" into non-natural amino acid polypeptides are described in the non-limiting examples 20.

[0040] The term "biotin analogue," or also referred to as "biotin mimic", as used herein, is any molecule, other than biotin, which bind with high affinity to avidin and/or streptavidin.

[0041] The term "carbonyl" as used herein refers to a group containing at a moiety selecting from the group consisting of -C(O)-, -S(O)-, -S(O)2-, and -C(S)-, including, but not limited to, groups containing a least one ketone group, and/or at least one aldehyde groups, and/or at least one ester group, and/or at least one carboxylic acid group, and/or at least one thioester group. Such carbonyl groups include ketones, aldehydes, carboxylic acids, esters, and thioesters. In addition, such groups may be part of linear, branched, or cyclic molecules.

[0042] The term "carboxy terminus modification group" refers to any molecule that can be attached to a terminal carboxy group. By way of example, such terminal carboxy groups may be at the end of polymeric molecules, wherein such polymeric molecules include, but are not limited to, polypeptides, polynucleotides, and polysaccharides. Terminus modification groups include but are not limited to, various water soluble polymers, peptides or proteins. By way of example only, terminus modification groups include polyethylene glycol or serum albumin. Terminus modification groups may be used to modify therapeutic characteristics of the polymeric molecule, including but not limited to increasing the serum half-life of peptides.

[0043] The term "chemically cleavable group," also referred to as "chemically labile", as used herein, refers to a group which breaks or cleaves upon exposure to acid, base, oxidizing agents, reducing agents, chemical inititiators, or radical initiators.

[0044] The term "chemiluminescent group," as used herein, refers to a group which emits light as a result of a chemical reaction without the addition of heat. By way of example only, luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) reacts with oxidants like hydrogen peroxide (H_2O_2) in the presence of a base and a metal catalyst to produce an excited state product (3-aminophthalate, 3-APA).

[0045] The term "chromophore," as used herein, refers to a molecule which absorbs light of visible wavelengths, UV wavelengths or IR wavelengths.

[0046] The term "cofactor," as used herein, refers to an atom or molecule essential for the action of a large molecule. Cofactors include, but are not limited to, inorganic ions, coenzymes, proteins, or some other factor necessary for the activity of enzymes. Examples include heme in hemoglobin, magnesium in chlorophyll, and metal ions for proteins.

[0047] "Cofolding," as used herein, refers to refolding processes, reactions, or methods which employ at least two molecules which interact with each other and result in the transformation of unfolded or improperly folded molecules to properly folded molecules. By way of example only, "cofolding," employ at least two polypeptides which interact with each other and result in the transformation of unfolded or improperly folded polypeptides to native, properly folded polypeptides. Such polypeptides may contain natural amino acids and/or at least one non-natural amino acid.

[0048] A "comparison window," as used herein, refers a segment of any one of contiguous positions used to compare a sequence to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Such contiguous positions include, but are not limited to a group consisting of from about 20 to about 600 sequential units, including about 50 to about 200 sequential units, and about 100 to about 150 sequential units. By way of example only, such sequences include polypeptides and polypeptides containing non-natural amino acids, with the sequential units include, but are not limited to natural and non-natural amino acids. In addition, by way of example only, such sequences include polynucleotides with nucleotides being the corresponding sequential units. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, including but not limited to, by the local homology algorithm of Smith and Waterman (1970) Adv. Appl. Math. 2:482c, by the homology alignment algorithm of Needleman and Wunsch (1970) J. Mol. Biol. 48:443, by the search for similarity method of Pearson and Lipman (1988) Proc. Nat'l. Acad. Sci. USA 85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (see, e.g., Ausubel et al., Current Protocols in Molecular Biology (1995 supplement)).

[0049] By way of example, an algorithm which may be used to determine percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al. (1997) Nuc. Acids Res. 25:3389-3402, and Altschul et al. (1990) J. Mol. Biol. 215:403-410, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) or 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff and Henikoff (1992) Proc. Natl. Acad. Sci. USA 89:10915) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands. The BLAST algorithm is typically performed with the "low complexity" filter turned off.

[0050] The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90:5873-5787). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, or less than about 0.01, or less than about 0.001.

[0051] The term "conservatively modified variants" applies to both natural and non-natural amino acid and natural and non-natural nucleic acid sequences, and combinations thereof. With respect to particular nucleic acid sequences, "conservatively modified variants" refers to those natural and non-natural nucleic acids which encode identical or essentially identical natural and non-natural amino acid sequences, or where the natural and non-natural nucleic acid does not encode a natural and non-natural amino acid sequence, to essentially identical sequences. By way of example, because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Thus by way of example every natural or non-natural nucleic acid sequence herein which encodes a natural or non-natural polypeptide also describes every possible silent variation of the natural or non-natural

nucleic acid. One of ordinary skill in the art will recognize that each codon in a natural or non-natural nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a natural and non-natural nucleic acid which encodes a natural and non-natural polypeptide is implicit in each described sequence.

[0052] As to amino acid sequences, individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single natural and non-natural amino acid or a small percentage of natural and non-natural amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the deletion of an amino acid, addition of an amino acid, or substitution of a natural and non-natural amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar natural amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the methods and compositions described herein.

[0053] Conservative substitution tables providing functionally similar amino acids are known to those of ordinary skill in the art. The following eight groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Glycine (G);
2) Aspartic acid (D), Glutamic acid (E);
3) Asparagine (N), Glutamine (Q);
4) Arginine (R), Lysine (K);
5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V);
6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W);
7) Serine (S), Threonine (T); and
8) Cysteine (C), Methionine (M)

(see, e.g., Creighton, Proteins:Structures and Molecular Properties (W H Freeman & Co.; 2nd edition (December 1993)

[0054] The terms "cycloalkyl" and "heterocycloalkyl", by themselves or in combination with other terms, represent, unless otherwise stated, cyclic versions of "alkyl" and "heteroalkyl", respectively. Thus, a cycloalkyl or heterocycloalkyl include saturated, partially unsaturated and fully unsaturated ring linkages. Additionally, for heterocycloalkyl, a heteroatom can occupy the position at which the heterocycle is attached to the remainder of the molecule. The heteroatom may include, but is not limited to, oxygen, nitrogen or sulfur. 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. Additionally, the term encompasses multicyclic structures, including but not limited to, bicyclic and tricyclic ring structures. Similarly, the term "heterocycloalkylene" by itself or as part of another molecule means a divalent radical derived from heterocycloalkyl, and the term "cycloalkylene" by itself or as part of another molecule means a divalent radical derived from cycloalkyl.

[0055] The term "cyclodextrin," as used herein, refers to cyclic carbohydrates consisting of at least six to eight glucose molecules in a ring formation. The outer part of the ring contains water soluble groups; at the center of the ring is a relatively nonpolar cavity able to accommodate small molecules.

[0056] The term "cytotoxic," as used herein, refers to a compound which harms cells.

[0057] "Denaturing agent" or "denaturant," as used herein, refers to any compound or material which will cause a reversible unfolding of a polymer. By way of example only, "denaturing agent" or "denaturants," may cause a reversible unfolding of a protein. The strength of a denaturing agent or denaturant will be determined both by the properties and the concentration of the particular denaturing agent or denaturant. By way of example, denaturing agents or denaturants include, but are not limited to, chaotropes, detergents, organic, water miscible solvents, phospholipids, or a combination thereof. Non-limiting examples of chaotropes include, but are not limited to, urea, guanidine, and sodium thiocyanate. Non-limiting examples of detergents may include, but are not limited to, strong detergents such as sodium dodecyl sulfate, or polyoxyethylene ethers (e.g. Tween or Triton detergents), Sarkosyl, mild non-ionic detergents (e.g., digitonin), mild cationic detergents such as N->2,3-(Dioleyoxy)-propyl-N,N,N-trimethylammonium, mild ionic detergents (e.g. sodium cholate or sodium deoxycholate) or zwitterionic detergents including, but not limited to, sulfobetaines (Zwittergent), 3-(3-chlolamidopropyl)dimethylammonio-1-propane sulfate (CHAPS), and 3-(3-chlolamidopropyl)dimethylammonio-2-hydroxy-1-propane sulfonate (CHAPSO). Non-limiting examples of organic, water miscible solvents include, but are not limited to, acetonitrile, lower alkanols (especially C2 - C4 alkanols such as ethanol or isopropanol), or lower alkandiols (C2 - C4 alkandiols such as ethylene-glycol) may be used as denaturants. Non-limiting examples of phospholipids include, but are not limited to, naturally occurring phospholipids such as phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, and phosphatidylinositol or synthetic phospholipid derivatives or variants such as dihexanoylphosphatidylcholine or diheptanoylphosphatidylcholine.

[0058] The term "desired functionality" as used herein refers to any group selected from a label; a dye; a polymer; a

water-soluble polymer; a derivative of polyethylene glycol; a photocrosslinker; a cytotoxic compound; a drug; an affinity label; a photoaffinity label; a reactive compound; a resin; a second protein or polypeptide or polypeptide analog; an antibody or antibody fragment; a metal chelator; a cofactor; a fatty acid; a carbohydrate; a polynucleotide; a DNA; a RNA; an antisense polynucleotide; a saccharide, a water-soluble dendrimer, a cyclodextrin, a biomaterial; a nanoparticle; a spin label; a fluorophore; a metal-containing moiety; a radioactive moiety; a novel functional group; a group that covalently or noncovalently interacts with other molecules; a photocaged moiety; an actinic radiation excitable moiety; a ligand; a photoisomerizable moiety; biotin; a biotin analogue; a moiety incorporating a heavy atom; a chemically cleavable group; a photocleavable group; an elongated side chain; a carbon-linked sugar; a redox-active agent; an amino thioacid; a toxic moiety; an isotopically labeled moiety; a biophysical probe; a phosphorescent group; a chemiluminescent group; an electron dense group; a magnetic group; an intercalating group; a chromophore; an energy transfer agent; a biologically active agent (in which case, the biologically active agent can include an agent with therapeutic activity and the non-natural amino acid polypeptide or modified non-natural amino acid can serve either as a co-therapeutic agent with the attached therapeutic agent or as a means for delivery the therapeutic agent to a desired site within an organism); a detectable label; a small molecule; an inhibitory ribonucleic acid; a radionucleotide; a neutron-capture agent; a derivative of biotin; quantum dot(s); a nanotransmitter; a radiotransmitter; an abzyme, an activated complex activator, a virus, an adjuvant, an aglycan, an allergan, an angiostatin, an antihormone, an antioxidant, an aptamer, a guide RNA, a saponin, a shuttle vector, a macromolecule, a mimotope, a receptor, a reverse micelle, and any combination thereof.

[0059] The term "diamine,"as used herein, refers to groups/molecules comprising at least two amine functional groups, including, but not limited to, a hydrazine group, an amidine group, an imine group, a 1,1-diamine group, a 1,2-diamine group, a 1,3-diamine group, and a 1,4-diamine group. In addition, such groups may be part of linear, branched, or cyclic molecules.

[0060] The term "detectable label," as used herein, refers to a label which may be observable using analytical techniques including, but not limited to, fluorescence, chemiluminescence, electron-spin resonance, ultraviolet/visible absorbance spectroscopy, mass spectrometry, nuclear magnetic resonance, magnetic resonance, and electrochemical methods.

[0061] The term "dicarbonyl" as used herein refers to a group containing at least two moieties selected from the group consisting of -C(O)-, -S(O)-, -$S(O)_2$-, and -C(S)-, including, but not limited to, 1,2-dicarbonyl groups, a 1,3-dicarbonyl groups, and 1,4-dicarbonyl groups, and groups containing a least one ketone group, and/or at least one aldehyde groups, and/or at least one ester group, and/or at least one carboxylic acid group, and/or at least one thioester group. Such dicarbonyl groups include diketones, ketoaldehydes, ketoacids, ketoesters, and ketothioesters. In addition, such groups may be part of linear, branched, or cyclic molecules. The two moieties in the dicarbonyl group may be the same or different, and may include substituents that would produce, by way of example only, an ester, a ketone, an aldehyde, a thioester, or an amide, at either of the two moieties.

[0062] The term "drug," as used herein, refers to any substance used in the prevention, diagnosis, alleviation, treatment, or cure of a disease or condition.

[0063] The term "dye," as used herein, refers to a soluble, coloring substance which contains a chromophore.

[0064] The term "effective amount," as used herein, refers to a sufficient amount of an agent or a compound being administered which will relieve to some extent one or more of the symptoms of the disease or condition being treated. The result can be reduction and/or alleviation of the signs, symptoms, or causes of a disease, or any other desired alteration of a biological system. By way of example, an agent or a compound being administered includes, but is not limited to, a natural amino acid polypeptide, non-natural amino acid polypeptide, modified natural amino acid polypeptide, or modified non-amino acid polypeptide. Compositions containing such natural amino acid polypeptides, non-natural amino acid polypeptides, modified natural amino acid polypeptides, or modified non-natural amino acid polypeptides can be administered for prophylactic, enhancing, and/or therapeutic treatments. An appropriate "effective" amount in any individual case may be determined using techniques, such as a dose escalation study.

[0065] The term "electron dense group," as used herein, refers to a group which scatters electrons when irradiated with an electron beam. Such groups include, but are not limited to, ammonium molybdate, bismuth subnitrate cadmium iodide, 99%, carbohydrazide, ferric chloride hexahydrate, hexamethylene tetramine, 98.5%, indium trichloride anhydrous, lanthanum nitrate, lead acetate trihydrate, lead citrate trihydrate, lead nitrate, periodic acid, phosphomolybdic acid, phosphotungstic acid, potassium ferricyanide, potassium ferrocyanide, ruthenium red, silver nitrate, silver proteinate (Ag Assay: 8.0-8.5%) "Strong", silver tetraphenylporphin (S-TPPS), sodium chloroaurate, sodium tungstate, thallium nitrate, thiosemicarbazide (TSC), uranyl acetate, uranyl nitrate, and vanadyl sulfate.

[0066] The term "energy transfer agent," as used herein, refers to a molecule which can either donate or accept energy from another molecule. By way of example only, fluorescence resonance energy transfer (FRET) is a dipole-dipole coupling process by which the excited-state energy of a fluorescence donor molecule is non-radiatively transferred to an unexcited acceptor molecule which then fluorescently emits the donated energy at a longer wavelength.

[0067] The terms "enhance" or "enhancing" means to increase or prolong either in potency or duration a desired effect. By way of example, "enhancing" the effect of therapeutic agents refers to the ability to increase or prolong, either in

potency or duration, the effect of therapeutic agents on during treatment of a disease, disorder or condition. An "enhancing-effective amount," as used herein, refers to an amount adequate to enhance the effect of a therapeutic agent in the treatment of a disease, disorder or condition. When used in a patient, amounts effective for this use will depend on the severity and course of the disease, disorder or condition, previous therapy, the patient's health status and response to the drugs, and the judgment of the treating physician.

[0068] As used herein, the term "eukaryote" refers to organisms belonging to the phylogenetic domain Eucarya, including but not limited to animals (including but not limited to, mammals, insects, reptiles, birds, etc.), ciliates, plants (including but not limited to, monocots, dicots, and algae), fungi, yeasts, flagellates, microsporidia, and protists.

[0069] The term "fatty acid," as used herein, refers to carboxylic acids with about C6 or longer hydrocarbon side chain.

[0070] The term "fluorophore," as used herein, refers to a molecule which upon excitation emits photons and is thereby fluorescent.

[0071] The terms "functional group", "active moiety", "activating group", "leaving group", "reactive site", "chemically reactive group" and "chemically reactive moiety," as used herein, refer to portions or units of a molecule at which chemical reactions occur. The terms are somewhat synonymous in the chemical arts and are used herein to indicate the portions of molecules that perform some function or activity and are reactive with other molecules.

[0072] The term "halogen" includes fluorine, chlorine, iodine, and bromine.

[0073] The term "haloacyl," as used herein, refers to acyl groups which contain halogen moieties, including, but not limited to, $-C(O)CH_3$, $-C(O)CF_3$, $-C(O)CH_2OCH_3$, and the like.

[0074] The term "haloalkyl," as used herein, refers to alkyl groups which contain halogen moieties, including, but not limited to, $-CF_3$ and $-CH_2CF_3$ and the like.

[0075] The term "heteroalkyl," as used herein, refers to straight or branched chain, or cyclic hydrocarbon radicals, or combinations thereof, consisting of an alkyl group and at least one heteroatom selected from the group consisting of O, N, Si and S, and wherein the nitrogen and sulfur atoms may optionally be oxidized and the nitrogen heteroatom may optionally be quaternized. The heteroatom(s) O, N and 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 $-CH=CH-N(CH_3)-CH_3$. In addition, up to two heteroatoms may be consecutive, such as, by way of example, $-CH_2-NH-OCH_3$ and $-CH_2-O-Si(CH_3)_3$.

[0076] The terms "heterocyclic-based linkage" or "heterocycle linkage" refers to a moiety formed from the reaction of a dicarbonyl group with a diamine group. The resulting reaction product is a heterocycle, including a heteroaryl group or a heterocycloalkyl group. The resulting heterocycle group serves as a chemical link between a non-natural amino acid or non-natural amino acid polypeptide and another functional group. In one embodiment, the heterocycle linkage includes a nitrogen-containing heterocycle linkage, including by way of example only a pyrazole linkage, a pyrrole linkage, an indole linkage, a benzodiazepine linkage, and a pyrazalone linkage.

[0077] Similarly, the term "heteroalkylene" refers to 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, the same or different heteroatoms can also occupy either or both of the chain termini (including but not limited to, alkyleneoxy, alkylenedioxy, alkyleneamino, alkylenediamino, aminooxyalkylene, and the like). 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. By way of example, the formula $-C(O)_2R'-$ represents both $-C(O)_2R'-$ and $-R'C(O)_2-$.

[0078] The term "heteroaryl" or "heteroaromatic," as used herein, refers to aryl groups which contain at least one heteroatom selected from N, O, and S; wherein the nitrogen and sulfur atoms may be optionally oxidized, and the nitrogen atom(s) may be optionally quaternized. Heteroaryl groups may be substituted or unsubstituted. A heteroaryl group may be attached to the remainder of the molecule through a heteroatom. Non-limiting examples of heteroaryl groups include 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.

[0079] The term "homoalkyl," as used herein refers to alkyl groups which are hydrocarbon groups.

[0080] The term "identical," as used herein, refers to two or more sequences or subsequences which are the same. In addition, the term "substantially identical," as used herein, refers to two or more sequences which have a percentage of sequential units which are the same when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using comparison algorithms or by manual alignment and visual inspection. By way of example only, two or more sequences may be "substantially identical" if the sequential units are about 60% identical, about 65% identical, about 70% identical, about 75% identical, about 80% identical, about 85% identical, about 90% identical, or about 95% identical over a specified region. Such percentages to describe the "percent identity" of two or more sequences. The identity of a sequence can exist over a region that is at least about 75-100 sequential units in length, over a region that is about 50 sequential units in length, or, where not specified, across the entire sequence.

This definition also refers to the complement of a test sequence. By way of example only, two or more polypeptide sequences are identical when the amino acid residues are the same, while two or more polypeptide sequences are "substantially identical" if the amino acid residues are about 60% identical, about 65% identical, about 70% identical, about 75% identical, about 80% identical, about 85% identical, about 90% identical, or about 95% identical over a specified region. The identity can exist over a region that is at least about 75 to about 100 amino acids in length, over a region that is about 50 amino acids in length, or, where not specified, across the entire sequence of a polypeptide sequence. In addition, by way of example only, two or more polynucleotide sequences are identical when the nucleic acid residues are the same, while two or more polynucleotide sequences are "substantially identical" if the nucleic acid residues are about 60% identical, about 65% identical, about 70% identical, about 75% identical, about 80% identical, about 85% identical, about 90% identical, or about 95% identical over a specified region. The identity can exist over a region that is at least about 75 to about 100 nucleic acids in length, over a region that is about 50 nucleic acids in length, or, where not specified, across the entire sequence of a polynucleotide sequence.

[0081] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

[0082] The term "immunogenicity," as used herein, refers to an antibody response to administration of a therapeutic drug. The immunogenicity toward therapeutic non-natural amino acid polypeptides can be obtained using quantitative and qualitative assays for detection of anti-non-natural amino acid polypeptides antibodies in biological fluids. Such assays include, but are not limited to, Radioimmunoassay (RIA), Enzyme-linked immunosorbent assay (ELISA), luminescent immunoassay (LIA), and fluorescent immunoassay (FIA). Analysis of immunogenicity toward therapeutic non-natural amino acid polypeptides involves comparing the antibody response upon administration of therapeutic non-natural amino acid polypeptides to the antibody response upon administration of therapeutic natural amino acid polypeptides.

[0083] The term "intercalating agent," also referred to as "intercalating group," as used herein, refers to a chemical that can insert into the intramolecular space of a molecule or the intermolecular space between molecules. By way of example only an intercalating agent or group may be a molecule which inserts into the stacked bases of the DNA double helix.

[0084] The term "isolated," as used herein, refers to separating and removing a component of interest from components not of interest. Isolated substances can be in either a dry or semi-dry state, or in solution, including but not limited to an aqueous solution. The isolated component can be in a homogeneous state or the isolated component can be a part of a pharmaceutical composition that comprises additional pharmaceutically acceptable carriers and/or excipients. Purity and homogeneity may be determined using analytical chemistry techniques including, but not limited to, polyacrylamide gel electrophoresis or high performance liquid chromatography. In addition, when a component of interest is isolated and is the predominant species present in a preparation, the component is described herein as substantially purified. The term "purified," as used herein, may refer to a component of interest which is at least 85% pure, at least 90% pure, at least 95% pure, at least 99% or greater pure. By way of example only, nucleic acids or proteins are "isolated" when such nucleic acids or proteins are free of at least some of the cellular components with which it is associated in the natural state, or that the nucleic acid or protein has been concentrated to a level greater than the concentration of its in vivo or in vitro production. Also, by way of example, a gene is isolated when separated from open reading frames which flank the gene and encode a protein other than the gene of interest.

[0085] The term "label," as used herein, refers to a substance which is incorporated into a compound and is readily detected, whereby its physical distribution may be detected and/or monitored.

[0086] The term "linkage," as used herein to refer to bonds or chemical moiety formed from a chemical reaction between the functional group of a linker and another molecule. Such bonds may include, but are not limited to, covalent linkages and non-covalent bonds, while such chemical moieties may include, but are not limited to, esters, carbonates, imines phosphate esters, hydrazones, acetals, orthoesters, peptide linkages, and oligonucleotide linkages. Hydrolytically stable linkages means that the linkages are substantially stable in water and do not react with water at useful pH values, including but not limited to, under physiological conditions for an extended period of time, perhaps even indefinitely. Hydrolytically unstable or degradable linkages mean that the linkages are degradable in water or in aqueous solutions, including for example, blood. Enzymatically unstable or degradable linkages mean that the linkage can be degraded by one or more enzymes. By way of example only, PEG and related polymers may include degradable linkages in the polymer backbone or in the linker group between the polymer backbone and one or more of the terminal functional groups of the polymer molecule. Such degradable linkages include, but are not limited to ester linkages formed by the reaction of PEG carboxylic acids or activated PEG carboxylic acids with alcohol groups on a biologically active agent, wherein such ester groups generally hydrolyze under physiological conditions to release the biologically active agent.

Other hydrolytically degradable linkages include but are not limited to carbonate linkages; imine linkages resulted from reaction of an amine and an aldehyde; phosphate ester linkages formed by reacting an alcohol with a phosphate group; hydrazone linkages which are reaction product of a hydrazide and an aldehyde; acetal linkages that are the reaction product of an aldehyde and an alcohol; orthoester linkages that are the reaction product of a formate and an alcohol; peptide linkages formed by an amine group, including but not limited to, at an end of a polymer such as PEG, and a carboxyl group of a peptide; and oligonucleotide linkages formed by a phosphoramidite group, including but not limited to, at the end of a polymer, and a 5' hydroxyl group of an oligonucleotide.

[0087] The terms "medium" or "media," as used herein, refer to any culture medium used to grow and harvest cells and/or products expressed and/or secreted by such cells. Such "medium" or "media" include, but are not limited to, solution, solid, semi-solid, or rigid supports that may support or contain any host cell, including, by way of example, bacterial host cells, yeast host cells, insect host cells, plant host cells, eukaryotic host cells, mammalian host cells, CHO cells, prokaryotic host cells, E. coli, or Pseudomonas host cells, and cell contents. Such "medium" or "media" includes, but is not limited to, medium or media in which the host cell has been grown into which a polypeptide has been secreted, including medium either before or after a proliferation step. Such "medium" or "media" also includes, but is not limited to, buffers or reagents that contain host cell lysates, by way of example a polypeptide produced intracellularly and the host cells are lysed or disrupted to release the polypeptide.

[0088] The term "metabolite," as used herein, refers to a derivative of a compound, by way of example natural amino acid polypeptide, a non-natural amino acid polypeptide, a modified natural amino acid polypeptide, or a modified non-natural amino acid polypeptide, that is formed when the compound, by way of example natural amino acid polypeptide, non-natural amino acid polypeptide, modified natural amino acid polypeptide, or modified non-natural amino acid polypeptide, is metabolized. The term "pharmaceutically active metabolite" or "active metabolite" refers to a biologically active derivative of a compound, by way of example natural amino acid polypeptide, a non-natural amino acid polypeptide, a modified natural amino acid polypeptide, or a modified non-natural amino acid polypeptide, that is formed when such a compound, by way of example a natural amino acid polypeptide, non-natural amino acid polypeptide, modified natural amino acid polypeptide, or modified non-natural amino acid polypeptide, is metabolized.

[0089] The term "metabolized," as used herein, refers to the sum of the processes by which a particular substance is changed by an organism. Such processes include, but are not limited to, hydrolysis reactions and reactions catalyzed by enzymes. Further information on metabolism may be obtained from The Pharmacological Basis of Therapeutics, 9th Edition, McGraw-Hill (1996). By way of example only, metabolites of natural amino acid polypeptides, non-natural amino acid polypeptides, modified natural amino acid polypeptides, or modified non-natural amino acid polypeptides may be identified either by administration of the natural amino acid polypeptides, non-natural amino acid polypeptides, modified natural amino acid polypeptides, or modified non-natural amino acid polypeptides to a host and analysis of tissue samples from the host, or by incubation of natural amino acid polypeptides, non-natural amino acid polypeptides, modified natural amino acid polypeptides, or modified non-natural amino acid polypeptides with hepatic cells in vitro and analysis of the resulting compounds.

[0090] The term "metal chelator," as used herein, refers to a molecule which forms a metal complex with metal ions. By way of example, such molecules may form two or more coordination bonds with a central metal ion and may form ring structures.

[0091] The term "metal-containing moiety," as used herein, refers to a group which contains a metal ion, atom or particle. Such moieties include, but are not limited to, cisplatin, chelated metals ions (such as nickel, iron, and platinum), and metal nanoparticles (such as nickel, iron, and platinum).

[0092] The term "moiety incorporating a heavy atom," as used herein, refers to a group which incorporates an ion of atom which is usually heavier than carbon. Such ions or atoms include, but are not limited to, silicon, tungsten, gold, lead, and uranium.

[0093] The term "modified," as used herein refers to the presence of a change to a natural amino acid, a non-natural amino acid, a natural amino acid polypeptide or a non-natural amino acid polypeptide. Such changes, or modifications, may be obtained by post synthesis modifications of natural amino acids, non-natural amino acids, natural amino acid polypeptides or non-natural amino acid polypeptides, or by co-translational, or by post-translational modification of natural amino acids, non-natural amino acids, natural amino acid polypeptides or non-natural amino acid polypeptides. The form "modified or unmodified" means that the natural amino acid, non-natural amino acid, natural amino acid polypeptide or non-natural amino acid polypeptide being discussed are optionally modified, that is, he natural amino acid, non-natural amino acid, natural amino acid polypeptide or non-natural amino acid polypeptide under discussion can be modified or unmodified.

[0094] As used herein, the term "modulated serum half-life" refers to positive or negative changes in the circulating half-life of a modified biologically active molecule relative to its non-modified form. By way of example, the modified biologically active molecules include, but are not limited to, natural amino acid, non-natural amino acid, natural amino acid polypeptide or non-natural amino acid polypeptide. By way of example, serum half-life is measured by taking blood samples at various time points after administration of the biologically active molecule or modified biologically active

molecule, and determining the concentration of that molecule in each sample. Correlation of the serum concentration with time allows calculation of the serum half-life. By way of example, modulated serum half-life may be an increased in serum half-life, which may enable an improved dosing regimens or avoid toxic effects. Such increases in serum may be at least about two fold, at least about three-fold, at least about five-fold, or at least about ten-fold. A non-limiting example of a method to evaluate increases in serum half-life is given in example 33. This method may be used for evaluating the serum half-life of any polypeptide.

[0095] The term "modulated therapeutic half-life," as used herein, refers to positive or negative change in the half-life of the therapeutically effective amount of a modified biologically active molecule, relative to its non-modified form. By way of example, the modified biologically active molecules include, but are not limited to, natural amino acid, non-natural amino acid, natural amino acid polypeptide or non-natural amino acid polypeptide. By way of example, therapeutic half-life is measured by measuring pharmacokinetic and/or pharmacodynamic properties of the molecule at various time points after administration. Increased therapeutic half-life may enable a particular beneficial dosing regimen, a particular beneficial total dose, or avoids an undesired effect. By way of example, the increased therapeutic half-life may result from increased potency, increased or decreased binding of the modified molecule to its target, an increase or decrease in another parameter or mechanism of action of the non-modified molecule, or an increased or decreased breakdown of the molecules by enzymes such as, by way of example only, proteases. A non-limiting example of a method to evaluate increases in therapeutic half-life is given in example 33. This method may be used for evaluating the therapeutic half-life of any polypeptide.

[0096] The term "nanoparticle," as used herein, refers to a particle which has a particle size between about 500 nm to about 1 nm.

[0097] The term "near-stoichiometric," as used herein, refers to the ratio of the moles of compounds participating in a chemical reaction being about 0.75 to about 1.5.

[0098] As used herein, the term "non-eukaryote" refers to non-eukaryotic organisms. By way of example, a non-eukaryotic organism may belong to the Eubacteria, (which includes but is not limited to, Escherichia coli, Thermus thermophilus, or Bacillus stearothermophilus, Pseudomonas fluorescens, Pseudomonas aeruginosa, Pseudomonas putida), phylogenetic domain, or the Archaea, which includes, but is not limited to, Methanococcus jannaschii, Methanobacterium thermoautotrophicum, Archaeoglobus fulgidus, Pyrococcus furiosus, Pyrococcus horikoshii, Aeuropyrum pernix, or Halobacterium such as Haloferax volcanii and Halobacterium species NRC-1, or phylogenetic domain.

[0099] A "non-natural amino acid" refers to an amino acid that is not one of the 20 common amino acids or pyrolysine or selenocysteine. Other terms that may be used synonymously with the term "non-natural amino acid" is "non-naturally encoded amino acid," "unnatural amino acid," "non-naturally-occurring amino acid," and variously hyphenated and non-hyphenated versions thereof. The term "non-natural amino acid" includes, but is not limited to, amino acids which occur naturally by modification of a naturally encoded amino acid (including but not limited to, the 20 common amino acids or pyrrolysine and selenocysteine) but are not themselves incorporated into a growing polypeptide chain by the translation complex. Examples of naturally-occurring amino acids that are not naturally-encoded include, but are not limited to, N-acetylglucosaminyl-L-serine, N-acetylglucosaminyl-L-threonine, and O-phosphotyrosine. Additionally, the term "non-natural amino acid" includes, but is not limited to, amino acids which do not occur naturally and may be obtained synthetically or may be obtained by modification of non-natural amino acids.

[0100] The term "nucleic acid," as used herein, refers to deoxyribonucleotides, deoxyribonucleosides, ribonucleosides or ribonucleotides and polymers thereof in either single- or double-stranded form. By way of example only, such nucleic acids and nucleic acid polymers include, but are not limited to, (i) analogues of natural nucleotides which have similar binding properties as a reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides; (ii) oligonucleotide analogs including, but are not limited to, PNA (peptidonucleic acid), analogs of DNA used in antisense technology (phosphorothioates, phosphoroamidates, and the like); (iii) conservatively modified variants thereof (including but not limited to, degenerate codon substitutions) and complementary sequences and sequence explicitly indicated. By way of example, 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); and Rossolini et al., Mol. Cell. Probes 8:91-98 (1994)).

[0101] The term "oxidizing agent," as used herein, refers to a compound or material which is capable of removing an electron from a compound being oxidized. By way of example oxidizing agents include, but are not limited to, oxidized glutathione, cystine, cystamine, oxidized dithiothreitol, oxidized erythreitol, and oxygen. A wide variety of oxidizing agents are suitable for use in the methods and compositions described herein.

[0102] The term "pharmaceutically acceptable", as used herein, refers to a material, including but not limited, to a salt, carrier or diluent, which does not abrogate the biological activity or properties of the compound, and is relatively nontoxic, i.e., the material may be administered to an individual without causing undesirable biological effects or interacting in a deleterious manner with any of the components of the composition in which it is contained.

[0103] The term "photoaffinity label," as used herein, refers to a label with a group, which, upon exposure to light,

forms a linkage with a molecule for which the label has an affinity. By way of example only, such a linkage may be covalent or non-covalent.

[0104] The term "photocaged moiety," as used herein, refers to a group which, upon illumination at certain wavelengths, covalently or non-covalently binds other ions or molecules.

[0105] The term "photocleavable group," as used herein, refers to a group which breaks upon exposure to light.

[0106] The term "photocrosslinker," as used herein, refers to a compound comprising two or more functional groups which, upon exposure to light, are reactive and form a covalent or non-covalent linkage with two or more monomeric or polymeric molecules.

[0107] The term "photoisomerizable moiety," as used herein, refers to a group wherein upon illumination with light changes from one isomeric form to another.

[0108] The term "polyalkylene glycol," as used herein, refers to linear or branched polymeric polyether polyols. Such polyalkylene glycols, including, but are not limited to, polyethylene glycol, polypropylene glycol, polybutylene glycol, and derivatives thereof. Other exemplary embodiments are listed, for example, in commercial supplier catalogs, such as Shearwater Corporation's catalog "Polyethylene Glycol and Derivatives for Biomedical Applications" (2001). By way of example only, such polymeric polyether polyols have average molecular weights between about 0.1 kDa to about 100 kDa. By way of example, such polymeric polyether polyols include, but are not limited to, between about 100 Da and about 100,000 Da or more. The molecular weight of the polymer may be between about 100 Da and about 100,000 Da, including but not limited to, about 100,000 Da, about 95,000 Da, about 90,000 Da, about 85,000 Da, about 80,000 Da, about 75,000 Da, about 70,000 Da, about 65,000 Da, about 60,000 Da, about 55,000 Da, about 50,000 Da, about 45,000 Da, about 40,000 Da, about 35,000 Da, about 30,000 Da, about 25,000 Da, about 20,000 Da, about 15,000 Da, about 10,000 Da, about 9,000 Da, about 8,000 Da, about 7,000 Da, about 6,000 Da, about 5,000 Da, about 4,000 Da, about 3,000 Da, about 2,000 Da, about 1,000 Da, about 900 Da, about 800 Da, about 700 Da, about 600 Da, about 500 Da, 400 Da, about 300 Da, about 200 Da, and about 100 Da. In some embodiments molecular weight of the polymer is between about 100 Da and about 50,000 Da. In some embodiments, the molecular weight of the polymer is between about 100 Da and about 40,000 Da. In some embodiments, the molecular weight of the polymer is between about 1,000 Da and about 40,000 Da. In some embodiments, the molecular weight of the polymer is between about 2,000 to about 50,000 Da. In some embodiments, the molecular weight of the polymer is between about 5,000 Da and about 40,000 Da. In some embodiments, the molecular weight of the polymer is between about 10,000 Da and about 40,000 Da. In some embodiments, the poly(ethylene glycol) molecule is a branched polymer. The molecular weight of the branched chain PEG may be between about 1,000 Da and about 100,000 Da, including but not limited to, about 100,000 Da, about 95,000 Da, about 90,000 Da, about 85,000 Da, about 80,000 Da, about 75,000 Da, about 70,000 Da, about 65,000 Da, about 60,000 Da, about 55,000 Da, about 50,000 Da, about 45,000 Da, about 40,000 Da, about 35,000 Da, about 30,000 Da, about 25,000 Da, about 20,000 Da, about 15,000 Da, about 10,000 Da, about 9,000 Da, about 8,000 Da, about 7,000 Da, about 6,000 Da, about 5,000 Da, about 4,000 Da, about 3,000 Da, about 2,000 Da, and about 1,000 Da. In some embodiments, the molecular weight of the branched chain PEG is between about 1,000 Da and about 50,000 Da. In some embodiments, the molecular weight of the branched chain PEG is between about 1,000 Da and about 40,000 Da. In some embodiments, the molecular weight of the branched chain PEG is between about 5,000 Da and about 40,000 Da. In some embodiments, the molecular weight of the branched chain PEG is between about 5,000 Da and about 20,000 Da. In other embodiments, the molecular weight of the branched chain PEG is between about 2,000 to about 50,000 Da.

[0109] The term "polymer," as used herein, refers to a molecule composed of repeated subunits. Such molecules include, but are not limited to, polypeptides, polynucleotides, or polysaccharides or polyalkylene glycols.

[0110] The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. That is, a description directed to a polypeptide applies equally to a description of a peptide and a description of a protein, and vice versa. The terms apply to naturally occurring amino acid polymers as well as amino acid polymers in which one or more amino acid residues is a non-natural amino acid. Additionally, such "polypeptides," "peptides" and "proteins" include amino acid chains of any length, including full length proteins, wherein the amino acid residues are linked by covalent peptide bonds.

[0111] The term "post-translationally modified" refers to any modification of a natural or non-natural amino acid which occurs after such an amino acid has been translationally incorporated into a polypeptide chain. Such modifications include, but are not limited to, co-translational in vivo modifications, co-translational in vitro modifications (such as in a cell-free translation system), post-translational in vivo modifications, and post-translational in vitro modifications.

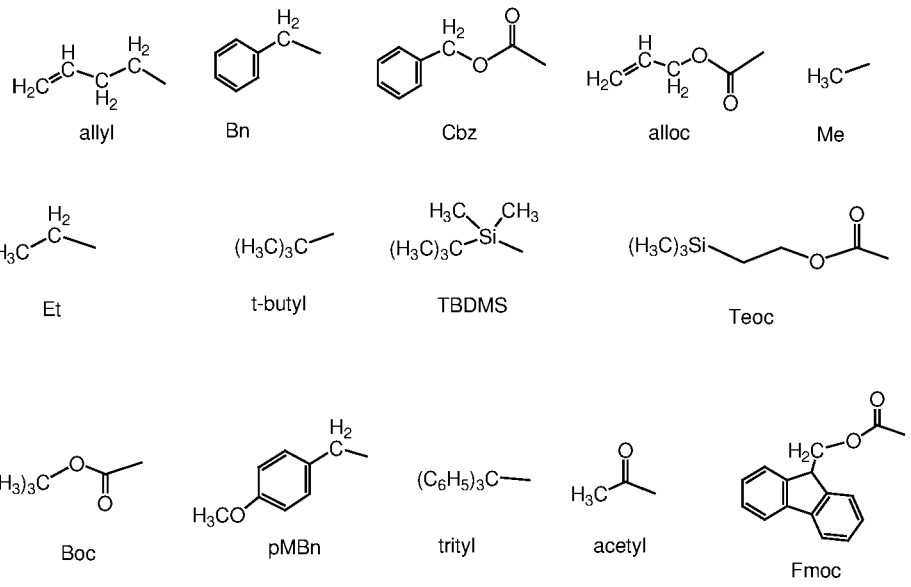
[0112] The terms "prodrug" or "pharmaceutically acceptable prodrug," as used herein, refers to an agent that is converted into the parent drug in vivo or in vitro, wherein which does not abrogate the biological activity or properties of the drug, and is relatively nontoxic, i.e., the material may be administered to an individual without causing undesirable biological effects or interacting in a deleterious manner with any of the components of the composition in which it is contained. Prodrugs are generally drug precursors that, following administration to a subject and subsequent absorption, are converted to an active, or a more active species via some process, such as conversion by a metabolic pathway.

Some prodrugs have a chemical group present on the prodrug that renders it less active and/or confers solubility or some other property to the drug. Once the chemical group has been cleaved and/or modified from the prodrug the active drug is generated. Prodrugs are converted into active drug within the body through enzymatic or non-enzymatic reactions. Prodrugs may provide improved physiochemical properties such as better solubility, enhanced delivery characteristics, such as specifically targeting a particular cell, tissue, organ or ligand, and improved therapeutic value of the drug. The benefits of such prodrugs include, but are not limited to, (i) ease of administration compared with the parent drug; (ii) the prodrug may be bioavailable by oral administration whereas the parent is not; and (iii) the prodrug may also have improved solubility in pharmaceutical compositions compared with the parent drug. A pro-drug includes a pharmacologically inactive, or reduced-activity, derivative of an active drug. Prodrugs may be designed to modulate the amount of a drug or biologically active molecule that reaches a desired site of action through the manipulation of the properties of a drug, such as physiochemical, biopharmaceutical, or pharmacokinetic properties. An example, without limitation, of a prodrug would be a non-natural amino acid polypeptide which is administered as an ester (the "prodrug") to facilitate transmittal across a cell membrane where water solubility is detrimental to mobility but which then is metabolically hydrolyzed to the carboxylic acid, the active entity, once inside the cell where water solubility is beneficial. Prodrugs may be designed as reversible drug derivatives, for use as modifiers to enhance drug transport to site-specific tissues.

[0113] The term "prophylactically effective amount," as used herein, refers that amount of a composition containing at least one non-natural amino acid polypeptide or at least one modified non-natural amino acid polypeptide prophylactically applied to a patient which will relieve to some extent one or more of the symptoms of a disease, condition or disorder being treated. In such prophylactic applications, such amounts may depend on the patient's state of health, weight, and the like. It is considered well within the skill of the art for one to determine such prophylactically effective amounts by routine experimentation, including, but not limited to, a dose escalation clinical trial.

[0114] The term "protected," as used herein, refers to the presence of a "protecting group" or moiety that prevents reaction of the chemically reactive functional group under certain reaction conditions. The protecting group will vary depending on the type of chemically reactive group being protected. By way of example only, (i) if the chemically reactive group is an amine or a hydrazide, the protecting group may be selected from tert-butyloxycarbonyl (t-Boc) and 9-fluorenylmethoxycarbonyl (Fmoc); (ii) if the chemically reactive group is a thiol, the protecting group may be orthopyridyldisulfide; and (iii) if the chemically reactive group is a carboxylic acid, such as butanoic or propionic acid, or a hydroxyl group, the protecting group may be benzyl or an alkyl group such as methyl, ethyl, or tert-butyl.

[0115] **By** way of example only, blocking/protecting groups may be selected from:



[0116] Additionally, protecting groups include, but are not limited to, including photolabile groups such as Nvoc and MeNvoc and other protecting groups known in the art. Other protecting groups are described in Greene and Wuts, Protective Groups in Organic Synthesis, 3rd Ed., John Wiley & Sons, New York, NY, 1999.

[0117] The term "radioactive moiety," as used herein, refers to a group whose nuclei spontaneously give off nuclear radiation, such as alpha, beta, or gamma particles; wherein, alpha particles are helium nuclei, beta particles are electrons, and gamma particles are high energy photons.

[0118] The term "reactive compound," as used herein, refers to a compound which under appropriate conditions is reactive toward another atom, molecule or compound.

[0119] The term "recombinant host cell," also referred to as "host cell," refers to a cell which includes an exogenous polynucleotide, wherein the methods used to insert the exogenous polynucleotide into a cell include, but are not limited to, direct uptake, transduction, f-mating, or other methods known in the art to create recombinant host cells. By way of example only, such exogenous polynucleotide may be a nonintegrated vector, including but not limited to a plasmid, or may be integrated into the host genome.

[0120] The term "redox-active agent," as used herein, refers to a molecule which oxidizes or reduces another molecule, whereby the redox active agent becomes reduced or oxidized. Examples of redox active agent include, but are not limited to, ferrocene, quinones, $RU^{2+/3+}$ complexes, $Co^{2+/3+}$ complexes, and $Os^{2+/3+}$ complexes.

[0121] The term "reducing agent," as used herein, refers to a compound or material which is capable of adding an electron to a compound being reduced. By way of example reducing agents include, but are not limited to, dithiothreitol (DTT), 2-mercaptoethanol, dithioerythritol, cysteine, cysteamine (2-aminoethanethiol), and reduced glutathione. Such reducing agents may be used, by way of example only, to maintain sulfhydryl groups in the reduced state and to reduce intra- or intermolecular disulfide bonds.

[0122] "Refolding," as used herein describes any process, reaction or method which transforms an improperly folded or unfolded state to a native or properly folded conformation. By way of example only, refolding transforms disulfide bond containing polypeptides from an improperly folded or unfolded state to a native or properly folded conformation with respect to disulfide bonds. Such disulfide bond containing polypeptides may be natural amino acid polypeptides or non-natural amino acid polypeptides.

[0123] The term "resin," as used herein, refers to high molecular weight, insoluble polymer beads. By way of example only, such beads may be used as supports for solid phase peptide synthesis, or sites for attachment of molecules prior to purification.

[0124] The term "saccharide," as used herein, refers to a series of carbohydrates including but not limited to sugars, monosaccharides, oligosaccharides, and polysaccharides.

[0125] The term "safety" or "safety profile," as used herein, refers to side effects that might be related to administration of a drug relative to the number of times the drug has been administered. By way of example, a drug which has been administered many times and produced only mild or no side effects is said to have an excellent safety profile. A non-limiting example of a method to evaluate the safety profile is given in example 26. This method may be used for evaluating the safety profile of any polypeptide.

[0126] The phrase "selectively hybridizes to" or "specifically hybridizes to," as used herein, refers to the binding, duplexing, or hybridizing of a molecule to a particular nucleotide sequence under stringent hybridization conditions when that sequence is present in a complex mixture including but not limited to, total cellular or library DNA or RNA.

[0127] The term "spin label," as used herein, refers to molecules which contain an atom or a group of atoms exhibiting an unpaired electron spin (i.e. a stable paramagnetic group) that can be detected by electron spin resonance spectroscopy and can be attached to another molecule. Such spin-label molecules include, but are not limited to, nitryl radicals and nitroxides, and may be single spin-labels or double spin-labels.

[0128] The term "stoichiometric," as used herein, refers to the ratio of the moles of compounds participating in a chemical reaction being about 0.9 to about 1.1.

[0129] The term "stoichiometric-like," as used herein, refers to a chemical reaction which becomes stoichiometric or near-stoichiometric upon changes in reaction conditions or in the presence of additives. Such changes in reaction conditions include, but are not limited to, an increase in temperature or change in pH. Such additives include, but are not limited to, accelerants.

[0130] The phrase "stringent hybridization conditions" refers to hybridization of sequences of DNA, RNA, PNA or other nucleic acid mimics, or combinations thereof, under conditions of low ionic strength and high temperature. By way of example, under stringent conditions a probe will hybridize to its target subsequence in a complex mixture of nucleic acid (including but not limited to, total cellular or library DNA or RNA) but does not hybridize to other sequences in the complex mixture. Stringent conditions are sequence-dependent and will be different in different circumstances. By way of example, longer sequences hybridize specifically at higher temperatures. Stringent hybridization conditions include, but are not limited to, (i) about 5-10 °C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH; (ii) the salt concentration is about 0.01 M to about 1.0 M at about pH 7.0 to about pH 8.3 and the temperature is at least about 30 °C for short probes (including but not limited to, about 10 to about 50 nucleotides) and at least about 60 °C for long probes (including but not limited to, greater than 50 nucleotides); (iii) the addition of destabilizing agents including, but not limited to, formamide, (iv) 50% formamide, 5X SSC, and 1% SDS, incubating at 42 °C, or 5X SSC, about 1% SDS, incubating at 65 °C, with wash in 0.2X SSC, and about 0.1% SDS at 65 °C for between about 5 minutes to about 120 minutes. By way of example only, detection of selective or specific hybridization, includes, but is not limited to, a positive signal at least two times background. An extensive guide to the hybridization of nucleic acids is found in Tijssen, Laboratory Techniques in Biochemistry and Molecular BiologyHybridization with Nucleic Probes, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993).

[0131] The term "subject" as used herein, refers to an animal which is the object of treatment, observation or experiment.

By way of example only, a subject may be, but is not limited to, a mammal including, but not limited to, a human.

[0132] The term "substantially purified," as used herein, refers to a component of interest that may be substantially or essentially free of other components which normally accompany or interact with the component of interest prior to purification. By way of example only, a component of interest may be "substantially purified" when the preparation of the component of interest contains less than about 30%, less than about 25%, less than about 20%, less than about 15%, less than about 10%, less than about 5%, less than about 4%, less than about 3%, less than about 2%, or less than about 1% (by dry weight) of contaminating components. Thus, a "substantially purified" component of interest may have a purity level of about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or greater. By way of example only, a natural amino acid polypeptide or a non-natural amino acid polypeptide may be purified from a native cell, or host cell in the case of recombinantly produced natural amino acid polypeptides or non-natural amino acid polypeptides. By way of example a preparation of a natural amino acid polypeptide or a non-natural amino acid polypeptide may be "substantially purified" when the preparation contains less than about 30%, less than about 25%, less than about 20%, less than about 15%, less than about 10%, less than about 5%, less than about 4%, less than about 3%, less than about 2%, or less than about 1% (by dry weight) of contaminating material. By way of example when a natural amino acid polypeptide or a non-natural amino acid polypeptide is recombinantly produced by host cells, the natural amino acid polypeptide or non-natural amino acid polypeptide may be present at about 30%, about 25%, about 20%, about 15%, about 10%, about 5%, about 4%, about 3%, about 2%, or about 1% or less of the dry weight of the cells. By way of example when a natural amino acid polypeptide or a non-natural amino acid polypeptide is recombinantly produced by host cells, the natural amino acid polypeptide or non-natural amino acid polypeptide may be present in the culture medium at about 5g/L, about 4g/L, about 3g/L, about 2g/L, about 1g/L, about 750mg/L, about 500mg/L, about 250mg/L, about 100mg/L, about 50mg/L, about 10mg/L, or about 1mg/L or less of the dry weight of the cells. By way of example, "substantially purified" natural amino acid polypeptides or non-natural amino acid polypeptides may have a purity level of about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 99% or greater as determined by appropriate methods, including, but not limited to, SDS/PAGE analysis, RP-HPLC, SEC, and capillary electrophoresis.

[0133] The term "substituents" also referred to as "non-interfering substituents" "refers to groups which may be used to replace another group on a molecule. Such groups include, but are not limited to, halo, C_1-C_{10} alkyl, C_2-C_{10} alkenyl, C_2-C_{10} alkynyl, C_1-C_{10} alkoxy, C_5-C_{12} aralkyl, C_3-C_{12} cycloalkyl, C_4-C_{12} cycloalkenyl, phenyl, substituted phenyl, toluolyl, xylenyl, biphenyl, C_2-C_{12} alkoxyalkyl, C_5-C_{12} alkoxyaryl, C_5-C_{12} aryloxyalkyl, C_7-C_{12} oxyaryl, C_1-C_6 alkylsulfinyl, C_1-C_{10} alkylsulfonyl, $-(CH_2)_m$-O-(C_1-C_{10} alkyl) wherein m is from 1 to 8, aryl, substituted aryl, substituted alkoxy, fluoroalkyl, heterocyclic radical, substituted heterocyclic radical, nitroalkyl, $-NO_2$, -CN, -NRC(O)-(C_1-C_{10} alkyl), -C(O)-(C_1-C_{10} alkyl), C_2-C_{10} alkthioalkyl, -C(O)O-(C_1-C_{10} alkyl), -OH, $-SO_2$, =S, -COOH, $-NR_2$, carbonyl, -C(O)-(C_1-C_{10} alkyl)-CF_3, -C(O)-CF_3, $-C(O)NR_2$, -(C_1-C_{10} aryl)-S-(C_6-C_{10} aryl), -C(O)-(C_6-C_{10} aryl), $-(CH_2)_m$-O-$(CH_2)_m$-O-(C_1-C_{10} alkyl) wherein each m is from 1 to 8, $-C(O)NR_2$, $-C(S)NR_2$, $-SO_2NR_2$, -NRC(O)NR_2, - NRC(S)NR_2, salts thereof, and the like. Each R group in the preceding list includes, but is not limited to, H, alkyl or substituted alkyl, aryl or substituted aryl, or alkaryl. Where substituent groups are specified by their conventional chemical formulas, written from left to right, they equally encompass the chemically identical substituents that would result from writing the structure from right to left; for example, $-CH_2O$- is equivalent to $-OCH_2$-.

[0134] By way of example only, substituents for alkyl and heteroalkyl radicals (including those groups referred to as alkylene, alkenyl, heteroalkylene, heteroalkenyl, alkynyl, cycloalkyl, heterocycloalkyl, cycloalkenyl, and heterocycloalkenyl) includes, but is not limited to: -OR, =O, =NR, =N-OR, $-NR_2$, -SR, - halogen, $-SiR_3$, -OC(O)R, -C(O)R, $-CO_2R$, $-CONR_2$, $-OC(O)NR_2$, -NRC(O)R, -NRC(O)NR_2, $-NR(O)_2R$, - NR-C(NR_2)=NR, -S(O)R, $-S(O)_2R$, $-S(O)_2NR_2$, $-NRSO_2R$, -CN and $-NO_2$. Each R group in the preceding list includes, but is not limited to, hydrogen, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, including but not limited to, aryl substituted with 1-3 halogens, substituted or unsubstituted alkyl, alkoxy or thioalkoxy groups, or aralkyl groups. When two R groups 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, $-NR_2$ is meant to include, but not be limited to, 1-pyrrolidinyl and 4-morpholinyl.

[0135] By way of example, substituents for aryl and heteroaryl groups include, but are not limited to, - OR, =O, =NR, =N-OR, $-NR_2$, -SR, -halogen, $-SiR_3$, -OC(O)R, -C(O)R, $-CO_2R$, $-CONR_2$, $-OC(O)NR_2$, - NRC(O)R, -NRC(O)NR_2, $-NR(O)_2R$, -NR-C(NR_2)=NR, -S(O)R, $-S(O)_2R$, $-S(O)_2NR_2$, $-NRSO_2R$, -CN, $-NO_2$, -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 each R group in the preceding list includes, but is not limited to, hydrogen, alkyl, heteroalkyl, aryl and heteroaryl.

[0136] The term "therapeutically effective amount," as used herein, refers to the amount of a composition containing at least one non-natural amino acid polypeptide and/or at least one modified non-natural amino acid polypeptide administered to a patient already suffering from a disease, condition or disorder, sufficient to cure or at least partially arrest, or relieve to some extent one or more of the symptoms of the disease, disorder or condition being treated. The effectiveness of such compositions depend conditions including, but not limited to, the severity and course of the disease,

disorder or condition, previous therapy, the patient's health status and response to the drugs, and the judgment of the treating physician. By way of example only, therapeutically effective amounts may be determined by routine experimentation, including but not limited to a dose escalation clinical trial.

[0137] The term "thioalkoxy," as used herein, refers to sulfur containing alkyl groups linked to molecules via an oxygen atom.

[0138] The term "thermal melting point" or Tm is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of probes complementary to a target hybridize to the target sequence at equilibrium.

[0139] The term "toxic moiety" or "toxic group" as used herein, refers to a compound which can cause harm, disturbances, or death. Toxic moieties include, but are not limited to, auristatin, DNA minor groove binding agent, DNA minor groove alkylating agent, enediyne, lexitropsin, duocarmycin, taxane, puromycin, dolastatin, maytansinoid, vinca alkaloid, AFP, MMAF, MMAE, AEB, AEVB, auristatin E, paclitaxel, docetaxel, CC-1065, SN-38, topotecan, morpholino-doxorubicin, rhizoxin, cyanomorpholino-doxorubicin, dolastatin-10, echinomycin, combretatstatin, chalicheamicin, maytansine, DM-1, netropsin, podophyllotoxin (e.g. etoposide, teniposide, etc.), baccatin and its derivatives, anti-tubulin agents, cryptophysin, combretastatin, auristatin E, vincristine, vinblastine, vindesine, vinorelbine, VP-16, camptothecin, epothilone A, epothilone B, nocodazole, colchicines, colcimid, estramustine, cemadotin, discodermolide, maytansine, eleutherobin, mechlorethamine, cyclophosphamide, melphalan, carmustine, lomustine, semustine, streptozocin, chlorozotocin, uracil mustard, chlormethine, ifosfamide, chlorambucil, pipobroman, triethylenemelamine, triethylenethiophosphoramine, busulfan, dacarbazine, and temozolomide, ytarabine, cytosine arabinoside, fluorouracil, floxuridine, 6-thioguanine, 6-mercaptopurine, pentostatin, 5-fluorouracil, methotrexate, 10-propargyl-5,8-dideazafolate, 5,8-dideazatetrahydrofolic acid, leucovorin, fludarabine phosphate, pentostatine, gemcitabine, Ara-C, paclitaxel, docetaxel, deoxycoformycin, mitomycin-C, L-asparaginase, azathioprine, brequinar, antibiotics (e.g., anthracycline, gentamicin, cefalotin, vancomycin, telavancin, daptomycin, azithromycin, erythromycin, rocithromycin, furazolidone, amoxicillin, ampicillin, carbenicillin, flucloxacillin, methicillin, penicillin, ciprofloxacin, moxifloxacin, ofloxacin, doxycycline, minocycline, oxytetracycline, tetracycline, streptomycin, rifabutin, ethambutol, rifaximin, etc.), antiviral drugs (e.g., abacavir, acyclovir, ampligen, cidofovir, delavirdine, didanosine, efavirenz, entecavir, fosfonet, ganciclovir, ibacitabine, imunovir, idoxuridine, inosine, lopinavir, methisazone, nexavir, nevirapine, oseltamivir, penciclovir, stavudine, trifluridine, truvada, valaciclovir, zanamivir, etc.), daunorubicin hydrochloride, daunomycin, rubidomycin, cerubidine, idarubicin, doxorubicin, epirubicin and morpholino derivatives, phenoxizone biscyclopeptides (e.g., dactinomycin), basic glycopeptides (e.g., bleomycin), anthraquinone glycosides (e.g., plicamycin, mithramycin), anthracenediones (e.g., mitoxantrone), azirinopyrrolo indolediones (e.g., mitomycin), macrocyclic immunosuppressants (e.g., cyclosporine, FK-506, tacrolimus, prograf, rapamycin etc.), navelbene, CPT-11, anastrazole, letrazole, capecitabine, reloxafine, cyclophosphamide, ifosamide, droloxafine, allocolchicine, Halichondrin B, colchicine, colchicine derivatives , maytansine, rhizoxin, paclitaxel, paclitaxel derivatives, docetaxel, thiocolchicine, trityl cysterin, vinblastine sulfate, vincristine sulfate, cisplatin, carboplatin, hydroxyurea, N-methylhydrazine, epidophyllotoxin, procarbazine, mitoxantrone, leucovorin, and tegafur. "Taxanes" include paclitaxel, as well as any active taxane derivative or pro-drug.

[0140] The terms "treat," "treating" or "treatment", as used herein, include alleviating, abating or ameliorating a disease or condition symptoms, preventing additional symptoms, ameliorating or preventing the underlying metabolic causes of symptoms, inhibiting the disease or condition, e.g., arresting the development of the disease or condition, relieving the disease or condition, causing regression of the disease or condition, relieving a condition caused by the disease or condition, or stopping the symptoms of the disease or condition. The terms "treat," "treating" or "treatment", include, but are not limited to, prophylactic and/or therapeutic treatments.

[0141] As used herein, the term "water soluble polymer" refers to any polymer that is soluble in aqueous solvents. Such water soluble polymers include, but are not limited to, polyethylene glycol, polyethylene glycol propionaldehyde, mono C_1-C_{10} alkoxy or aryloxy derivatives thereof (described in U.S. Patent No. 5,252,714), monomethoxy-polyethylene glycol, polyvinyl pyrrolidone, polyvinyl alcohol, polyamino acids, divinylether maleic anhydride, N-(2-Hydroxypropyl)-methacrylamide, dextran, dextran derivatives including dextran sulfate, polypropylene glycol, polypropylene oxide/ethylene oxide copolymer, polyoxyethylated polyol, heparin, heparin fragments, polysaccharides, oligosaccharides, glycans, cellulose and cellulose derivatives, including but not limited to methylcellulose and carboxymethyl cellulose, serum albumin, starch and starch derivatives, polypeptides, polyalkylene glycol and derivatives thereof, copolymers of polyalkylene glycols and derivatives thereof, polyvinyl ethyl ethers, and alpha-beta-poly[(2-hydroxyethyl)-DL-aspartamide, and the like, or mixtures thereof. By way of example only, coupling of such water soluble polymers to natural amino acid polypeptides or non-natural polypeptides may result in changes including, but not limited to, increased water solubility, increased or modulated serum half-life, increased or modulated therapeutic half-life relative to the unmodified form, increased bioavailability, modulated biological activity, extended circulation time, modulated immunogenicity, modulated physical association characteristics including, but not limited to, aggregation and multimer formation, altered receptor binding, altered binding to one or more binding partners, and altered receptor dimerization or multimerization. In addition, such water soluble polymers may or may not have their own biological activity.

[0142] Unless otherwise indicated, conventional methods of mass spectroscopy, NMR, HPLC, protein chemistry,

biochemistry, recombinant DNA techniques and pharmacology, within the skill of the art are employed.

[0143] Compounds, (including, but not limited to non-natural amino acids, non-natural amino acid polypeptides, modified non-natural amino acid polypeptides, and reagents for producing the aforementioned compounds) presented herein include isotopically-labeled compounds, which are identical to those recited in the various formulas and structures presented herein, but for the fact that one or more atoms are replaced by an atom having an atomic mass or mass number different from the atomic mass or mass number usually found in nature. Examples of isotopes that can be incorporated into the present compounds include isotopes of hydrogen, carbon, nitrogen, oxygen, fluorine and chlorine, such as ^{2}H, ^{3}H, ^{13}C, ^{14}C, ^{15}N, ^{18}O, ^{17}O, ^{35}S, ^{18}F, ^{36}Cl, respectively. Certain isotopically-labeled compounds described herein, for example those into which radioactive isotopes such as ^{3}H and ^{14}C are incorporated, are useful in drug and/or substrate tissue distribution assays. Further, substitution with isotopes such as deuterium, i.e., ^{2}H, can afford certain therapeutic advantages resulting from greater metabolic stability, for example increased in vivo half-life or reduced dosage requirements.

[0144] Some of the compounds herein (including, but not limited to non-natural amino acids, non-natural amino acid polypeptides and modified non-natural amino acid polypeptides, and reagents for producing the aforementioned compounds) have asymmetric carbon atoms and can therefore exist as enantiomers or diastereomers. Diastereomeric mixtures can be separated into their individual diastereomers on the basis of their physical chemical differences by methods known, for example, by chromatography and/or fractional crystallization. Enantiomers can be separated by converting the enantiomeric mixture into a diastereomeric mixture by reaction with an appropriate optically active compound (e.g., alcohol), separating the diastereomers and converting (e.g., hydrolyzing) the individual diastereomers to the corresponding pure enantiomers. All such isomers, including diastereomers, enantiomers, and mixtures thereof are considered as part of the compositions described herein.

[0145] The compounds described herein (including, but not limited to non-natural amino acids, non-natural amino acid polypeptides and modified non-natural amino acid polypeptides, and reagents for producing the aforementioned compounds) are metabolized upon administration to an organism in need to produce a metabolite that is then used to produce a desired effect, including a desired therapeutic effect.

[0146] The methods and formulations described herein include the use of N-oxides, crystalline forms (also known as polymorphs), or pharmaceutically acceptable salts of non-natural amino acids, non-natural amino acid polypeptides and modified non-natural amino acid polypeptides. In certain embodiments, non-natural amino acids, non-natural amino acid polypeptides and modified non-natural amino acid polypeptides may exist as tautomers. All tautomers are included within the scope of the non-natural amino acids, non-natural amino acid polypeptides and modified non-natural amino acid polypeptides presented herein. In addition, the non-natural amino acids, non-natural amino acid polypeptides and modified non-natural amino acid polypeptides described herein can exist in unsolvated as well as solvated forms with pharmaceutically acceptable solvents such as water, ethanol, and the like. The solvated forms of the non-natural amino acids, non-natural amino acid polypeptides and modified non-natural amino acid polypeptides presented herein are also considered to be disclosed herein.

[0147] Some of the compounds herein (including, but not limited to non-natural amino acids, non-natural amino acid polypeptides and modified non-natural amino acid polypeptides and reagents for producing the aforementioned compounds) may exist in several tautomeric forms. All such tautomeric forms are considered as part of the compositions described herein. Also, for example all enol-keto forms of any compounds (including, but not limited to non-natural amino acids, non-natural amino acid polypeptides and modified non-natural amino acid polypeptides and reagents for producing the aforementioned compounds) herein are considered as part of the compositions described herein.

[0148] Some of the compounds herein (including, but not limited to non-natural amino acids, non-natural amino acid polypeptides and modified non-natural amino acid polypeptides and reagents for producing either of the aforementioned compounds) are acidic and may form a salt with a pharmaceutically acceptable cation. Some of the compounds herein (including, but not limited to non-natural amino acids, non-natural amino acid polypeptides and modified non-natural amino acid polypeptides and reagents for producing the aforementioned compounds) can be basic and accordingly, may form a salt with a pharmaceutically acceptable anion. All such salts, including di-salts are within the scope of the compositions described herein and they can be prepared by conventional methods. For example, salts can be prepared by contacting the acidic and basic entities, in either an aqueous, non-aqueous or partially aqueous medium. The salts are recovered by using at least one of the following techniques: filtration, precipitation with a non-solvent followed by filtration, evaporation of the solvent, or, in the case of aqueous solutions, lyophilization.

[0149] Pharmaceutically acceptable salts of the non-natural amino acid polypeptides disclosed herein may be formed when an acidic proton present in the parent non-natural amino acid polypeptides either is replaced by a metal ion, by way of example an alkali metal ion, an alkaline earth ion, or an aluminum ion; or coordinates with an organic base. In addition, the salt forms of the disclosed non-natural amino acid polypeptides can be prepared using salts of the starting materials or intermediates. The non-natural amino acid polypeptides described herein may be prepared as a pharmaceutically acceptable acid addition salt (which is a type of a pharmaceutically acceptable salt) by reacting the free base form of non-natural amino acid polypeptides described herein with a pharmaceutically acceptable inorganic or organic

acid. Alternatively, the non-natural amino acid polypeptides described herein may be prepared as pharmaceutically acceptable base addition salts (which are a type of a pharmaceutically acceptable salt) by reacting the free acid form of non-natural amino acid polypeptides described herein with a pharmaceutically acceptable inorganic or organic base.

[0150] The type of pharmaceutical acceptable salts, include, but are not limited to: (1) acid addition salts, formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, and the like; or formed with organic acids such as acetic acid, propionic acid, hexanoic acid, cyclopentanepropionic acid, glycolic acid, pyruvic acid, lactic acid, malonic acid, succinic acid, malic acid, maleic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, 3-(4-hydroxybenzoyl)benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, 1,2-ethanedisulfonic acid, 2-hydroxyethanesulfonic acid, benzenesulfonic acid, 2-naphthalenesulfonic acid, 4-methylbicyclo-[2.2.2]oct-2-ene-1-carboxylic acid, glucoheptonic acid, 4,4'-methylenebis-(3-hydroxy-2-ene-1-carboxylic acid), 3-phenylpropionic acid, trimethylacetic acid, tertiary butylacetic acid, lauryl sulfuric acid, gluconic acid, glutamic acid, hydroxynaphthoic acid, salicylic acid, stearic acid, muconic acid, and the like; (2) salts formed when an acidic proton present in the parent compound either is replaced by a metal ion, e.g., an alkali metal ion, an alkaline earth ion, or an aluminum ion; or coordinates with an organic base. Acceptable organic bases include ethanolamine, diethanolamine, triethanolamine, tromethamine, N-methylglucamine, and the like. Acceptable inorganic bases include aluminum hydroxide, calcium hydroxide, potassium hydroxide, sodium carbonate, sodium hydroxide, and the like.

[0151] The corresponding counterions of the non-natural amino acid polypeptide pharmaceutical acceptable salts may be analyzed and identified using various methods including, but not limited to, ion exchange chromatography, ion chromatography, capillary electrophoresis, inductively coupled plasma, atomic absorption spectroscopy, mass spectrometry, or any combination thereof. In addition, the therapeutic activity of such non-natural amino acid polypeptide pharmaceutical acceptable salts may be tested using the techniques and methods described in examples 87-91.

[0152] It should be understood that a reference to a salt includes the solvent addition forms or crystal forms thereof, particularly solvates or polymorphs. Solvates contain either stoichiometric or non-stoichiometric amounts of a solvent, and are often formed during the process of crystallization with pharmaceutically acceptable solvents such as water, ethanol, and the like. Hydrates are formed when the solvent is water, or alcoholates are formed when the solvent is alcohol. Polymorphs include the different crystal packing arrangements of the same elemental composition of a compound. Polymorphs usually have different X-ray diffraction patterns, infrared spectra, melting points, density, hardness, crystal shape, optical and electrical properties, stability, and solubility. Various factors such as the recrystallization solvent, rate of crystallization, and storage temperature may cause a single crystal form to dominate.

[0153] The screening and characterization of non-natural amino acid polypeptide pharmaceutical acceptable salts polymorphs and/or solvates may be accomplished using a variety of techniques including, but not limited to, thermal analysis, x-ray diffraction, spectroscopy, vapor sorption, and microscopy. Thermal analysis methods address thermo chemical degradation or thermo physical processes including, but not limited to, polymorphic transitions, and such methods are used to analyze the relationships between polymorphic forms, determine weight loss, to find the glass transition temperature, or for excipient compatibility studies. Such methods include, but are not limited to, Differential scanning calorimetry (DSC), Modulated Differential Scanning Calorimetry (MDCS), Thermogravimetric analysis (TGA), and Thermogravi-metric and Infrared analysis (TG/IR). X-ray diffraction methods include, but are not limited to, single crystal and powder diffractometers and synchrotron sources. The various spectroscopic techniques used include, but are not limited to, Raman, FTIR, UVIS, and NMR (liquid and solid state). The various microscopy techniques include, but are not limited to, polarized light microscopy, Scanning Electron Microscopy (SEM) with Energy Dispersive X-Ray Analysis (EDX), Environmental Scanning Electron Microscopy with EDX (in gas or water vapor atmosphere), IR microscopy, and Raman microscopy.

BRIEF DESCRIPTION OF THE DRAWINGS

[0154] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

Figure 1 presents a graphical illustration of Her-Tox binding to the Her2 receptor.

Figure 2 presents a graphical illustration of the expression of Anti-Her2 variants determined by ELISA analysis.

Figure 3 presents a graphical illustration of the expression of Anti-Her2 variants determined by ELISA analysis.

Figure 4 presents a graphical illustration of the cell proliferation assay with HCC 1954 breast cancer cell line and dolastatin linker derivatives.

Figure 5 presents a graphical illustration of the analysis of the cell proliferation assay with HCC 1954 breast cancer cell line and trastuzumab-tox conjugates.

Figure 6 presents a graphical illustration of the analysis of the cell proliferation assay with SKOV-3 ovarian cancer

cell line and dolastatin linker derivatives.

Figure 7 presents a graphical illustration of the analysis of the cell proliferation assay with SKOV-3 ovarian cancer cell line and trastuzumab-tox conjugates.

Figure 8 presents a graphical illustration of the analysis of the cell proliferation assay with MDA-MB-468 breast cancer cell line and dolastatin linker derivatives.

Figure 9 presents a graphical illustration of the analysis of the cell proliferation assay with MDA-MB-468 breast cancer line and trastuzumab-tox conjugates.

Figure 10 presents a graphical illustration of tumor volume measurement (mm^3) after a single IC dose (3.3 mg/kg, 10 mg/kg, 20mg/kg) of trastuzumab-linked dolastatin derivatives.

Figure 11 presents assay formats used to measure trastuzumab-linked dolastatin derivatives concentration in SD rat serum

Figure 12 presents graphical illustrations of serum concentrations (ng/mL) of trastuzumab-linked dolastatin derivatives after single IV injections.

Figure 13 presents a graphical illustration of serum concentrations (ng/mL) of trastuzumab-linked dolastatin derivatives after single IV injections. This assay detects antibody binding to the ErbB2 receptor.

Figure 14 presents a graphical illustration of of serum concentrations (ng/mL) of trastuzumab-linked dolastatin derivatives after IV injection. The in vivo stability measurements detect at least two dolastatin derivatives linked to rastuzumab.

Figure 15 presents graphical illustrations of the change in rat body weight and tumor volume after treatment with trastuzumab-linked dolastatin derivatives.

Figure 16 presents graphical illustrations of anti-tumor efficacy of trastuzumab, Her2-HS122-NCD1 and Her2-HS122/LK145-HCD1 against established tumors of HCC1954 in SCID-bg mice. Mice were administered a single IV injection on day 1 (arrow). Data points represent group average tumor volume and error bars represent standard error of the mean (SEM).

Figure 17 presents graphical illustrations of anti-tumor efficacy of dolastatin linker derivatives in the MDA361DYT2 Breast (2+) Xenograft model.

Figure 18 presents graphical illustrations of anti-tumor efficacy of dolastatin linker derivatives in the MDA361DYT2 Breast (2+) Xenograft model.

DETAILED DESCRIPTION OF THE INVENTION

I. Introduction

[0155] Recently, an entirely new technology in the protein sciences has been reported, which promises to overcome many of the limitations associated with site-specific modifications of proteins. Specifically, new components have been added to the protein biosynthetic machinery of the prokaryote *Escherichia coli (E. coli)* (e.g., L. Wang, et al., (2001), Science 292:498-500) and the eukaryote *Sacchromyces cerevisiae (S. cerevisiae)* (e.g., J. Chin et al., Science 301:964-7 (2003)), which has enabled the incorporation of non-natural amino acids to proteins *in vivo*. A number of new amino acids with novel chemical, physical or biological properties, including photoaffinity labels and photoisomerizable amino acids, keto amino acids, and glycosylated amino acids have been incorporated efficiently and with high fidelity into proteins in *E. coli* and in yeast in response to the amber codon, TAG, using this methodology. Studies have demonstrated that it is possible to selectively and routinely introduce chemical functional groups that are not found in proteins, that are chemically inert to all of the functional groups found in the 20 common, genetically-encoded amino acids and that may be used to react efficiently and selectively to form stable covalent linkages.

II. Overview

[0156] At one level, described herein are the tools (methods, compositions, techniques) for creating and using dolastatin linker derivatives or analogs comprising at least one carbonyl, dicarbonyl, oxime, hydroxylamine, aldehyde, protected aldehyde, ketone, protected ketone, thioester, ester, dicarbonyl, hydrazine, azide, amidine, imine, diamine, keto-amine, keto-alkyne, alkyne, cycloalkyne, or ene-dione. At another level, described herein are the tools (methods, compositions, techniques) for creating and using dolastatin linker derivatives or analogs comprising at least one non-natural amino acid or modified non- natural amino acid with an oxime, aromatic amine, heterocycle (e.g., indole, quinoxaline, phenazine, pyrazole, triazole, etc.).

[0157] Such dolastatin linker derivatives comprising non-natural amino acids may contain further functionality, including but not limited to, a polymer; a water-soluble polymer; a derivative of polyethylene glycol; a second protein or polypeptide or polypeptide analog; an antibody or antibody fragment; and any combination thereof. Note that the various aforementioned functionalities are not meant to imply that the members of one functionality cannot be classified as members of

another functionality. Indeed, there will be overlap depending upon the particular circumstances. By way of example only, a water-soluble polymer overlaps in scope with a derivative of polyethylene glycol, however the overlap is not complete and thus both functionalities are cited above.

[0158] Provided herein in some embodiments, is a toxic group linker derivative comprising a carbonyl, dicarbonyl, oxime, hydroxylamine, aldehyde, protected aldehyde, ketone, protected ketone, thioester, ester, dicarbonyl, hydrazine, azide, amidine, imine, diamine, keto-amine, keto-alkyne, alkyne, cycloalkyne, or ene-dione. In some embodiments, the toxic group derivative comprises any of the linkers disclosed herein. In other embodiments, described herein are the tools (methods, compositions, techniques) for creating and using toxic group derivatives or analogs comprising at least one non-natural amino acid or modified non-natural amino acid with an oxime, aromatic amine, heterocycle (e.g., indole, quinoxaline, phenazine, pyrazole, triazole, etc.).

[0159] In some embodiments, such toxic derivatives comprising non-natural amino acids may contain further functionality, including but not limited to, a polymer; a water-soluble polymer; a derivative of polyethylene glycol; a second protein or polypeptide or polypeptide analog; an antibody or antibody fragment; and any combination thereof. In specific embodiments, the toxic group is dolastatin or auristatin. In certain specific embodiments, the toxic group is dolastatin-10. Note that the various aforementioned functionalities are not meant to imply that the members of one functionality cannot be classified as members of another functionality. Indeed, there will be overlap depending upon the particular circumstances. By way of example only, a water-soluble polymer overlaps in scope with a derivative of polyethylene glycol, however the overlap is not complete and thus both functionalities are cited above.

[0160] Certain embodiments of the present invention describe preparations of certain toxic moieties with linkers that reduce the toxicity of the moiety in vivo while the toxic moiety retains pharmacological activity. In some embodiments, the toxicity of the linked toxic group, when administered to an animal or human, is reduced or eliminated compared to the free toxic group or toxic group derivatives comprising labile linkages, while retaining pharmacological activity. In some embodiments, increased doses of the linked toxic group (e.g., dolastatin linker derivatives, non-natural amino acid linked dolastatin derivatives) may be administered to animals or humans with greater safety. In certain embodiments, the non-natural amino acid polypeptides linked to a toxic moiety (e.g., dolastatin derivative) provides in vitro and in vivo stability. In some embodiments, the non-natural amino acid polypeptides linked to a toxic moiety (e.g., dolastatin-10 derivative) are efficacious and less toxic compared to the free toxic moiety (e.g., dolastatin-10).

III. Dolastatin Linker Derivatives

[0161] At one level, described herein are the tools (methods, compositions, techniques) for creating and using a dolastatin linker derivatives or analogs comprising at least one non-natural amino acid or modified non-natural amino acid with a carbonyl, dicarbonyl, oxime or hydroxylamine group. Such dolastatin linker derivatives comprising non-natural amino acids may contain further functionality, including but not limited to, a polymer; a water-soluble polymer; a derivative of polyethylene glycol; a second protein or polypeptide or polypeptide analog; an antibody or antibody fragment; and any combination thereof. Note that the various aforementioned functionalities are not meant to imply that the members of one functionality cannot be classified as members of another functionality. Indeed, there will be overlap depending upon the particular circumstances. By way of example only, a water-soluble polymer overlaps in scope with a derivative of polyethylene glycol, however the overlap is not complete and thus both functionalities are cited above.

[0162] In one aspect are methods for selecting and designing a dolastatin linker derivative to be modified using the methods, compositions and techniques described herein. The new dolastatin linker derivative may be designed de novo, including by way of example only, as part of high-throughput screening process (in which case numerous polypeptides may be designed, synthesized, characterized and/or tested) or based on the interests of the researcher. The new dolastatin linker derivative may also be designed based on the structure of a known or partially characterized polypeptide. By way of example only, dolastatin has been the subject of intense study by the scientific community; a new compound may be designed based on the structure of dolastatin. The principles for selecting which amino acid(s) to substitute and/or modify are described separately herein. The choice of which modification to employ is also described herein, and can be used to meet the need of the experimenter or end user. Such needs may include, but are not limited to, manipulating the therapeutic effectiveness of the polypeptide, improving the safety profile of the polypeptide, adjusting the pharmacokinetics, pharmacologics and/or pharmacodynamics of the polypeptide, such as, by way of example only, increasing water solubility, bioavailability, increasing serum half-life, increasing therapeutic half-life, modulating immunogenicity, modulating biological activity, or extending the circulation time. In addition, such modifications include, by way of example only, providing additional functionality to the polypeptide, incorporating an antibody, and any combination of the aforementioned modifications.

[0163] Also described herein are dolastatin linker derivatives that have or can be modified to contain an oxime, carbonyl, dicarbonyl, or hydroxylamine group. Included with this aspect are methods for producing, purifying, characterizing and using such dolastatin linker derivatives

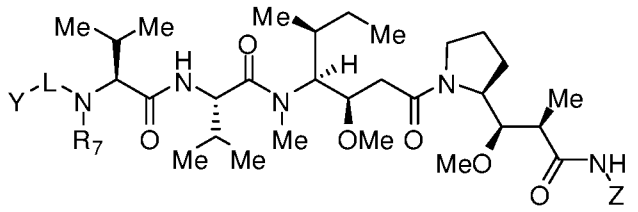
[0164] The dolastatin linker derivative may contain at least one, at least two, at least three, at least four, at least five,

at least six, at least seven, at least eight, at least nine, or ten or more of a carbonyl or dicarbonyl group, oxime group, hydroxylamine group, or protected forms thereof. The dolastatin linker derivative can be the same or different, for example, there can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more different sites in the derivative that comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more different reactive groups.

A. Structure and Synthesis of Dolastatin Linker Derivatives: Electrophilic and Nucleophilic Groups

[0165] Dolastatin derivatives with linkers containing a hydroxylamine (also called an aminooxy) group allow for reaction with a variety of electrophilic groups to form conjugates (including but not limited to, with PEG or other water soluble polymers). Like hydrazines, hydrazides and semicarbazides, the enhanced nucleophilicity of the aminooxy group permits it to react efficiently and selectively with a variety of molecules that contain carbonyl- or dicarbonyl-groups, including but not limited to, ketones, aldehydes or other functional groups with similar chemical reactivity. Whereas the result of reaction with a hydrazine group is the corresponding hydrazone, however, an oxime results generally from the reaction of an aminooxy group with a carbonyl- or dicarbonyl-containing group such as, by way of example, a ketones, aldehydes or other functional groups with similar chemical reactivity. In some embodiments, dolastatin derivatives with linkers comprising an azide, alkyne or cycloalkyne allow for linking of molecules via cycloaddition reactions (e.g., 1,3-dipolar cycloadditions, azide-alkyne Huisgen cycloaddition, etc.)

[0166] Thus, in certain embodiments described herein are dolastatin derivatives with linkers comprising a hydroxylamine, aldehyde, protected aldehyde, ketone, protected ketone, thioester, ester, dicarbonyl, hydrazine, amidine, imine, diamine, keto-amine, keto-alkyne, and ene-dione hydroxylamine group, a hydroxylamine-like group (which has reactivity similar to a hydroxylamine group and is structurally similar to a hydroxylamine group), a masked hydroxylamine group (which can be readily converted into a hydroxylamine group), or a protected hydroxylamine group (which has reactivity similar to a hydroxylamine group upon deprotection). In some embodiments, the dolastatin derivatives with linkers comprise azides, alkynes or cycloalkynes. The present invention provides compounds having the structure of Formula (I):

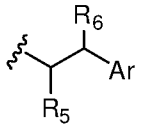


(I)

;

wherein:

Z has the structure of:



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R_5 is H, COR_8, C_1-C_6alkyl, or thiazole;
R_8 is OH;
R_6 is OH or H;
Ar is phenyl or pyridine;

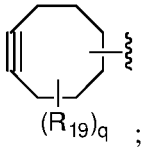
R_7 is C_1-C_6 alkyl or hydrogen;
Y is selected from the group consisting of an hydroxylamine, azide, alkyne, and cycloalkyne;
L is-alkylene- or -(alkylene-O)$_n$-alkylene-; and
n is an integer greater than or equal to one;
wherein alkyl means a straight or branched chain, or cyclic hydrocarbon radical, or combination thereof, which may be fully saturated, mono- or polyunsaturated and can include di- and multivalent radicals and also includes heteroalkyl and haloalkyl;
wherein alkylene includes heteroalkylene.

Such dolastatin linker derivatives may be in the form of a salt, or may be incorporated into a non-natural amino acid

polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[0167] In certain embodiments of compounds of Formula (I), R_5 is thiazole or carboxylic acid. In certain embodiments of compounds of Formula (I), R_5 is hydrogen. In certain embodiments of compounds of Formula (I), R_5 is methyl, ethyl, propyl, iso-propyl, butyl, iso-butyl, sec-butyl, tert-butyl, pentyl, or hexyl.

[0168] In some embodiments, Y is azide. In other embodiments, Y is cycloalkyne. In specific embodiments, the cyclooctyne has a structure of:



;

each R_{19} is independently selected from the group consisting of C_1-C_6 alkyl, C_1-C_6 alkoxy, ester, ether, thioether, aminoalkyl, halogen, alkyl ester, aryl ester, amide, aryl amide, alkyl halide, alkyl amine, alkyl sulfonic acid, alkyl nitro, thioester, sulfonyl ester, halosulfonyl, nitrile, alkyl nitrile, and nitro; and
q is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11.

[0169] In certain embodiments of compounds of Formula (I), R_6 is H. In some embodiments of compounds of Formula (I), R_6 is hydroxy.

[0170] In certain embodiments of compounds of Formula (I), Ar is phenyl.

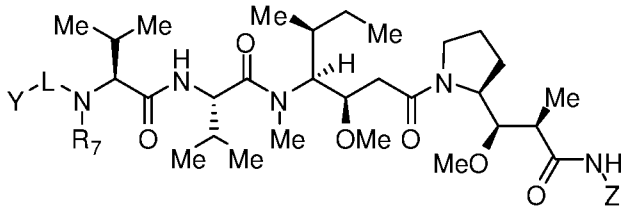
[0171] In certain embodiments of compounds of Formula (I), R_7 is methyl, ethyl, propyl, iso-propyl, butyl, sec-butyl iso-butyl, tert-butyl, pentyl, or hexyl. In certain embodiments of compounds of Formula (I), R_7 is hydrogen.

[0172] In certain embodiments of compounds of Formula (I), Y is hydroxylamine.

[0173] In certain embodiments of compounds of Formula (I), each alkylene independently is $-CH_2-$, $-CH_2CH_2-$, $-CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$, or $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$. In certain embodiments of compounds of Formula (I), n is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100.

B. Structure and Synthesis of Dolastatin Linker Derivatives: Hydroxylamine Groups

[0174] Thus the dolastatin derivatives of the invention may have linkers comprising a hydroxylamine group. Such dolastatin linker derivatives include compounds having the structure of Formula (I):

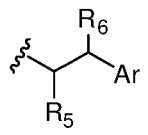


(I)

;

wherein:

Z has the structure of:



;

R_5 is H, COR_8, C_1-C_6alkyl, or thiazole;

R_8 is OH;
R_6 is OH or H;
Ar is phenyl or pyridine;

R_7 is C_1-C_6 alkyl or hydrogen;
Y is NH_2-O-;
L is-alkylene- or -(alkylene-O)$_n$-alkylene-; and
n is an integer greater than or equal to one;

wherein alkyl means 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 and also includes heteroalkyl and haloalkyl;

wherein alkylene includes heteroalkylene.

Such dolastatin linker derivatives may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[0175] In certain embodiments of compounds of Formula (I), R_5 is thiazole. In certain embodiments of compounds of Formula (I), R_5 is hydrogen. In certain embodiments of compounds of Formula (I), R_5 is methyl, ethyl, propyl, iso-propyl, butyl, iso-butyl, sec-butyl, tert-butyl, pentyl, or hexyl.

[0176] In certain embodiments of compounds of Formula (I), R_6 is H. In some embodiments of compounds of Formula (I), R_6 is hydroxy.

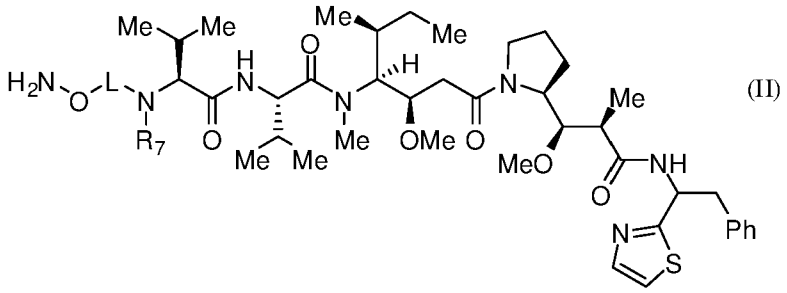
[0177] In certain embodiments of compounds of Formula (I), Ar is phenyl.

[0178] In certain embodiments of compounds of Formula (I), R_7 is methyl, ethyl, propyl, iso-propyl, butyl, sec-butyl iso-butyl, tert-butyl, pentyl, or hexyl. In certain embodiments of compounds of Formula (I), R_7 is hydrogen.

[0179] In certain embodiments of compounds of Formula (I), Y is hydroxylamine.

[0180] In certain embodiments of compounds of Formula (I), alkylene is $-CH_2-$, $--CH_2CH_2-$,$-CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$,or $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$. In certain embodiments of compounds of Formula (I), n is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100.

[0181] **In** certain embodiments, dolastatin linker derivatives include compounds having the structure of Formula (II):



(II)

In some embodiments of compounds of Formula (II), L is -(alkylene-O)$_n$-alkylene-. In some embodiments, each alkylene is $-CH_2CH_2-$, n is equal to 3, and R_7 is methyl. In some embodiments, L is - alkylene-. In some embodiments of compounds of Formula (II), each alkylene is $-CH_2CH_2-$ and R_7 is methyl or hydrogen. Such dolastatin linker derivatives may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[0182] In certain embodiments of compounds of Formula (II), R_7 is methyl, ethyl, propyl, iso-propyl, butyl, sec-butyl iso-butyl, tert-butyl, pentyl, or hexyl. In certain embodiments of compounds of Formula (II), R_7 is hydrogen.

[0183] In certain embodiments of compounds of Formula (II), alkylene is $-CH_2-$, $-CH_2CH_2-$, $-CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$, or

$-CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$. In certain embodiments of compounds of Formula (II), n is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100.

[0184] In certain embodiments, compounds of Formula (I) are stable in aqueous solution for at least 1 month under mildly acidic conditions. In certain embodiments, compounds of Formula (I) are stable for at least 2 weeks under mildly acidic conditions. In certain embodiments, compound of Formula (I) are stable for at least 5 days under mildly acidic conditions. In certain embodiments, such acidic conditions are pH 2 to 8.

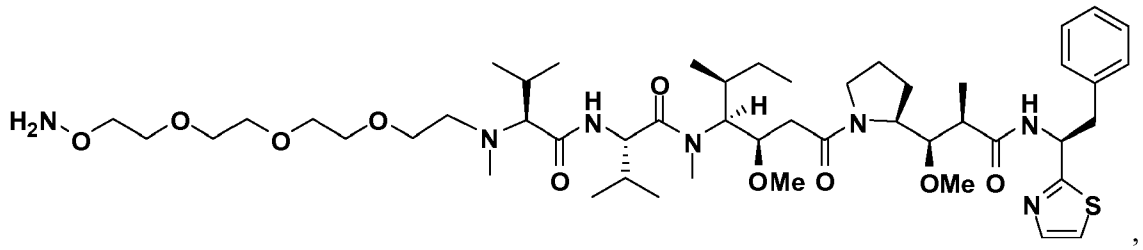
[0185] The methods and compositions provided and described herein include polypeptides comprising dolastatin linker derivative containing at least one carbonyl or dicarbonyl group, oxime group, hydroxylamine group, or protected or masked forms thereof. Introduction of at least one reactive group into a dolastatin linker derivative can allow for the application of conjugation chemistries that involve specific chemical reactions, including, but not limited to, with one or more dolastatin linker derivative(s) while not reacting with the commonly occurring amino acids. Once incorporated, the dolastatin linker derivative side chains can also be modified by utilizing chemistry methodologies described herein or suitable for the particular functional groups or substituents present in the dolastatin linker derivative.

[0186] The dolastatin linker derivative methods and compositions described herein provide conjugates of substances having a wide variety of functional groups, substituents or moieties, with other substances including but not limited to a polymer; a water-soluble polymer; a derivative of polyethylene glycol; a second protein or polypeptide or polypeptide analog; an antibody or antibody fragment; and any combination thereof.

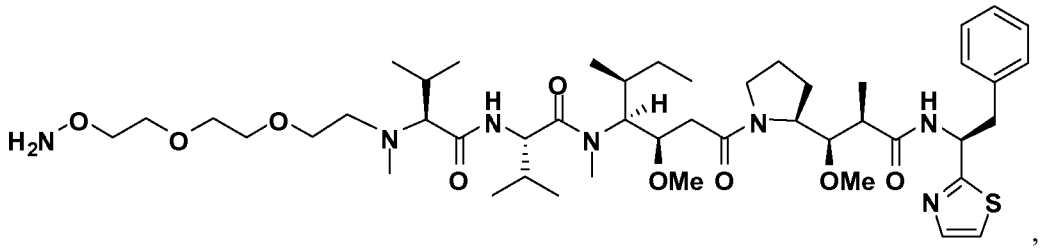
[0187] In certain embodiments, the dolastatin linker derivatives, linkers and reagents described herein, including compounds of Formulas (I) are stable in aqueous solution under mildly acidic conditions (including but not limited to pH 2 to 8). In other embodiments, such compounds are stable for at least one month under mildly acidic conditions. In other embodiments, such compounds are stable for at least 2 weeks under mildly acidic conditions. In other embodiments, such compounds are stable for at least 5 days under mildly acidic conditions.

[0188] In another aspect of the compositions, methods, techniques and strategies described herein are methods for studying or using any of the aforementioned "modified or unmodified" non-natural amino acid dolastatin linker derivatives. Included within this aspect, by way of example only, are therapeutic, diagnostic, assay-based, industrial, cosmetic, plant biology, environmental, energy-production, consumer-products, and/or military uses which would benefit from a dolastatin linker derivative comprising a "modified or unmodified" non-natural amino acid polypeptide or protein.

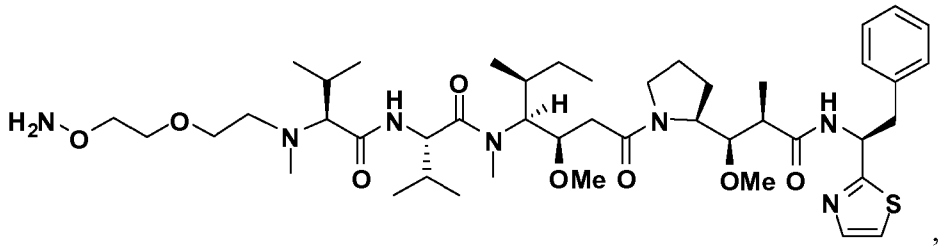
[0189] Non-limiting examples of dolastatin linker derivatives include:



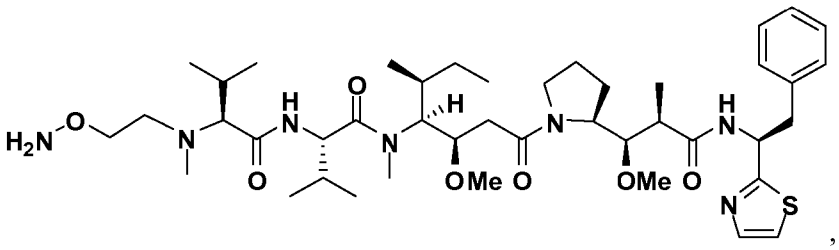
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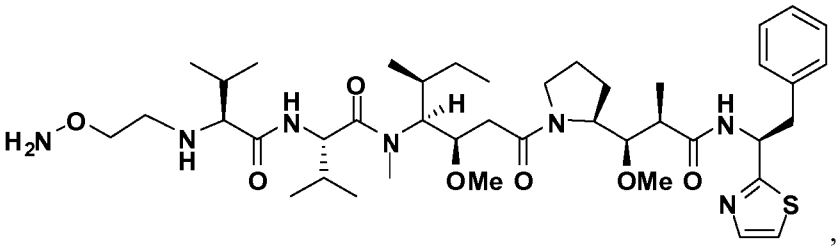
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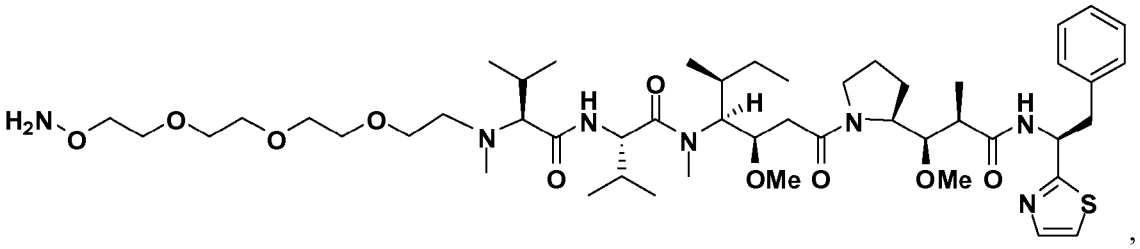
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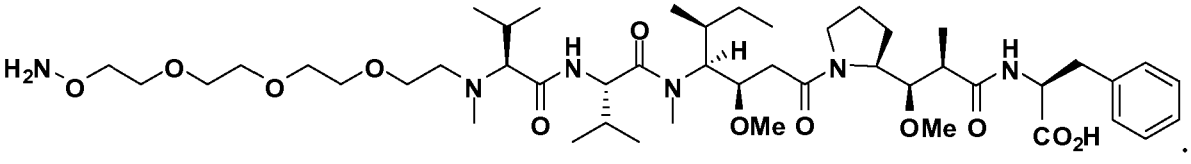


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IV. Non-Natural Amino Acid Derivatives

[0190] The non-natural amino acids used in the methods and compositions described herein have at least one of the following four properties: (1) at least one functional group on the sidechain of the non-natural amino acid has at least one characteristics and/or activity and/or reactivity orthogonal to the chemical reactivity of the 20 common, genetically-encoded amino acids (i.e., alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine), or at least orthogonal to the chemical reactivity of the naturally occurring amino acids present in the polypeptide that includes the non-natural amino acid; (2) the introduced non-natural amino acids are substantially chemically inert

toward the 20 common, genetically-encoded amino acids; (3) the non-natural amino acid can be stably incorporated into a polypeptide, preferably with the stability commensurate with the naturally-occurring amino acids or under typical physiological conditions, and further preferably such incorporation can occur via an *in vivo* system; and (4) the non-natural amino acid includes an oxime functional group or a functional group that can be transformed into an oxime group by reacting with a reagent, preferably under conditions that do not destroy the biological properties of the polypeptide that includes the non-natural amino acid (unless of course such a destruction of biological properties is the purpose of the modification/transformation), or where the transformation can occur under aqueous conditions at a pH between about 4 and about 8, or where the reactive site on the non-natural amino acid is an electrophilic site. Any number of non-natural amino acids can be introduced into the polypeptide. Non-natural amino acids may also include protected or masked oximes or protected or masked groups that can be transformed into an oxime group after deprotection of the protected group or unmasking of the masked group. Non-natural amino acids may also include protected or masked carbonyl or dicarbonyl groups, which can be transformed into a carbonyl or dicarbonyl group after deprotection of the protected group or unmasking of the masked group and thereby are available to react with hydroxylamines or oximes to form oxime groups.

[0191] Non-natural amino acids that may be used in the methods and compositions described herein include, but are not limited to, amino acids comprising a amino acids with novel functional groups, amino acids that covalently or non-covalently interact with other molecules, glycosylated amino acids such as a sugar substituted serine, other carbohydrate modified amino acids, keto-containing amino acids, aldehyde-containing amino acids, amino acids comprising polyethylene glycol or other polyethers, heavy atom substituted amino acids, chemically cleavable and/or photocleavable amino acids, amino acids with an elongated side chains as compared to natural amino acids, including but not limited to, polyethers or long chain hydrocarbons, including but not limited to, greater than about 5 or greater than about 10 carbons, carbon-linked sugar-containing amino acids, redox-active amino acids, amino thioacid containing amino acids, and amino acids comprising one or more toxic moiety.

[0192] In some embodiments, non-natural amino acids comprise a saccharide moiety. Examples of such amino acids include *N*-acetyl-L-glucosaminyl-L-serine, *N*-acetyl-L-galactosannnyl-L-serine, *N*-acetyl-L-glucosaminyl-L-threonine, *N*-acetyl-L-glucosaminyl-L-asparagine and *O*-mannosaminyl-L-serine. Examples of such amino acids also include examples where the naturally-occurring N- or O-linkage between the amino acid and the saccharide is replaced by a covalent linkage not commonly found in nature - including but not limited to, an alkene, an oxime, a thioether, an amide and the like. Examples of such amino acids also include saccharides that are not commonly found in naturally-occurring proteins such as 2-deoxy-glucose, 2-deoxygalactose and the like.

[0193] The chemical moieties incorporated into polypeptides via incorporation of non-natural amino acids into such polypeptides offer a variety of advantages and manipulations of polypeptides. For example, the unique reactivity of a carbonyl or dicarbonyl functional group (including a keto- or aldehyde- functional group) allows selective modification of proteins with any of a number of hydrazine- or hydroxylamine-containing reagents *in vivo* and *in vitro*. A heavy atom non-natural amino acid, for example, can be useful for phasing x-ray structure data. The site-specific introduction of heavy atoms using non-natural amino acids also provides selectivity and flexibility in choosing positions for heavy atoms. Photoreactive non-natural amino acids (including but not limited to, amino acids with benzophenone and arylazides (including but not limited to, phenylazide) side chains), for example, allow for efficient *in vivo* and *in vitro* photocrosslinking of polypeptides. Examples of photoreactive non-natural amino acids include, but are not limited to, p-azido-phenylalanine and p-benzoyl-phenylalanine. The polypeptide with the photoreactive non-natural amino acids may then be crosslinked at will by excitation of the photoreactive group-providing temporal control. In a non-limiting example, the methyl group of a non-natural amino can be substituted with an isotopically labeled, including but not limited to, with a methyl group, as a probe of local structure and dynamics, including but not limited to, with the use of nuclear magnetic resonance and vibrational spectroscopy.

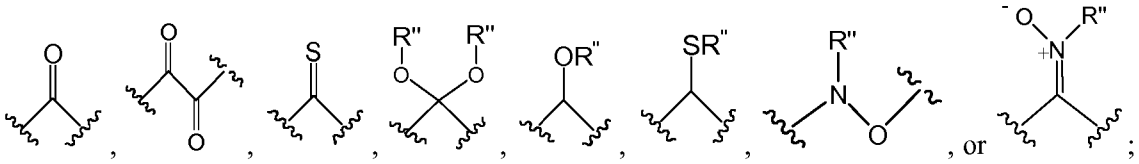
A. Structure and Synthesis of Non-Natural Amino Acid Derivatives: Carbonyl, Carbonyl like, Masked Carbonyl, and Protected Carbonyl Groups

[0194] Amino acids with an electrophilic reactive group allow for a variety of reactions to link molecules via various chemical reactions, including, but not limited to, nucleophilic addition reactions. Such electrophilic reactive groups include a carbonyl- or dicarbonyl-group (including a keto- or aldehyde group), a carbonyl-like- or dicarbonyl-like-group (which has reactivity similar to a carbonyl- or dicarbonyl-group and is structurally similar to a carbonyl- or dicarbonyl-group), a masked carbonyl- or masked dicarbonyl-group (which can be readily converted into a carbonyl- or dicarbonyl-group), or a protected carbonyl- or protected dicarbonyl-group (which has reactivity similar to a carbonyl- or dicarbonyl-group upon deprotection). Such amino acids include amino acids having the structure of Formula (XXXVII):


(XXXVII)

wherein:

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;
B is optional, and when present is a linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene, -O-, -O-(alkylene or substituted alkylene)-, -S-, -S-(alkylene or substituted alkylene)-, $-S(O)_k-$ where k is 1, 2, or 3, $-S(O)_k$(alkylene or substituted alkylene)-, -C(O)-, $-NS(O)_2-$, $-OS(O)_2-$, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -C(S)-(alkylene or substituted alkylene)-, -N(R')-, -NR'-(alkylene or substituted alkylene)-, -C(O)N(R')-, -CON(R')-(alkylene or substituted alkylene)-, -CSN(R')-, -CSN(R')-(alkylene or substituted alkylene)-, -N(R')CO-(alkylene or substituted alkylene)-, -N(R')C(O)O-, $-S(O)_kN(R')-$, -N(R')C(O)N(R')-, -N(R')C(S)N(R')-, $-N(R')S(O)_kN(R')-$, -N(R')-N=, -C(R')=N-, -C(R')=N-N(R')-, -C(R')=N-N=, $-C(R')_2-N=N-$, and $-C(R')_2-N(R')-N(R')-$, where each R' is independently H, alkyl, or substituted alkyl;
K is



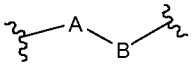
R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;
each R" is independently H, alkyl, substituted alkyl, or a protecting group, or when more than one R" group is present, two R" optionally form a heterocycloalkyl;
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and
R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;
each of R_3 and R_4 is independently H, halogen, lower alkyl, or substituted lower alkyl, or R_3 and R_4 or two R_3 groups optionally form a cycloalkyl or a heterocycloalkyl;
or the -A-B-K-R groups together form a bicyclic or tricyclic cycloalkyl or heterocycloalkyl comprising at least one carbonyl group, including a dicarbonyl group, protected carbonyl group, including a protected dicarbonyl group, or masked carbonyl group, including a masked dicarbonyl group;
or the -K-R group together forms a monocyclic or bicyclic cycloalkyl or heterocycloalkyl comprising at least one carbonyl group, including a dicarbonyl group, protected carbonyl group, including a protected dicarbonyl group, or masked carbonyl group, including a masked dicarbonyl group;
with a proviso that when A is phenylene and each R_3 is H, B is present; and that when A is $-(CH_2)_4-$ and each R_3 is H, B is not $-NHC(O)(CH_2CH_2)-$; and that when A and B are absent and each R_3 is H, R is not methyl. Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[0195] In certain embodiments, compounds of Formula (XXXVII) are stable in aqueous solution for at least 1 month under mildly acidic conditions. In certain embodiments, compounds of Formula (XXXVII) are stable for at least 2 weeks under mildly acidic conditions. In certain embodiments, compound of Formula (XXXVII) are stable for at least 5 days under mildly acidic conditions. In certain embodiments, such acidic conditions are pH 2 to 8.

[0196] In certain embodiments of compounds of Formula (XXXVII), B is lower alkylene, substituted lower alkylene, -O-(alkylene or substituted alkylene)-, -C(R')=N-N(R')-, -N(R')CO-, -C(O)-, -C(R')=N-, -C(O)-(alkylene or substituted alkylene)-, -CON(R')-(alkylene or substituted alkylene)-, -S(alkylene or substituted alkylene)-, -S(O)(alkylene or substituted alkylene)-, or $-S(O)_2$(alkylene or substituted alkylene)-. In certain embodiments of compounds of Formula (XXXVII), B is $-O(CH_2)-$, -CH=N-, -CH=N-NH-, $-NHCH_2-$, -NHCO-, -C(O)-, $-C(O)-(CH_2)-$, $-CONH-(CH_2)-$, $-SCH_2-$, $-S(=O)CH_2-$, or $-S(O)_2CH_2-$. In certain embodiments of compounds of Formula (XXXVII), R is C_{1-6} alkyl or cycloalkyl. In certain embod-

iments of compounds of Formula (XXXVII) R is -CH_3, -$CH(CH_3)_2$, or cyclopropyl. In certain embodiments of compounds of Formula (XXXVII), R_1 is H, tert-butyloxycarbonyl (Boc), 9-Fluorenylmethoxycarbonyl (Fmoc), N-acetyl, tetrafluoroacetyl (TFA), or benzyloxycarbonyl (Cbz). In certain embodiments of compounds of Formula (XXXVII), R_1 is a resin, amino acid, polypeptide, antibody, or polynucleotide. In certain embodiments of compounds of Formula (XXXVII), R_2 is OH, O-methyl, O-ethyl, or O-*t*-butyl. In certain embodiments of compounds of Formula (XXXVII), R_2 is a resin, amino acid, polypeptide, antibody, or polynucleotide. In certain embodiments of compounds of Formula (XXXVII), R_2 is a polynucleotide. In certain embodiments of compounds of Formula (XXXVII), R_2 is ribonucleic acid (RNA).

[0197] In certain embodiments of compounds of Formula (XXXVII),



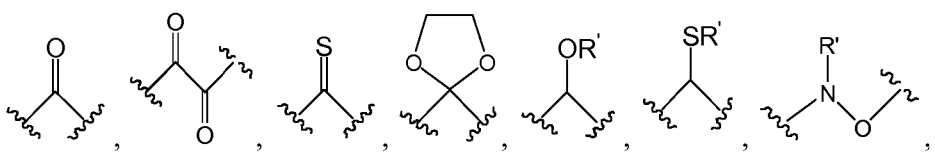
is selected from the group consisting of:

(i) A is substituted lower alkylene, C_4-arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene; B is optional, and when present is a divalent linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, -O-, -O-(alkylene or substituted alkylene)-, -S-, -S(O)-, -$S(O)_2$-, -$NS(O)_2$-,-$OS(O)_2$-, -C(O)-, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -N(R')-, -C(O)N(R')-, -CON(R')-(alkylene or substituted alkylene)-, -CSN(R')-, -N(R')CO-(alkylene or substituted alkylene)-, -N(R')C(O)O-, -N(R')C(S)-, -S(O)N(R'), -$S(O)_2$N(R'), -N(R')C(O)N(R')-, -N(R')C(S)N(R')-, -N(R')S(O)N(R')-, -N(R')$S(O)_2$N(R')-, -N(R')-N=, -C(R')=N-N(R')-, -C(R')=N-N=, -$C(R')_2$-N=N-, and -$C(R')_2$-N(R')-N(R')-;

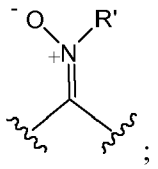
(ii) A is optional, and when present is substituted lower alkylene, C_4-arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;
B is a divalent linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, -O-, -O-(alkylene or substituted alkylene)-, -S-, -S(O)-, -$S(O)_2$-, -$NS(O)_2$-, -$OS(O)_2$-, -C(O)-, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -N(R')-, -C(O)N(R')-, -CON(R')-(alkylene or substituted alkylene)-, -CSN(R')-, -N(R')CO-(alkylene or substituted alkylene)-, -N(R')C(O)O-, -N(R')C(S)-, -S(O)N(R'), -$S(O)_2$N(R'), -N(R')C(O)N(R')-, -N(R')C(S)N(R')-, -N(R')S(O)N(R')-, -N(R')$S(O)_2$N(R')-, -N(R')-N=, -C(R')=N-N(R')-, -C(R')=N-N=, -$C(R')_2$-N=N-, and -$C(R')_2$-N(R')-N(R')-;

(iii) A is lower alkylene;
B is optional, and when present is a divalent linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, -O-, -O-(alkylene or substituted alkylene)-, -S-, -S(O)-, -$S(O)_2$-, -$NS(O)_2$-,-$OS(O)_2$-, -C(O)-, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -N(R')-, -C(O)N(R')-, -CSN(R')-, -CON(R')-(alkylene or substituted alkylene)-, -N(R')C(O)O-, -N(R')C(S)-, -S(O)N(R'), -$S(O)_2$N(R'), -N(R')C(O)N(R')-, -N(R')C(S)N(R')-, -N(R')S(O)N(R')-,-N(R')$S(O)_2$N(R')-, -N(R')-N=, -C(R')=N-N(R')-, -C(R')=N-N=, -$C(R')_2$-N=N-, and -$C(R')_2$-N(R')-N(R')-; and

(iv) A is phenylene;
B is a divalent linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, -O-, -O-(alkylene or substituted alkylene)-, -S-, -S(O)-, -$S(O)_2$-, -$NS(O)_2$-, -$OS(O)_2$-, -C(O)-, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -N(R')-, -C(O)N(R')-, -CON(R')-(alkylene or substituted alkylene)-, -CSN(R')-, -N(R')CO-(alkylene or substituted alkylene)-, -N(R')C(O)O-, -N(R')C(S)-, -S(O)N(R'), -$S(O)_2$N(R'), -N(R')C(O)N(R')-, -N(R')C(S)N(R')-, -N(R')S(O)N(R')-, -N(R')$S(O)_2$N(R')-, -N(R')-N=, -C(R')=N-N(R')-, -C(R')=N-N=, -$C(R')_2$-N=N-, and -$C(R')_2$-N(R')-N(R')-;
K is

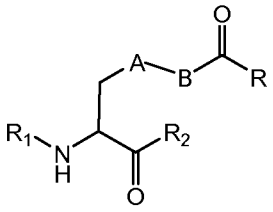


or



each R' is independently H, alkyl, or substituted alkyl;
R_1 is optional, and when present, is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and
R_2 is optional, and when present, is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide; and
each R_3 and R_4 is independently H, halogen, lower alkyl, or substituted lower alkyl;
R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;

[0198] In addition, amino acids having the structure of Formula (XXXVIII) are included:

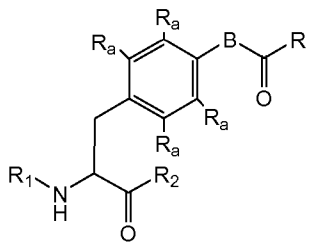


(XXXVIII),

wherein:

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;
B is optional, and when present is a linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene, -O-, -O-(alkylene or substituted alkylene)-, -S-, -S-(alkylene or substituted alkylene)-, $-S(O)_k-$ where k is 1, 2, or 3, $-S(O)_k$(alkylene or substituted alkylene)-, -C(O)-, $-NS(O)_2-$, $-OS(O)_2-$, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -C(S)-(alkylene or substituted alkylene)-, -N(R')-, -NR'-(alkylene or substituted alkylene)-, -C(O)N(R')-, -CON(R')-(alkylene or substituted alkylene)-, -CSN(R')-, -CSN(R')-(alkylene or substituted alkylene)-, -N(R')CO-(alkylene or substituted alkylene)-, -N(R')C(O)O-, $-S(O)_kN(R')-$, -N(R')C(O)N(R')-, -N(R')C(S)N(R')-, $-N(R')S(O)_kN(R')-$, -N(R')-N=, -C(R')=N-, -C(R')=N-N(R')-, -C(R')=N-N=, $-C(R')_2-N=N-$, and $-C(R')_2-N(R')-N(R')-$, where each R' is independently H, alkyl, or substituted alkyl;
R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;
R_1 is H, an amino protecting group, resin, at least one amino acid, polypeptide, or polynucleotide; and
R_2 is OH, an ester protecting group, resin, at least one amino acid, polypeptide, or polynucleotide;
with a proviso that when A is phenylene, B is present; and that when A is $-(CH_2)_4-$, B is not-$NHC(O)(CH_2CH_2)-$; and that when A and B are absent, R is not methyl. Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[0199] In addition, amino acids having the structure of Formula (XXXIX) are included:

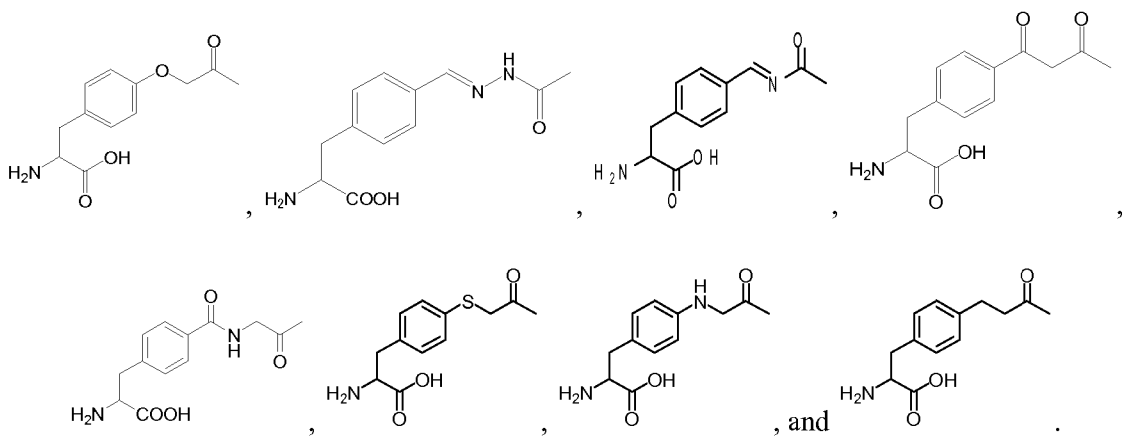


(XXXIX),

wherein:

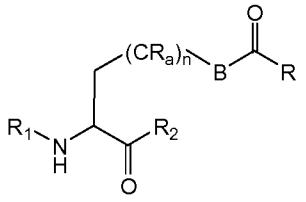
B is a linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene, -O-, -O-(alkylene or substituted alkylene)-, -S-, -S-(alkylene or substituted alkylene)-, $-S(O)_k-$ where k is 1, 2, or 3, $-S(O)_k$(alkylene or substituted alkylene)-, -C(O)-, $-NS(O)_2-$, $-OS(O)_2-$, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -C(S)-(alkylene or substituted alkylene)-, -N(R')-, -NR'-(alkylene or substituted alkylene)-, -C(O)N(R')-, -CON(R')-(alkylene or substituted alkylene)-, -CSN(R')-, -CSN(R')-(alkylene or substituted alkylene)-, -N(R')CO-(alkylene or substituted alkylene)-, $-N(R')C(O)O-$, $-S(O)_kN(R')-$, $-N(R')C(O)N(R')-$, $-N(R')C(S)N(R')-$, $-N(R')S(O)_kN(R')-$, -N(R')-N=, -C(R')=N-, -C(R')=N-N(R')-, -C(R')=N-N=, $-C(R')_2-N=N-$, and $-C(R')_2-N(R')-N(R')-$, where each R' is independently H, alkyl, or substituted alkyl;
R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and
R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;
each R_a is independently selected from the group consisting of H, halogen, alkyl, substituted alkyl,$-N(R')_2$, $-C(O)_kR'$ where k is 1, 2, or 3, $-C(O)N(R')_2$, -OR', and $-S(O)_kR'$, where each R' is independently H, alkyl, or substituted alkyl. Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[0200] In addition, the following amino acids are included:



Such non-natural amino acids may be are optionally amino protected group, carboxyl protected and/or in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[0201] In addition, the following amino acids having the structure of Formula (XXXX) are included:

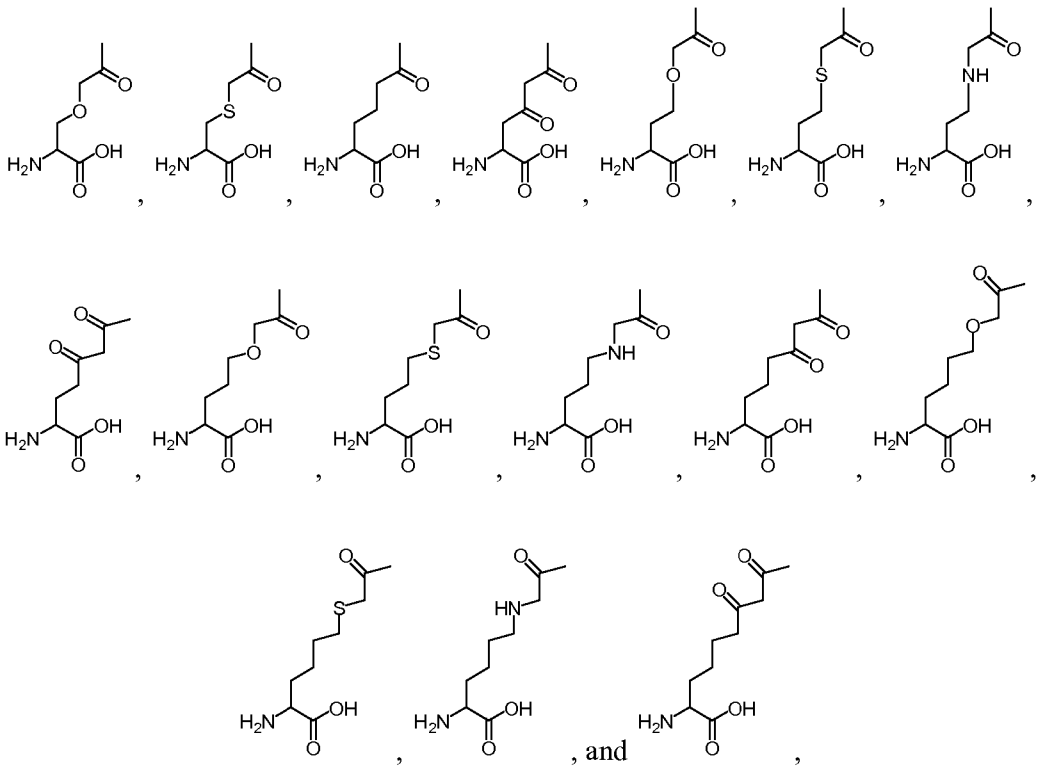


(XXXX)

wherein

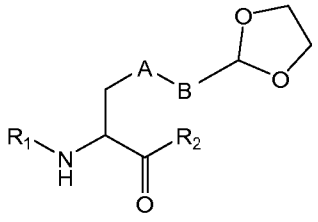
$-NS(O)_2-$, $-OS(O)_2-$, optional, and when present is a linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene, -O-, -O-(alkylene or substituted alkylene)-, -S-, -S-(alkylene or substituted alkylene)-, $-S(O)_k-$ where k is 1, 2, or 3, $-S(O)_k$(alkylene or substituted alkylene)-, -C(O)-, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -C(S)-(alkylene or substituted alkylene)-, -N(R')-, -NR'-(alkylene or substituted alkylene)-, -C(O)N(R')-, -CON(R')-(alkylene or substituted alkylene)-, -CSN(R')-, -CSN(R')-(alkylene or substituted alkylene)-, -N(R')CO-(alkylene or substituted alkylene)-, $-N(R')C(O)O-$, $-S(O)_kN(R')-$, $-N(R')C(O)N(R')-$, $-N(R')C(S)N(R')-$, $-N(R')S(O)_kN(R')-$, $-N(R')-N=$, $-C(R')=N-$, $-C(R')=N-N(R')-$, $-C(R')=N-N=$, $-C(R')_2-N=N-$, and $-C(R')_2-N(R')-N(R')-$, where each R' is independently H, alkyl, or substituted alkyl;
R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and
R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;
each R_a is independently selected from the group consisting of H, halogen, alkyl, substituted alkyl,$-N(R')_2$, $-C(O)_kR'$ where k is 1, 2, or 3, $-C(O)N(R')_2$, -OR', and $-S(O)_kR'$, where each R' is independently H, alkyl, or substituted alkyl; and n is 0 to 8;
with a proviso that when A is $-(CH_2)_4-$, B is not $-NHC(O)(CH_2CH_2)-$. Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[0202] In addition, the following amino acids are included:



wherein such compounds are optionally amino protected, optionally carboxyl protected, optionally amino protected and carboxyl protected, or a salt thereof, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[0203] In addition, the following amino acids having the structure of Formula (XXXXI) are included:



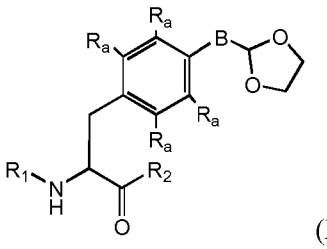
(XXXXI),

wherein,

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;
B is optional, and when present is a linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene, -O-, -O-(alkylene or substituted alkylene)-, -S-, -S-(alkylene or substituted alkylene)-, $-S(O)_k-$ where k is 1, 2, or 3, $-S(O)_k$(alkylene or substituted alkylene)-, -C(O)-, $-NS(O)_2-$, $-OS(O)_2-$, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -C(S)-(alkylene or substituted alkylene)-, -N(R')-, -NR'-(alkylene or substituted alkylene)-, -C(O)N(R')-, -CON(R')-(alkylene or substituted alkylene)-, -CSN(R')-, -CSN(R')-(alkylene or substituted alkylene)-, -N(R')CO-(alkylene or substituted alkylene)-, -N(R')C(O)O-, $-S(O)_kN(R')-$, -N(R')C(O)N(R')-, -N(R')C(S)N(R')-, $-N(R')S(O)_kN(R')-$, -N(R')-N=, -C(R')=N-, -C(R')=N-N(R')-, -C(R')=N-N=, $-C(R')_2-N=N-$, and $-C(R')_2-N(R')-N(R')-$, where each R' is independently H, alkyl, or substituted alkyl;
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and
R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide.

Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[0204] In addition, the following amino acids having the structure of Formula (XXXXII) are included:



(XXXXII),

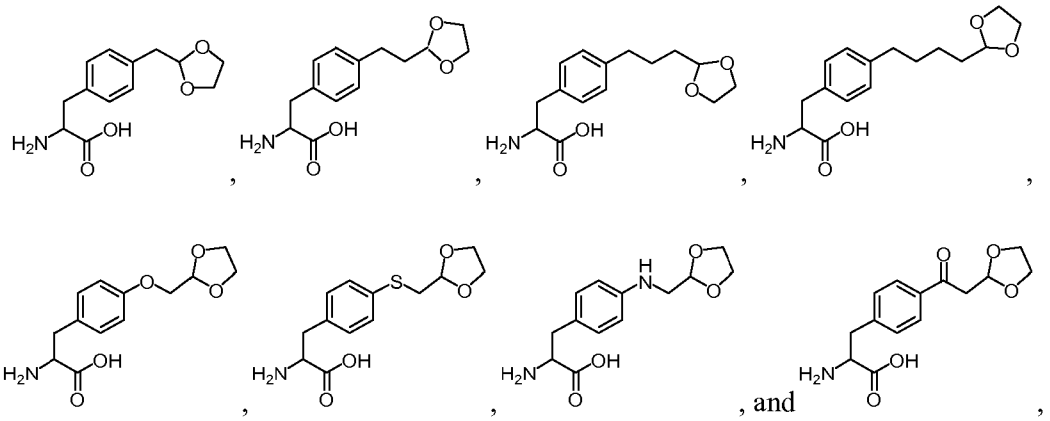
wherein,

B is optional, and when present is a linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene, -O-, -O-(alkylene or substituted alkylene)-, -S-, -S-(alkylene or substituted alkylene)-, $-S(O)_k-$ where k is 1, 2, or 3, $-S(O)_k$(alkylene or substituted alkylene)-, -C(O)-, $-NS(O)_2-$, $-OS(O)_2-$, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -C(S)-(alkylene or substituted alkylene)-, -N(R')-, -NR'-(alkylene or substituted alkylene)-, -C(O)N(R')-, -CON(R')-(alkylene or substituted alkylene)-, -CSN(R')-, -CSN(R')-(alkylene or substituted alkylene)-, -N(R')CO-(alkylene or substituted alkylene)-, -N(R')C(O)O-, $-S(O)_kN(R')-$, -N(R')C(O)N(R')-, -N(R')C(S)N(R')-, $-N(R')S(O)_kN(R')-$, -N(R')-N=, -C(R')=N-, -C(R')=N-N(R')-, -C(R')=N-N=, $-C(R')_2-N=N-$, and $-C(R')_2-N(R')-N(R')-$, where each R' is independently H, alkyl, or substituted alkyl;
R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and

R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide; wherein each R_a is independently selected from the group consisting of H, halogen, alkyl, substituted alkyl, $-N(R')_2$, $-C(O)_kR'$ where k is 1, 2, or 3, $-C(O)N(R')_2$, -OR', and $-S(O)_kR'$, where each R' is independently H, alkyl, or substituted alkyl.

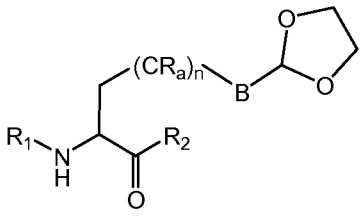
Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[0205] In addition, the following amino acids are included:



wherein such compounds are optionally amino protected, optionally carboxyl protected, optionally amino protected and carboxyl protected, or a salt thereof, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[0206] In addition, the following amino acids having the structure of Formula (XXXXIV) are included:



(XXXXIV),

wherein,

B is optional, and when present is a linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene, -O-, -O-(alkylene or substituted alkylene)-, -S-, -S-(alkylene or substituted alkylene)-, $-S(O)_k-$ where k is 1, 2, or 3, $-S(O)_k$(alkylene or substituted alkylene)-, -C(O)-, $-NS(O)_2-$, $-OS(O)_2-$, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -C(S)-(alkylene or substituted alkylene)-, -N(R')-, -NR'-(alkylene or substituted alkylene)-, -C(O)N(R')-, -CON(R')-(alkylene or substituted alkylene)-, -CSN(R')-, -CSN(R')-(alkylene or substituted alkylene)-, -N(R')CO-(alkylene or substituted alkylene)-, -N(R')C(O)O-, $-S(O)_kN(R')-$, -N(R')C(O)N(R')-, -N(R')C(S)N(R')-, $-N(R')S(O)_kN(R')-$, -N(R')-N=, -C(R')=N-, -C(R')=N-N(R')-, -C(R')=N-N=, $-C(R')_2-N=N-$, and $-C(R')_2-N(R')-N(R')-$, where each R' is independently H, alkyl, or substituted alkyl;

R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;

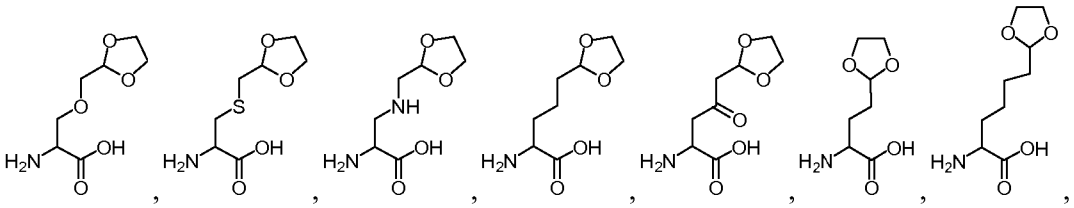
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and

R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;

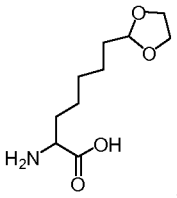
each R_a is independently selected from the group consisting of H, halogen, alkyl, substituted alkyl,$-N(R')_2$, $-C(O)_kR'$ where k is 1, 2, or 3, $-C(O)N(R')_2$, -OR', and $-S(O)_kR'$, where each R' is independently H, alkyl, or substituted alkyl; and n is 0 to 8.

Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[0207] In addition, the following amino acids are included:

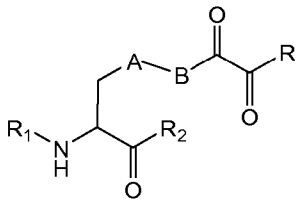


and



wherein such compounds are optionally amino protected, optionally carboxyl protected, optionally amino protected and carboxyl protected, or a salt thereof, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[0208] In addition to monocarbonyl structures, the non-natural amino acids described herein may include groups such as dicarbonyl, dicarbonyl like, masked dicarbonyl and protected dicarbonyl groups. For example, the following amino acids having the structure of Formula (XXXXV) are included:



(XXXXV),

wherein,

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;

B is optional, and when present is a linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene, -O-, -O-(alkylene or substituted alkylene)-, -S-, -S-(alkylene or substituted alkylene)-, $-S(O)_k-$ where k is 1, 2, or 3, $-S(O)_k$(alkylene or substituted alkylene)-, -C(O)-, $-NS(O)_2-$, $-OS(O)_2-$, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -C(S)-(alkylene or substituted alkylene)-, -N(R')-, -NR'-(alkylene or substituted alkylene)-, -C(O)N(R')-, -CON(R')-(alkylene or substituted alkylene)-, -CSN(R')-, -CSN(R')-(alkylene or substituted alkylene)-, -N(R')CO-(alkylene or substituted alkylene)-, -N(R')C(O)O-, $-S(O)_kN(R')-$, -N(R')C(O)N(R')-, -N(R')C(S)N(R')-, $-N(R')S(O)_kN(R')-$, -N(R')-N=, -C(R')=N-, -C(R')=N-N(R')-, -C(R')=N-N=, $-C(R')_2-N=N-$, and $-C(R')_2-N(R')-N(R')-$, where each R' is independently H, alkyl, or substituted alkyl;

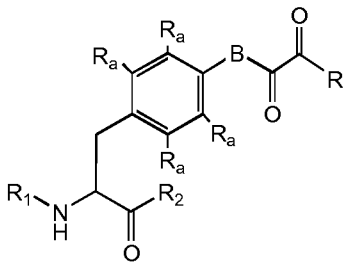
R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;

R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and

R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide.

Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[0209] In addition, the following amino acids having the structure of Formula (XXXXVI) are included:



(XXXXVI),

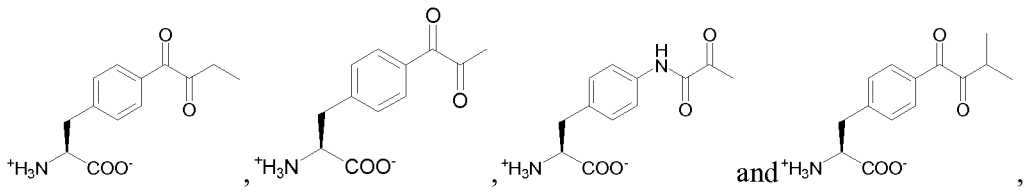
wherein,

B is optional, and when present is a linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene, -O-, -O-(alkylene or substituted alkylene)-, -S-, -S-(alkylene or substituted alkylene)-, $-S(O)_k-$ where k is 1, 2, or 3, $-S(O)_k$(alkylene or substituted alkylene)-, -C(O)-, $-NS(O)_2-$, $-OS(O)_2-$, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -C(S)-(alkylene or substituted alkylene)-, -N(R')-, -NR'-(alkylene or substituted alkylene)-, -C(O)N(R')-, -CON(R')-(alkylene or substituted alkylene)-, -CSN(R')-, -CSN(R')-(alkylene or substituted alkylene)-, -N(R')CO-(alkylene or substituted alkylene)-, -N(R')C(O)O-, $-S(O)_kN(R')-$, -N(R')C(O)N(R')-, -N(R')C(S)N(R')-, $-N(R')S(O)_kN(R')-$, -N(R')-N=, -C(R')=N-, -C(R')=N-N(R')-, -C(R')=N-N=, $-C(R')_2-N=N-$, and $-C(R')_2-N(R')-N(R')-$, where each R' is independently H, alkyl, or substituted alkyl;
R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and
R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;

wherein each R_a is independently selected from the group consisting of H, halogen, alkyl, substituted alkyl, $-N(R')_2$, $-C(O)_kR'$ where k is 1, 2, or 3, $-C(O)N(R')_2$, -OR', and $-S(O)_kR'$,
where each R' is independently H, alkyl, or substituted alkyl.

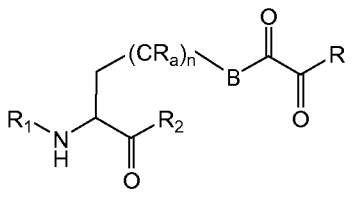
Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[0210] In addition, the following amino acids are included:



wherein such compounds are optionally amino protected and carboxyl protected, or a salt thereof. Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[0211] In addition, the following amino acids having the structure of Formula (XXXXVII) are included:



(XXXXVII),

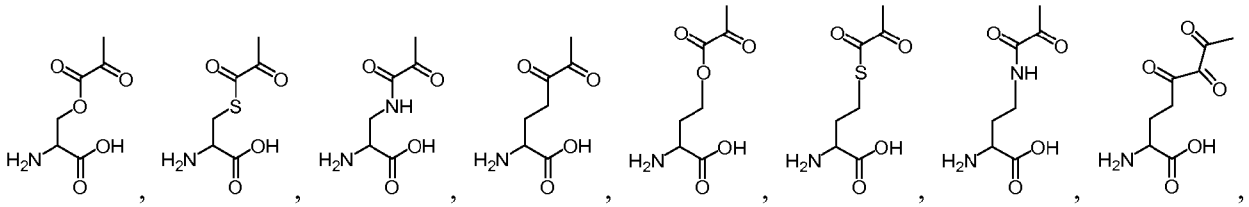
wherein,

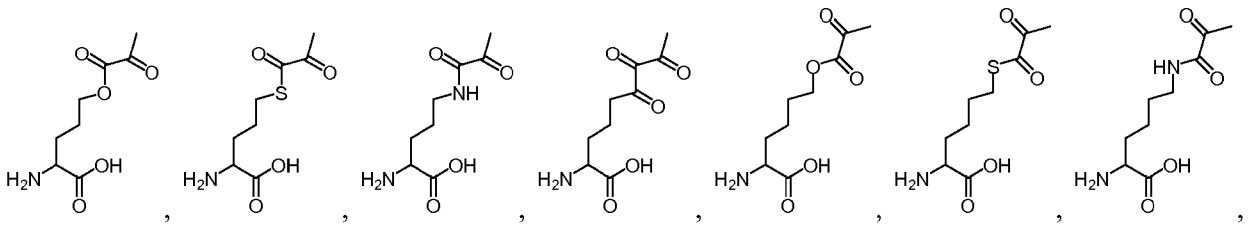
B is optional, and when present is a linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene,

-O-, -O-(alkylene or substituted alkylene)-, -S-, -S-(alkylene or substituted alkylene)-, -$S(O)_k$- where k is 1, 2, or 3, -$S(O)_k$(alkylene or substituted alkylene)-, -C(O)-, -$NS(O)_2$-, -$OS(O)_2$-, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -C(S)-(alkylene or substituted alkylene)-, -N(R')-, -NR'-(alkylene or substituted alkylene)-, -C(O)N(R')-, -CON(R')-(alkylene or substituted alkylene)-, -CSN(R')-, -CSN(R')-(alkylene or substituted alkylene)-, -N(R')CO-(alkylene or substituted alkylene)-, -N(R')C(O)O-, -$S(O)_k$N(R')-, -N(R')C(O)N(R')-, -N(R')C(S)N(R')-, -N(R')$S(O)_k$N(R')-, -N(R')-N=, -C(R')=N-, -C(R')=N-N(R')-, -C(R')=N-N=, -$C(R')_2$-N=N-, and -$C(R')_2$-N(R')-N(R')-, where each R' is independently H, alkyl, or substituted alkyl;
R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and
R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;
each R_a is independently selected from the group consisting of H, halogen, alkyl, substituted alkyl, -$N(R')_2$, -$C(O)_kR'$ where k is 1, 2, or 3, -$C(O)N(R')_2$, -OR', and -$S(O)_kR'$, where each R' is independently H, alkyl, or substituted alkyl; and n is 0 to 8.

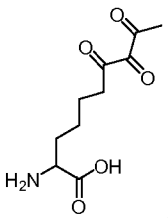
Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[0212] In addition, the following amino acids are included:



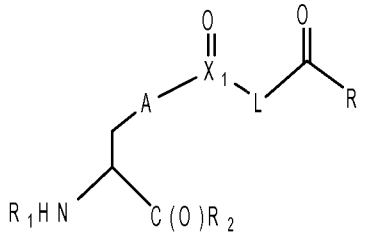


and



wherein such compounds are optionally amino protected and carboxyl protected, or a salt thereof, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[0213] In addition, the following amino acids having the structure of Formula (XXXXVIII) are included:



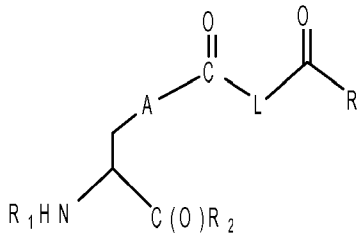
(XXXXVIII);

wherein:

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;
R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and
R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;
X_1 is C, S, or S(O); and L is alkylene, substituted alkylene, N(R')(alkylene) or N(R')(substituted alkylene), where R' is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl.

Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[0214] In addition, the following amino acids having the structure of Formula (XXXXIX) are included:



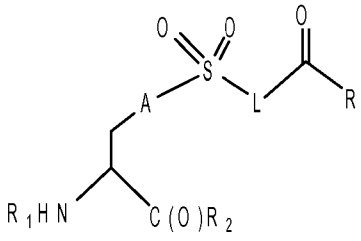
(XXXXIX)

wherein:

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;
R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and
R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;
L is alkylene, substituted alkylene, N(R')(alkylene) or N(R')(substituted alkylene), where R' is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl.

Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[0215] In addition, the following amino acids having the structure of Formula (XXXXX) are included:



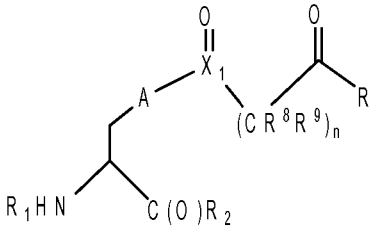
(XXXXX)

wherein:

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;
R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and
R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;
L is alkylene, substituted alkylene, N(R')(alkylene) or N(R')(substituted alkylene), where R' is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl.

Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[0216] In addition, the following amino acids having the structure of Formula (XXXXXI) are included:



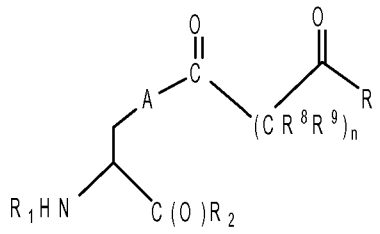
(XXXXXI);

wherein:

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;
R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and
R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;
X_1 is C, S, or S(O); and n is 0, 1, 2, 3, 4, or 5; and each R^8 and R^9 on each CR^8R^9 group is independently selected from the group consisting of H, alkoxy, alkylamine, halogen, alkyl, aryl, or any R^8 and R^9 can together form =O or a cycloalkyl, or any to adjacent R^8 groups can together form a cycloalkyl.

Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[0217] In addition, the following amino acids having the structure of Formula (XXXXXII) are included:



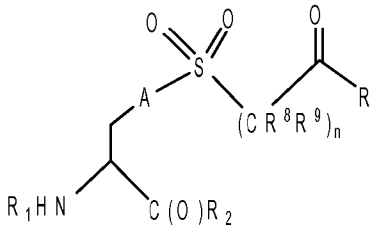
(XXXXXII)

wherein:

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;
R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and
R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide; n is 0, 1, 2, 3, 4, or 5; and each R^8 and R^9 on each CR^8R^9 group is independently selected from the group consisting of H, alkoxy, alkylamine, halogen, alkyl, aryl, or any R^8 and R^9 can together form =O or a cycloalkyl, or any to adjacent R^8 groups can together form a cycloalkyl.

Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[0218] In addition, the following amino acids having the structure of Formula (XXXXXIII) are included:



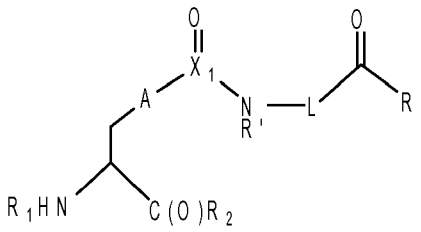
(XXXXXIII)

wherein:

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;
R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and
R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide; n is 0, 1, 2, 3, 4, or 5; and each R^8 and R^9 on each CR^8R^9 group is independently selected from the group consisting of H, alkoxy, alkylamine, halogen, alkyl, aryl, or any R^8 and R^9 can together form =O or a cycloalkyl, or any to adjacent R^8 groups can together form a cycloalkyl.

Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[0219] In addition, the following amino acids having the structure of Formula (XXXXXIV) are included:



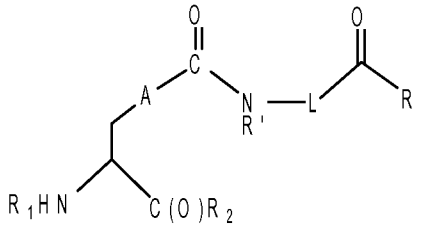
(XXXXXIV);

wherein:

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;
R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and
R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;
X_1 is C, S, or S(O); and L is alkylene, substituted alkylene, N(R')(alkylene) or N(R')(substituted alkylene), where R' is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl.

Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[0220] In addition, the following amino acids having the structure of Formula (XXXXXV) are included:



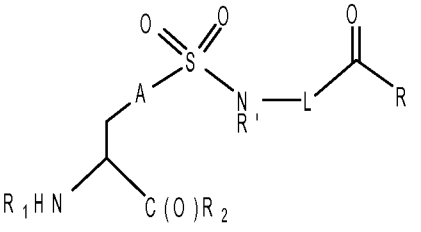
(XXXXXV)

wherein:

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;
R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and
R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;
L is alkylene, substituted alkylene, N(R')(alkylene) or N(R')(substituted alkylene), where R' is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl.

Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[0221] In addition, the following amino acids having the structure of Formula (XXXXXVI) are included:



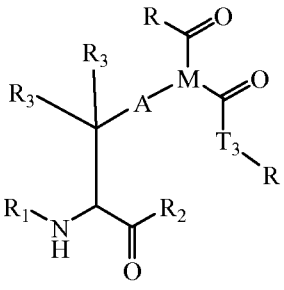
(XXXXXVI)

wherein:

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;
R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and
R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;
L is alkylene, substituted alkylene, N(R')(alkylene) or N(R')(substituted alkylene), where R' is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl.

Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

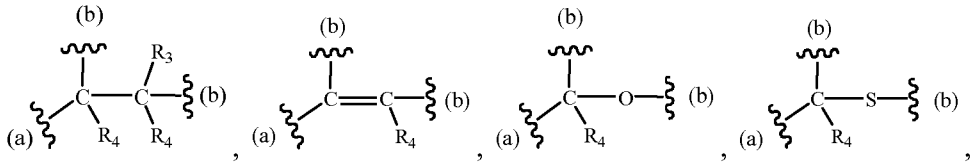
[0222] In addition, amino acids having the structure of Formula (XXXXXVII) are included:

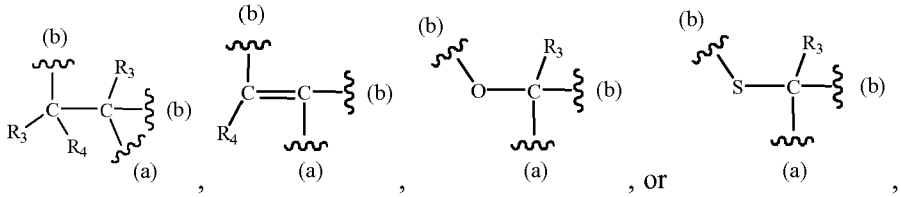


(XXXXXVII),

wherein:

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;
M is $-C(R_3)-$,

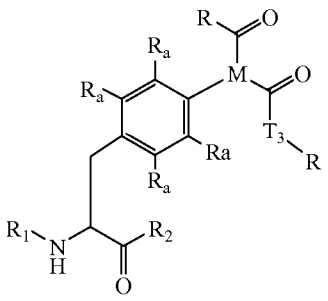




where (a) indicates bonding to the A group and (b) indicates bonding to respective carbonyl groups, R_3 and R_4 are independently chosen from H, halogen, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl, or R_3 and R_4 or two R_3 groups or two R_4 groups optionally form a cycloalkyl or a heterocycloalkyl;
R is H, halogen, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;
T_3 is a bond, C(R)(R), O, or S, and R is H, halogen, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and
R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide.

Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

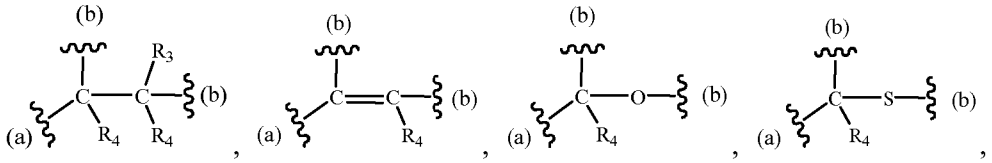
[0223] In addition, amino acids having the structure of Formula (XXXXXVIII) are included:

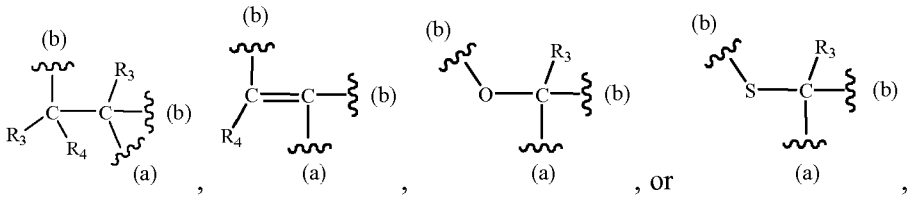


(XXXXXVIII),

wherein:

M is -C(R_3)-,



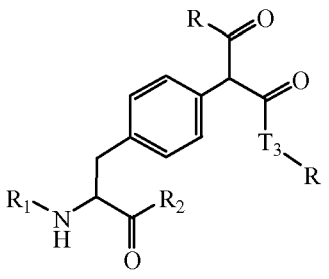


, or

where (a) indicates bonding to the A group and (b) indicates bonding to respective carbonyl groups, R_3 and R_4 are independently chosen from H, halogen, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl, or R_3 and R_4 or two R_3 groups or two R_4 groups optionally form a cycloalkyl or a heterocycloalkyl;
R is H, halogen, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;
T_3 is a bond, C(R)(R), O, or S, and R is H, halogen, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and
R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide; each R_a is independently selected from the group consisting of H, halogen, alkyl, substituted alkyl, -N(R')$_2$, -C(O)$_k$R' where k is 1, 2, or 3, -C(O)N(R')$_2$, -OR', and -S(O)$_k$R', where each R' is independently H, alkyl, or substituted alkyl.

Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[0224] In addition, amino acids having the structure of Formula (XXXXXIX) are included:



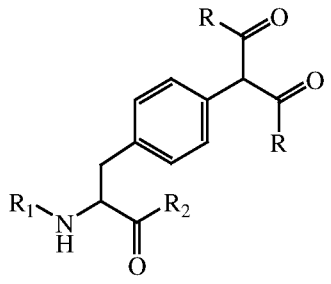
(XXXXXIX),

wherein:

R is H, halogen, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl; and
T_3 is O, or S.

Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

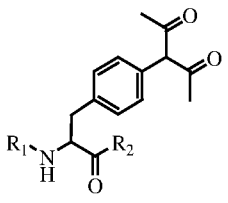
[0225] In addition, amino acids having the structure of Formula (XXXXXX) are included:



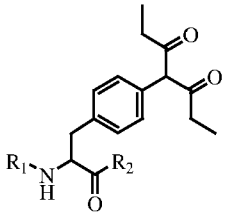
(XXXXXX),

wherein:
R is H, halogen, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl.

[0226] In addition, the following amino acids having structures of Formula (XXXXXX) are included:



, and



.

Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[0227] The carbonyl or dicarbonyl functionality can be reacted selectively with a hydroxylamine-containing reagent under mild conditions in aqueous solution to form the corresponding oxime linkage that is stable under physiological conditions. See, e.g., Jencks, W. P., J. Am. Chem. Soc. 81, 475-481 (1959); Shao, J. and Tam, J. P., J. Am. Chem. Soc. 117(14):3893-3899 (1995). Moreover, the unique reactivity of the carbonyl or dicarbonyl group allows for selective modification in the presence of the other amino acid side chains. See, e.g., Cornish, V. W., et al., J. Am. Chem. Soc. 118:8150-8151 (1996); Geoghegan, K. F. & Stroh, J. G., Bioconjug. Chem. 3:138-146 (1992); Mahal, L. K., et al., Science 276:1125-1128 (1997).

[0228] The synthesis of p-acetyl-(+/-)-phenylalanine and m-acetyl-(+/-)-phenylalanine is described in Zhang, Z., et al., Biochemistry 42: 6735-6746 (2003). Other carbonyl- or dicarbonyl-containing amino acids can be similarly prepared.

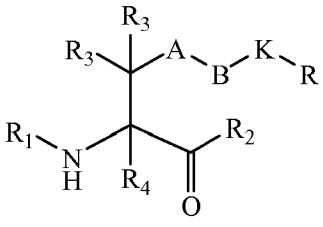
[0229] In some embodiments, a polypeptide comprising a non-natural amino acid is chemically modified to generate a reactive carbonyl or dicarbonyl functional group. For instance, an aldehyde functionality useful for conjugation reactions can be generated from a functionality having adjacent amino and hydroxyl groups. Where the biologically active molecule is a polypeptide, for example, an N-terminal serine or threonine (which may be normally present or may be exposed via chemical or enzymatic digestion) can be used to generate an aldehyde functionality under mild oxidative cleavage conditions using periodate. See, e.g., Gaertner, et. al., Bioconjug. Chem. 3: 262-268 (1992); Geoghegan, K. & Stroh, J., Bioconjug. Chem. 3:138-146 (1992); Gaertner et al., J. Biol. Chem. 269:7224-7230 (1994). However, methods known in the art are restricted to the amino acid at the N-terminus of the peptide or protein.

[0230] Additionally, by way of example a non-natural amino acid bearing adjacent hydroxyl and amino groups can be incorporated into a polypeptide as a "masked" aldehyde functionality. For example, 5-hydroxylysine bears a hydroxyl group adjacent to the epsilon amine. Reaction conditions for generating the aldehyde typically involve addition of molar excess of sodium metaperiodate under mild conditions to avoid oxidation at other sites within the polypeptide. The pH of the oxidation reaction is typically about 7.0. A typical reaction involves the addition of about 1.5 molar excess of sodium meta periodate to a buffered solution of the polypeptide, followed by incubation for about 10 minutes in the dark. See,

e.g. U.S. Patent No. 6,423,685.

B. Structure and Synthesis of Non-Natural Amino Acids: Dicarbonyl, Dicarbonyl-like, Masked Dicarbonyl, and Protected Dicarbonyl Groups

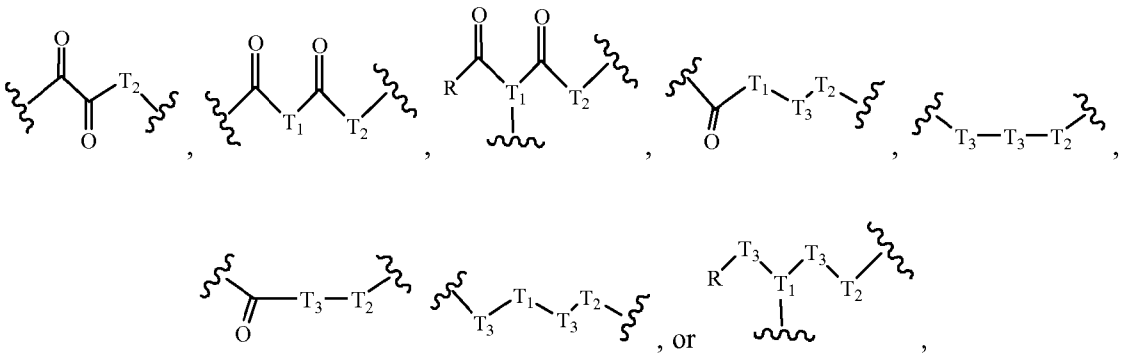
[0231] Amino acids with an electrophilic reactive group allow for a variety of reactions to link molecules via nucleophilic addition reactions among others. Such electrophilic reactive groups include a dicarbonyl group (including a diketone group, a ketoaldehyde group, a ketoacid group, a ketoester group, and a ketothioester group), a dicarbonyl-like group (which has reactivity similar to a dicarbonyl group and is structurally similar to a dicarbonyl group), a masked dicarbonyl group (which can be readily converted into a dicarbonyl group), or a protected dicarbonyl group (which has reactivity similar to a dicarbonyl group upon deprotection). Such amino acids include amino acids having the structure of Formula (XXXVII):



(XXXVII),

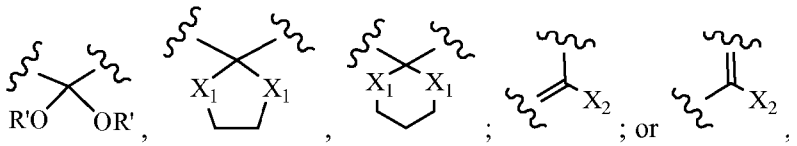
wherein:

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;
B is optional, and when present is a linker linked at one end to a diamine containing moiety, the linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene, -O-(alkylene or substituted alkylene)-, -S-(alkylene or substituted alkylene)-, -C(O)R"-,-$S(O)_k$(alkylene or substituted alkylene)-, where k is 1, 2, or 3, -C(O)-(alkylene or substituted alkylene)-, -C(S)-(alkylene or substituted alkylene)-, -NR"-(alkylene or substituted alkylene)-, -CON(R")-(alkylene or substituted alkylene)-, -CSN(R")-(alkylene or substituted alkylene)-, and -N(R")CO-(alkylene or substituted alkylene)-, where each R" is independently H, alkyl, or substituted alkyl;
K is



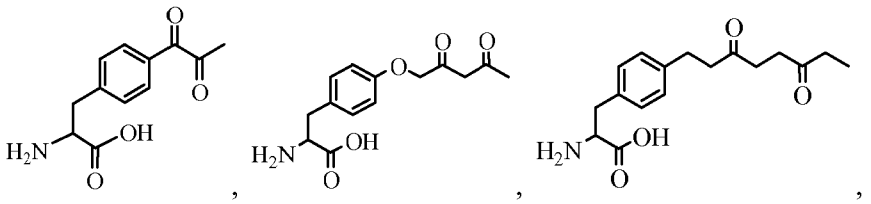
where,
T_1 is a bond, optionally substituted C_1-C_4 alkylene, optionally substituted C_1-C_4 alkenylene, or optionally substituted heteroalkyl;
wherein each optional substituents is independently selected from lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;

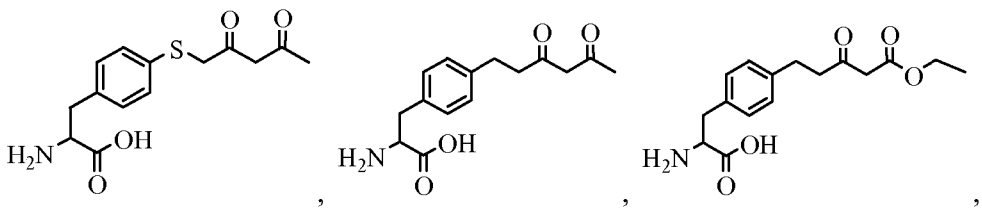
T_2, is selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene, -O-, -O-(alkylene or substituted alkylene)-, -S-, -S-(alkylene or substituted alkylene)-, -S(O)$_k$- where k is 1, 2, or 3, -S(O)$_k$(alkylene or substituted alkylene)-, -C(O)-, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -C(S)-(alkylene or substituted alkylene)-, -N(R')-, -NR'-(alkylene or substituted alkylene)-, -C(O)N(R')-, -CON(R')-(alkylene or substituted alkylene)-, -CSN(R')-, -CSN(R')-(alkylene or substituted alkylene)-, -N(R')CO-(alkylene or substituted alkylene)-, -N(R')C(O)O-, -S(O)$_k$N(R')-, -N(R')C(O)N(R')-, -N(R')C(S)N(R')-, -N(R')S(O)$_k$N(R')-, -N(R')-N=, -C(R')=N-, -C(R')=N-N(R')-, -C(R')=N-N=, -C(R')$_2$-N=N-, and -C(R')$_2$-N(R')-N(R')-, where each R' is independently H, alkyl, or substituted alkyl;
T_3 is

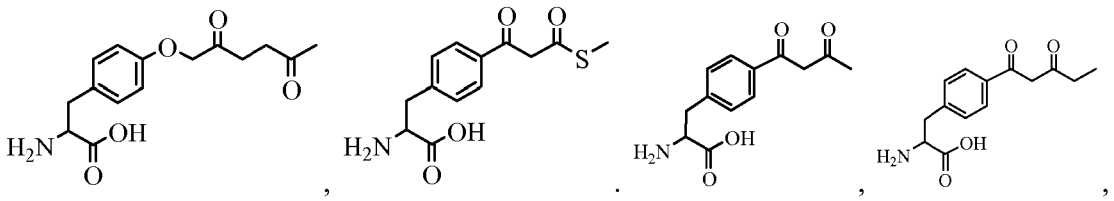


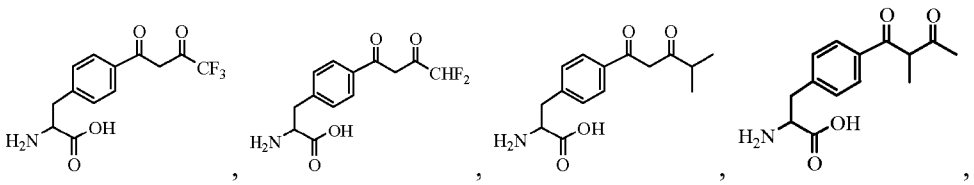
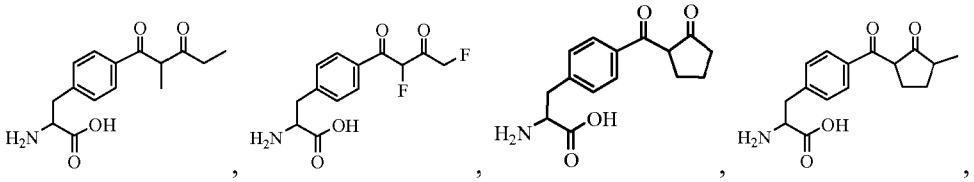
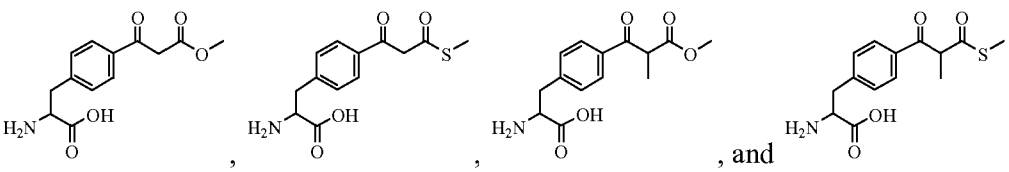
where each X_1 is independently selected from the group consisting of -O-, -S-, -N(H)-, -N(R)-, -N(Ac)-, and-N(OMe)-; X_2 is -OR, -OAc, -SR, -N(R)$_2$, -N(R)(Ac), -N(R)(OMe), or N_3, and where each R' is independently H, alkyl, or substituted alkyl;
R is H, halogen, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and
R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;
or the -A-B-K-R groups together form a bicyclic or tricyclic cycloalkyl or heterocycloalkyl comprising at least one carbonyl group, including a dicarbonyl group, protected carbonyl group, including a protected dicarbonyl group, or masked carbonyl group, including a masked dicarbonyl group;
or the -K-R group together forms a monocyclic or bicyclic cycloalkyl or heterocycloalkyl comprising at least one carbonyl group, including a dicarbonyl group, protected carbonyl group, including a protected dicarbonyl group, or masked carbonyl group, including a masked dicarbonyl group.

[0232] Non-limiting example of dicarbonyl amino acids having the structure of Formula (XXXVII) include:

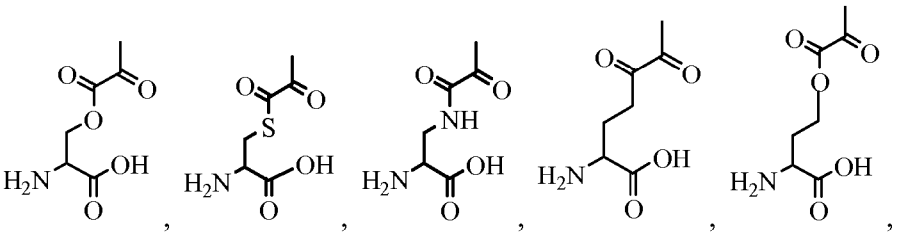
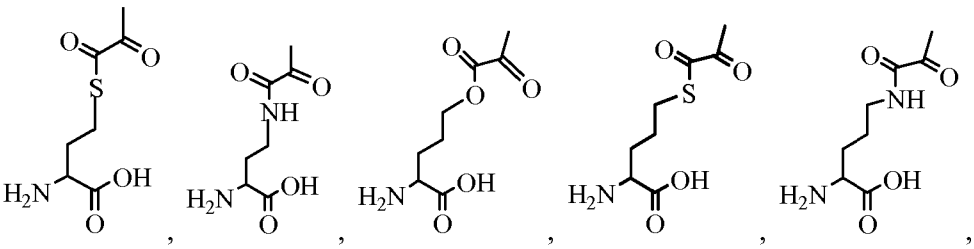
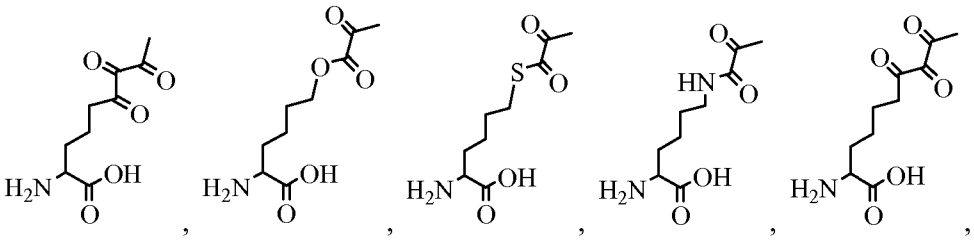
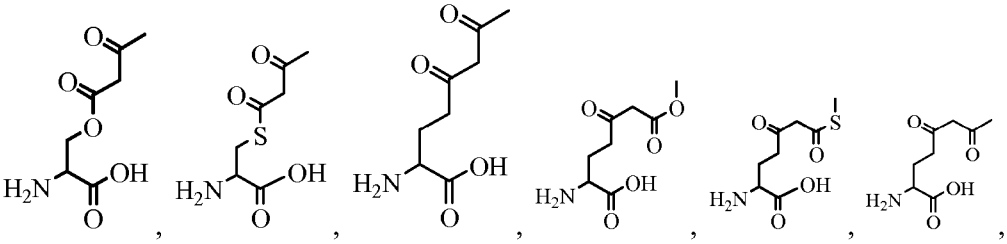


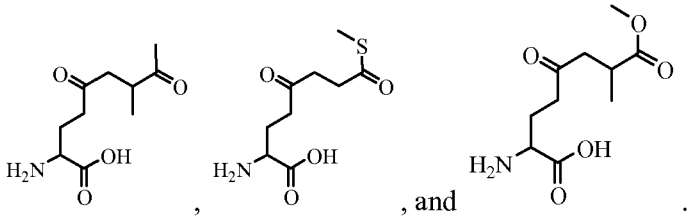




[0233] The following amino acids having structures of Formula (XXXVII) are also included:

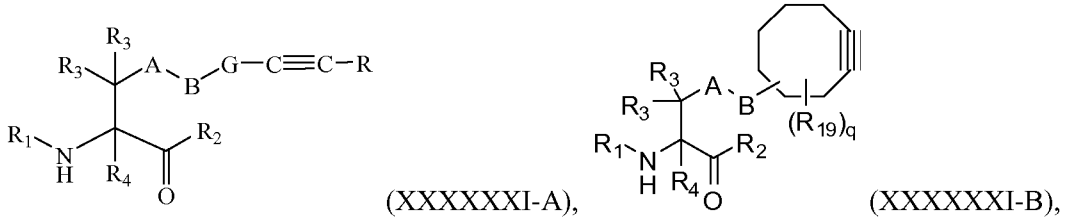







Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

C. Structure and Synthesis of Non-Natural Amino Acids: Ketoalkyne, Ketoalkyne - like, Masked Ketoalkyne, Protected Ketoalkyne Groupk, Alkyne, and Cycloalkyne Groups

[0234] Amino acids containing reactive groups with dicarbonyl-like reactivity allow for the linking of molecules via nucleophilic addition reactions. Such electrophilic reactive groups include a ketoalkyne group, a ketoalkyne-like group (which has reactivity similar to a ketoalkyne group and is structurally similar to a ketoalkyne group), a masked ketoalkyne group (which can be readily converted into a ketoalkyne group), or a protected ketoalkyne group (which has reactivity similar to a ketoalkyne group upon deprotection). In some embodiments, amino acids containing reactive groups with a terminal alkyne, internal alkyne or cycloalkyne allow for linking of molecules via cycloaddition reactions (e.g., 1,3-dipolar cycloadditions, azide-alkyne Huisgen cycloaddition, etc.) Such amino acids include amino acids having the structure of Formula (XXXXXXI-A) or (XXXXXXI-B):



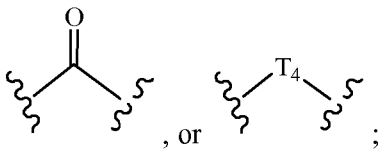
(XXXXXXI-A), (XXXXXXI-B),

wherein:

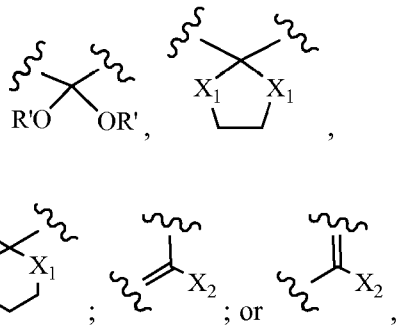
A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;

B is optional, and when present is a linker linked at one end to a diamine containing moiety, the linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene, -O-(alkylene or substituted alkylene)-, -S-(alkylene or substituted alkylene)-, -C(O)R"-,-$S(O)_k$(alkylene or substituted alkylene)-, where k is 1, 2, or 3, -C(O)-(alkylene or substituted alkylene)-, -C(S)-(alkylene or substituted alkylene)-, -NR"-(alkylene or substituted alkylene)-, -CON(R")-(alkylene or substituted alkylene)-, -CSN(R")-(alkylene or substituted alkylene)-, and -N(R")CO-(alkylene or substituted alkylene)-, where each R" is independently H, alkyl, or substituted alkyl;

G is optional, and when present is



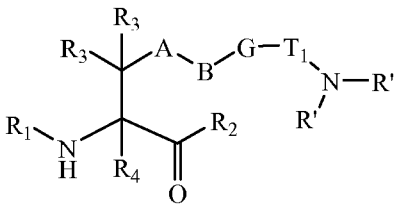
T_4 is a carbonyl protecting group including, but not limited to,



where each X_1 is independently selected from the group consisting of -O-, -S-, -N(H)-, -N(R)-, -N(Ac)-, and -N(OMe)-; X_2 is -OR, -OAc, -SR, $-N(R)_2$, -N(R)(Ac), -N(R)(OMe), or N_3, and where each R' is independently H, alkyl, or substituted alkyl;
R is H, halogen, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide;
R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;
each of R_3 and R_4 is independently H, halogen, lower alkyl, or substituted lower alkyl, or R_3 and R_4 or two R_3 groups optionally form a cycloalkyl or a heterocycloalkyl;
each R_{19} is independently selected from the group consisting of C_1-C_6 alkyl, C_1-C_6 alkoxy, ester, ether, thioether, aminoalkyl, halogen, alkyl ester, aryl ester, amide, aryl amide, alkyl halide, alkyl amine, alkyl sulfonic acid, alkyl nitro, thioester, sulfonyl ester, halosulfonyl, nitrile, alkyl nitrile, and nitro; and
q is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11.

D. Structure and Synthesis of Non-Natural Amino Acids: Ketoamine, Ketoamine-like, Masked Ketoamine, and Protected Ketoamine Groups

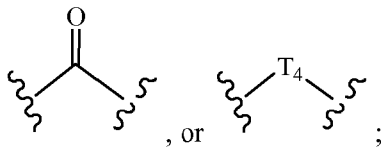
[0235] Amino acids containing reactive groups with dicarbonyl-like reactivity allow for the linking of molecules via nucleophilic addition reactions. Such reactive groups include a ketoamine group, a ketoamine-like group (which has reactivity similar to a ketoamine group and is structurally similar to a ketoamine group), a masked ketoamine group (which can be readily converted into a ketoamine group), or a protected ketoamine group (which has reactivity similar to a ketoamine group upon deprotection). Such amino acids include amino acids having the structure of Formula (XXXXXXII):



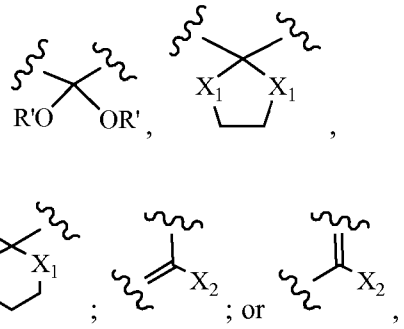
(XXXXXXII)

wherein:

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;
B is optional, and when present is a linker linked at one end to a diamine containing moiety, the linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene, -O-(alkylene or substituted alkylene)-, -S-(alkylene or substituted alkylene)-, -C(O)R"-,-$S(O)_k$(alkylene or substituted alkylene)-, where k is 1, 2, or 3, -C(O)-(alkylene or substituted alkylene)-, -C(S)-(alkylene or substituted alkylene)-, -NR"-(alkylene or substituted alkylene)-, -CON(R")-(alkylene or substituted alkylene)-, -CSN(R")-(alkylene or substituted alkylene)-, and -N(R")CO-(alkylene or substituted alkylene)-, where each R" is independently H, alkyl, or substituted alkyl;
G is

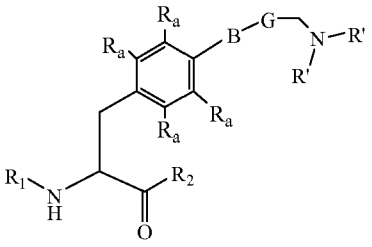


T_1 is an optionally substituted C_1-C_4 alkylene, an optionally substituted C_1-C_4 alkenylene, or an optionally substituted heteroalkyl;
T_4 is a carbonyl protecting group including, but not limited to,

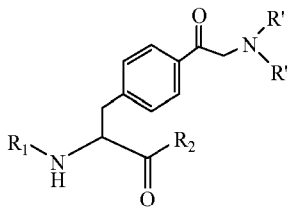


where each X_1 is independently selected from the group consisting of -O-, -S-, -N(H)-, -N(R')-, -N(Ac)-, and -N(OMe)-; X_2 is -OR, -OAc, -SR', -N(R')$_2$, -N(R')(Ac), -N(R')(OMe), or N_3, and where each R' is independently H, alkyl, or substituted alkyl;
R is H, halogen, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and
R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;
each of R_3 and R_4 is independently H, halogen, lower alkyl, or substituted lower alkyl, or R_3 and R_4 or two R_3 groups optionally form a cycloalkyl or a heterocycloalkyl.

[0236] Amino acids having the structure of Formula (XXXXXXII) include amino acids having the structure of Formula (XXXXXXIII) and Formula (XXXXXXIV):



(XXXXXXIII),



(XXXXXXIV)

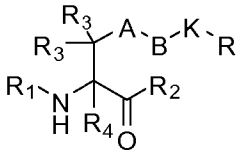
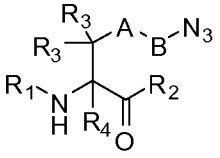
wherein each R_a is independently selected from the group consisting of H, halogen, alkyl, substituted alkyl, -N(R')$_2$, -C(O)$_k$R' where k is 1, 2, or 3, -C(O)N(R')$_2$, -OR', and -S(O)$_k$R', where each R' is independently H, alkyl, or substituted alkyl.

E. Structure and Synthesis of Non-Natural Amino Acids: Diamine, Diamine-like, Masked Diamine, Protected Amines and Azides

[0237] Amino acids with a nucleophilic reactive group allow for a variety of reactions to link molecules via electrophilic addition reactions among others. Such nucleophilic reactive groups include a diamine group (including a hydrazine group, an amidine group, an imine group, a 1,1-diamine group, a 1,2-diamine group, a 1,3-diamine group, and a 1,4-diamine group), a diamine-like group (which has reactivity similar to a diamine group and is structurally similar to a diamine group), a masked diamine group (which can be readily converted into a diamine group), or a protected diamine group (which has reactivity similar to a diamine group upon deprotection). In some embodiments, amino acids containing reactive groups with azides allow for linking of molecules via cycloaddition reactions (e.g., 1,3-dipolar cycloadditions, azide-alkyne Huisgen cycloaddition, etc.).

[0238] In another aspect are methods for the chemical synthesis of hydrazine-substituted molecules for the derivatization of carbonyl-substituted dolastatin derivatives. In one embodiment, the hydrazine-substituted molecule can dolastatin linked derivatives. In one embodiment are methods for the preparation of hydrazine-substituted molecules suitable for the derivatization of carbonyl-containing non-natural amino acid polypeptides, including by way of example only, ketone-, or aldehyde-containing non-natural amino acid polypeptides. In a further or additional embodiment, the non-natural amino acids are incorporated site-specifically during the in vivo translation of proteins. In a further or additional embodiment, the hydrazine-substituted dolastatin derivatives allow for the site-specific derivatization of carbonyl-containing non-natural amino acids via nucleophilic attack of each carbonyl group to form a heterocycle-derivatized polypeptide, including a nitrogen-containing heterocycle-derivatized polypeptide in a site-specific fashion. In a further or additional embodiment, the method for the preparation of hydrazine-substituted dolastatin derivatives provides access to a wide variety of site-specifically derivatized polypeptides. In a further or additional embodiment are methods for synthesizing hydrazine-functionalized polyethyleneglycol (PEG) linked dolastatin derivatives.

[0239] Such amino acids include amino acids having the structure of Formula (XXXVII-A) or (XXXVII-B):

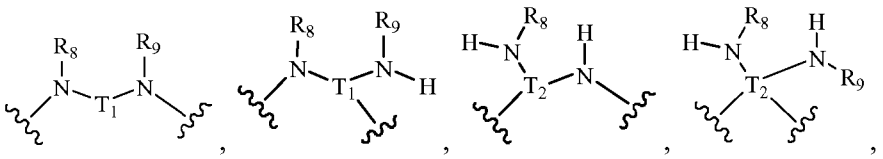

(XXXVII),

(XXXVII-B),

wherein:

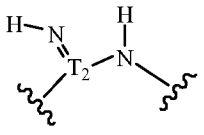
A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;

B is optional, and when present is a linker linked at one end to a diamine containing moiety, the linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene, -O-(alkylene or substituted alkylene)-, -S-(alkylene or substituted alkylene)-, -C(O)R"-, -C(O)R"-, -S(O)$_k$(alkylene or substituted alkylene)-, where k is 1, 2, or 3, -C(O)-(alkylene or substituted alkylene)-, -C(S)-(alkylene or substituted alkylene)-, -NR"-(alkylene or substituted alkylene)-, -CON(R")-(alkylene or substituted alkylene)-, -CSN(R")-(alkylene or substituted alkylene)-, and -N(R")CO-(alkylene or substituted alkylene)-, where each R" is independently H, alkyl, or substituted alkyl;

K is



or


;

where:

R_8 and R_9 are independently selected from H, alkyl, substituted alkyl, cycloalkyl, substituted cycloalkyl, or amine protecting group;

T_1 is a bond, optionally substituted C_1-C_4 alkylene, optionally substituted C_1-C_4 alkenylene, or optionally substituted heteroalkyl;

T_2 is optionally substituted C_1-C_4 alkylene, optionally substituted C_1-C_4 alkenylene, optionally substituted het-

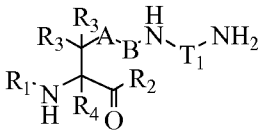
eroalkyl, optionally substituted aryl, or optionally substituted heteroaryl;
wherein each optional substituents is independently selected from lower alkyl, substituted lower alkyl, lower cycloalkyl, substituted lower cycloalkyl, lower alkenyl, substituted lower alkenyl, alkynyl, lower heteroalkyl, substituted heteroalkyl, lower heterocycloalkyl, substituted lower heterocycloalkyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, alkaryl, substituted alkaryl, aralkyl, or substituted aralkyl;

R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and
R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;
each of R_3 and R_4 is independently H, halogen, lower alkyl, or substituted lower alkyl, or R_3 and R_4 or two R_3 groups optionally form a cycloalkyl or a heterocycloalkyl;

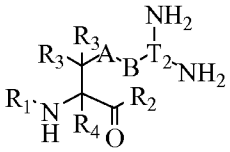
or the -A-B-K-R groups together form a bicyclic or tricyclic cycloalkyl or heterocycloalkyl comprising at least one diamine group, protected diamine group or masked diamine group;
or the -B-K-R groups together form a bicyclic or tricyclic cycloalkyl or cycloaryl or heterocycloalkyl comprising at least one diamine group, protected diamine group or masked diamine group;
or the -K-R group together forms a monocyclic or bicyclic cycloalkyl or heterocycloalkyl comprising at least one diamine group, protected diamine group or masked diamine group;

wherein at least one amine group on -A-B-K-R is optionally a protected amine.

[0240] In one aspect are compounds comprising the structures 1 or 2:



1



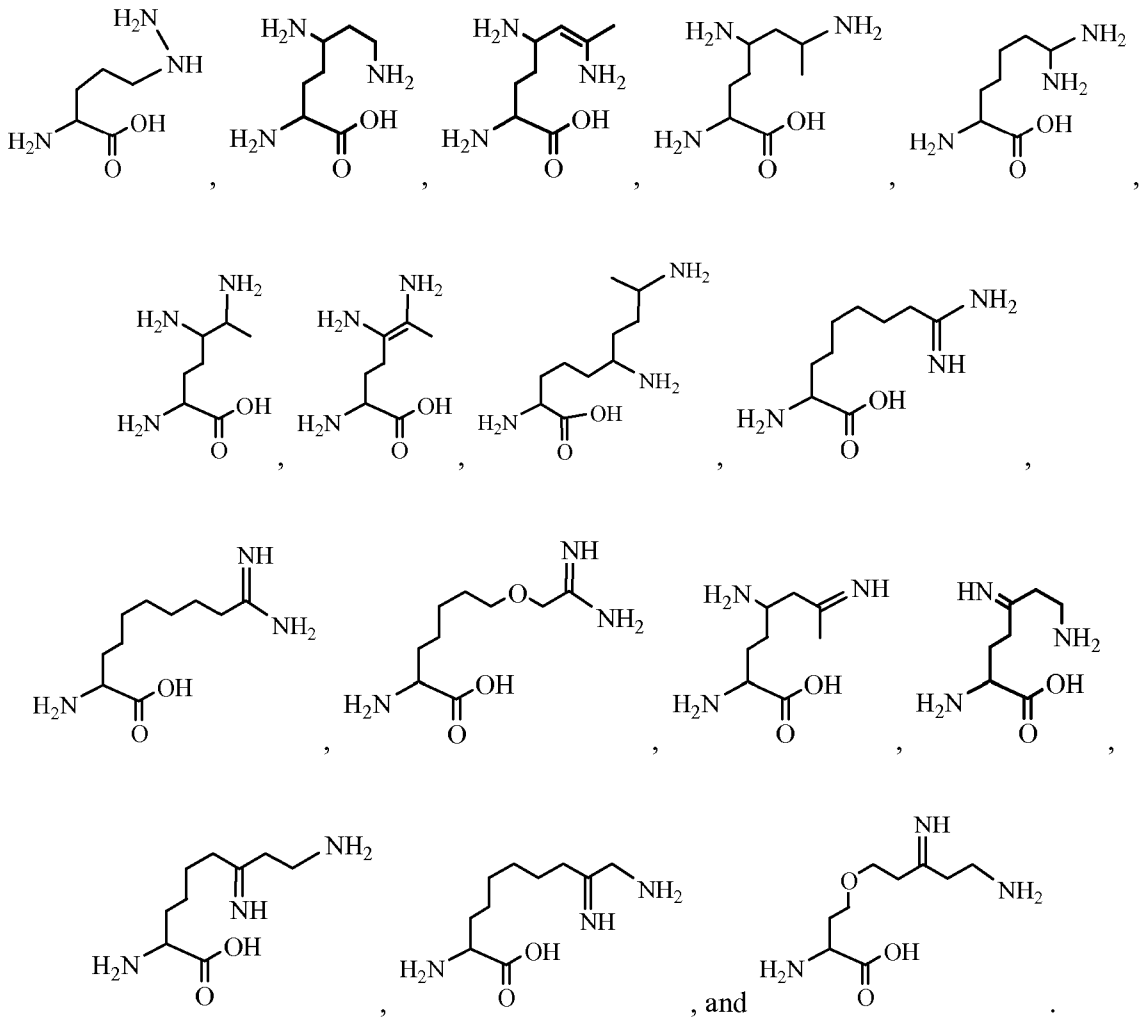
2

wherein:

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;
B is optional, and when present is a linker linked at one end to a diamine containing moiety, the linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene, -O-(alkylene or substituted alkylene)-, -S-(alkylene or substituted alkylene)-, -C(O)R"-, $-S(O)_k$(alkylene or substituted alkylene)-, where k is 1, 2, or 3, -C(O)-(alkylene or substituted alkylene)-, -C(S)-(alkylene or substituted alkylene)-, -NR"-(alkylene or substituted alkylene)-, -CON(R")-(alkylene or substituted alkylene)-, -CSN(R")-(alkylene or substituted alkylene)-, and -N(R")CO-(alkylene or substituted alkylene)-, where each R" is independently H, alkyl, or substituted alkyl;
T_1 is a bond or CH_2; and T_2 is CH;
wherein each optional substituents is independently selected from lower alkyl, substituted lower alkyl, lower cycloalkyl, substituted lower cycloalkyl, lower alkenyl, substituted lower alkenyl, alkynyl, lower heteroalkyl, substituted heteroalkyl, lower heterocycloalkyl, substituted lower heterocycloalkyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, alkaryl, substituted alkaryl, aralkyl, or substituted aralkyl;
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide; and
R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;
each of R_3 and R_4 is independently H, halogen, lower alkyl, or substituted lower alkyl, or R_3 and R_4 or two R_3 groups optionally form a cycloalkyl or a heterocycloalkyl;
or the -A-B-diamine containing moiety together form a bicyclic cycloalkyl or heterocycloalkyl comprising at least one diamine group, protected diamine group or masked diamine group;
or the -B-diamine containing moiety groups together form a bicyclic or tricyclic cycloalkyl or cycloaryl or heterocycloalkyl comprising at least one diamine group, protected diamine group or masked diamine group;
wherein at least one amine group on -A-B-diamine containing moiety is optionally a protected amine;

or an active metabolite, salt, or a pharmaceutically acceptable prodrug or solvate thereof.

[0241] The following non-limiting examples of amino acids having the structure of Formula (XXXVII) are included:

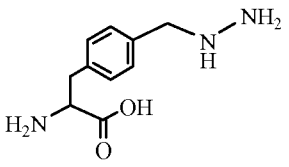
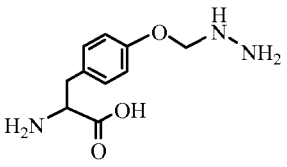
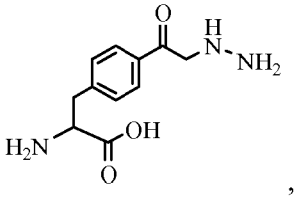
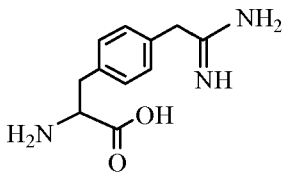
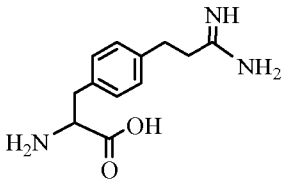
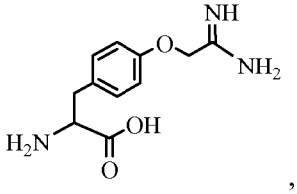
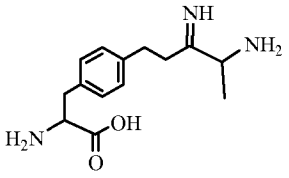
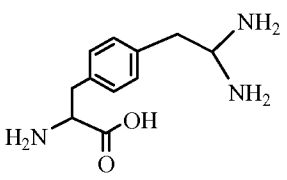
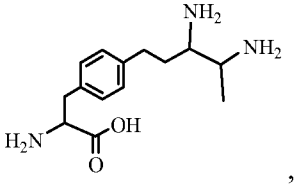
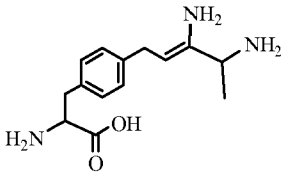
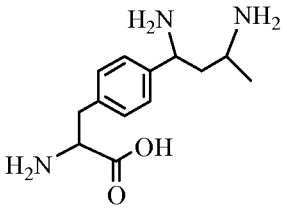
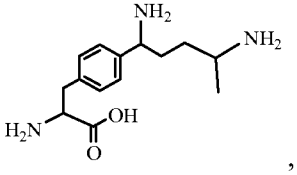
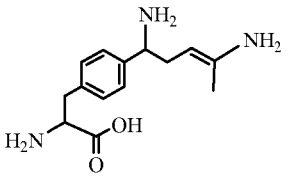
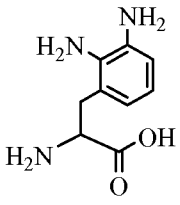


Such non-natural amino acids may also be in the form of a salt or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and/or optionally post translationally modified.

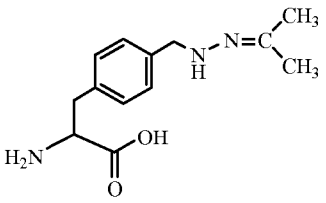
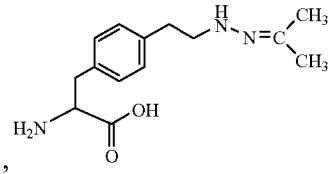
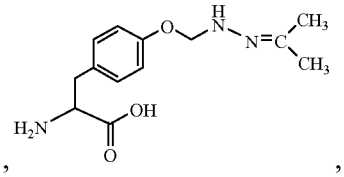
[0242] In certain embodiments, compounds of Formula (XXXVII) are stable in aqueous solution for at least 1 month under mildly acidic conditions. In certain embodiments, compounds of Formula (XXXVII) are stable for at least 2 weeks under mildly acidic conditions. In certain embodiments, compound of Formula (XXXVII) are stable for at least 5 days under mildly acidic conditions. In certain embodiments, such acidic conditions are pH about 2 to about 8.

[0243] In certain embodiments of compounds of Formula (XXXVII), B is lower alkylene, substituted lower alkylene, O-(alkylene or substituted alkylene)-, C(R')=NN(R')-, -N(R')CO-, C(O)-, -C(R')=N-, C(O)-(alkylene or substituted alkylene)-, CON(R')(alkylene or substituted alkylene)-, -S(alkylene or substituted alkylene)-, -S(O)(alkylene or substituted alkylene)-, or -$S(O)_2$(alkylene or substituted alkylene)-. In certain embodiments of compounds of Formula (XXXVII), B is -$O(CH_2)$-, -CH=N-, CH=NNH-, -$NHCH_2$-, -NHCO-, C(O)-, $C(O)(CH_2)$-, $CONH(CH_2)$-, -SCH_2-, -$S(=O)CH_2$-, or -$S(O)_2CH_2$-. In certain embodiments of compounds of Formula (XXXVII), R is C_1-$_6$ alkyl or cycloalkyl. In certain embodiments of compounds of Formula (XXXVII) R is -CH_3, -$CH(CH3)_2$, or cyclopropyl. In certain embodiments of compounds of Formula (XXXVII), R_1 is H, tert-butyloxycarbonyl (Boc), 9-Fluorenylmethoxycarbonyl (Fmoc), N-acetyl, tetrafluoroacetyl (TFA), or benzyloxycarbonyl (Cbz). In certain embodiments of compounds of Formula (XXXVII), R_1 is a resin, amino acid, polypeptide, or polynucleotide. In certain embodiments of compounds of Formula (XXXVII), R_1 is an antibody, antibody fragment or monoclonal antibody. In certain embodiments of compounds of Formula (XXXVII), R_2 is OH, O-methyl, O-ethyl, or O-t-butyl. In certain embodiments of compounds of Formula (XXXVII), R_2 is a resin, at least one amino acid, polypeptide, or polynucleotide. In certain embodiments of compounds of Formula (XXXVII), R_2 is an antibody, antibody fragment or monoclonal antibody.

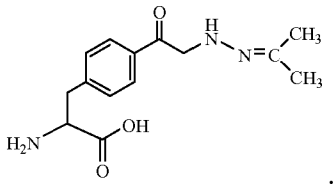
[0244] The following non-limiting examples of amino acids having the structure of Formula (XXXVII) are also included:


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[0245] Non-limiting examples of protected amino acids having the structure of Formula (XXXVII) include:

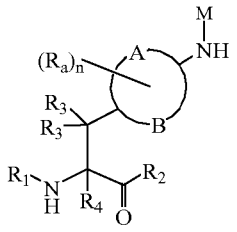

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and



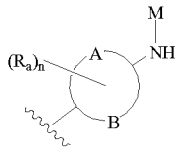
F. Structure and Synthesis of Non-Natural Amino Acids: Aromatic Amines

[0246] Non-natural amino acids with nucleophilic reactive groups, such as, by way of example only, an aromatic amine group (including secondary and tertiary amine groups), a masked aromatic amine group (which can be readily converted into a aromatic amine group), or a protected aromatic amine group (which has reactivity similar to an aromatic amine group upon deprotection) allow for a variety of reactions to link molecules via various reactions, including but not limited to, reductive alkylation reactions with aldehyde containing dolastatin linker derivatives. Such aromatic amine containing non-natural amino acids include amino acids having the structure of Formula (XXXXXXV):



(XXXXXXV)

wherein:



is selected from the group consisting of a monocyclic aryl ring, a bicyclic aryl ring, a multicyclic aryl ring, a monocyclic heteroaryl ring, a bicyclic heteroaryl ring, and a multicyclic heteroaryl ring;

A is independently CR_a, or N;

B is independently CR_a, N, O, or S;

each R_a is independently selected from the group consisting of H, halogen, alkyl, $-NO_2$, -CN, substituted alkyl, $-N(R')_2$, $-C(O)_kR'$, $-C(O)N(R')_2$, -OR', and $-S(O)_kR'$, where k is 1, 2, or 3; and n is 0, 1, 2, 3, 4, 5, or 6;

R_1 is H, an amino protecting group, resin, at least one amino acid, polypeptide, or polynucleotide; and

R_2 is OH, an ester protecting group, resin, at least one amino acid, polypeptide, or polynucleotide;

each of R_3 and R_4 is independently H, halogen, lower alkyl, or substituted lower alkyl, or R_3 and R_4 or two R_3 groups optionally form a cycloalkyl or a heterocycloalkyl;

M is H or $-CH_2R_5$; or the M-N-C(R_5) moiety may form a 4 to 7 membered ring structure;

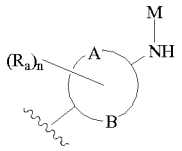
R_5 is alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, alkoxy, substituted alkoxy, alkylalkoxy, substituted alkylalkoxy, polyalkylene oxide, substituted polyalkylene oxide, cycloalkyl, substituted cycloalkyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocycle, substituted heterocycle, alkaryl, substituted alkaryl, aralkyl, substituted aralkyl, -C(O)R", -C(O)OR", $-C(O)N(R")_2$, $-C(O)NHCH(R")_2$, -(alkylene or substituted alkylene)-$N(R")_2$, -(alkenylene or substituted alkenylene)-$N(R")_2$, -(alkylene or substituted alkylene)-(aryl or substituted aryl), -(alkenylene or substituted alkenylene)-(aryl or substituted aryl), -(alkylene or substituted alkylene)-$ON(R")_2$, -(alkylene or substituted alkylene)-C(O)SR", -(alkylene or substituted alkylene)-S-S-(aryl or substituted aryl), wherein each R" is independently hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkoxy, substituted alkoxy, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocycle, substituted heterocycle, alkaryl, substituted alkaryl, aralkyl, substituted aralkyl, or -C(O)OR';

or two R_5 groups optionally form a cycloalkyl or a heterocycloalkyl;

or R_5 and any R_a optionally form a cycloalkyl or a heterocycloalkyl; and

each R' is independently H, alkyl, or substituted alkyl.

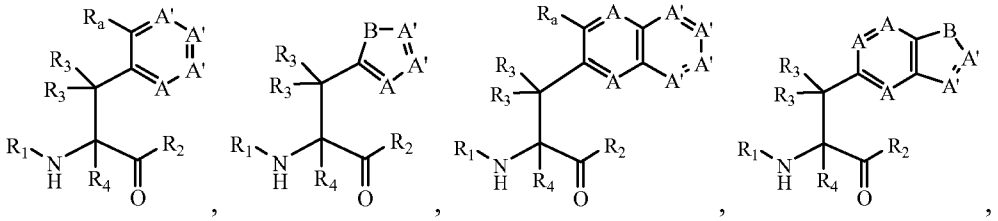
Such non-natural amino acids may also be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally reductively alkylated.
The structure



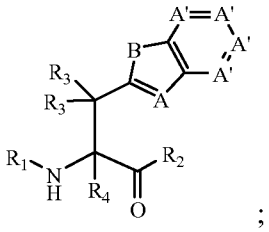
(as presented in all examples herein) does not present the relative orientations of "A," "B," "NH-M" and "R_a"; rather these four features of this structure may be oriented in any chemically-sound manner (along with other features of this structure), as illustrated by example herein.

[0247] Non-natural amino acids containing an aromatic amine moiety having the structure of Formula

(A) include non-natural amino acids having the structures:

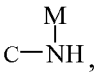


and

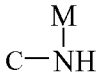


;

wherein, each A' is independently selected from CR_a, N, or



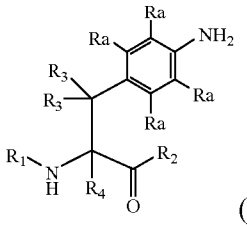
and up to two A' may be



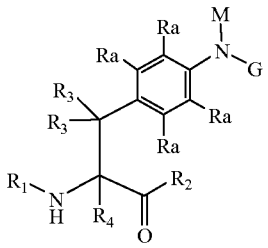
with the remaining A' selected from CR_a, or N.

Such non-natural amino acids may also be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally reductively alkylated.

[0248] Non-limiting examples of non-natural amino acids containing an aromatic amine moiety having the structure of Formula (XXXXXXV) include non-natural amino acids having the structure of Formula (XXXXXXVI), and Formula (XXXXXXVII),

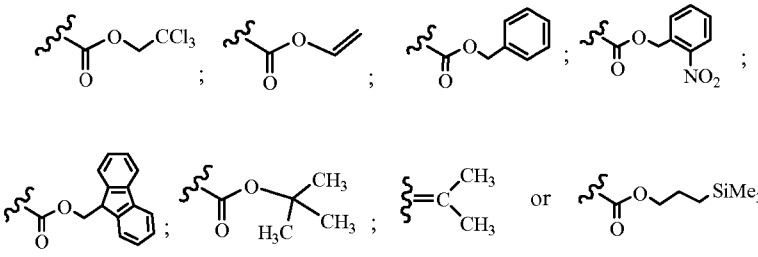


(XXXXXXVI),



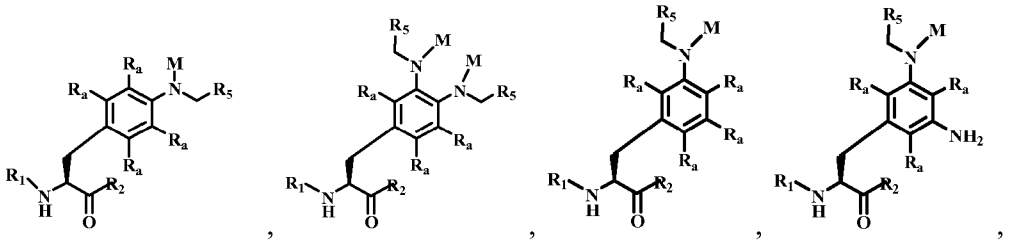
(XXXXXXVII),

wherein; G is an amine protecting group, including, but not limited to,

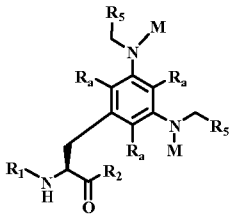


Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally reductively alkylated.

[0249] Non-natural amino acids containing an aromatic amine moiety have the following structures:



and



;

wherein each R_a is independently selected from the group consisting of H, halogen, alkyl, $-NO_2$, -CN, substituted alkyl, $-N(R')_2$, $-C(O)_kR'$, $-C(O)N(R')_2$, -OR', and $-S(O)_kR'$, where k is 1, 2, or 3;
M is H or $-CH_2R_5$; or the M-N-C(R_5) moiety may form a 4 to 7 membered ring structure;
R_1 is H, an amino protecting group, resin, amino acid, polypeptide, or polynucleotide;
R_2 is OH, an ester protecting group, resin, amino acid, polypeptide, or polynucleotide;
R_5 is alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, alkoxy, substituted alkoxy, alkylalkoxy, substituted alkylalkoxy, polyalkylene oxide, substituted polyalkylene oxide, cycloalkyl, substituted cycloalkyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocycle, substituted heterocycle, alkaryl, substituted alkaryl, aralkyl, substituted aralkyl, -C(O)R", -C(O)OR", $-C(O)N(R")_2$, $-C(O)NHCH(R")_2$, -(alkylene or substituted alkylene)-$N(R")_2$, -(alkenylene or substituted alkenylene)-$N(R")_2$, -(alkylene or substituted alkylene)-(aryl or substituted aryl), -(alkenylene or substituted alkenylene)-(aryl or substituted aryl), -(alkylene or substituted alkylene)-$ON(R")_2$, -(alkylene or substituted alkylene)-C(O)SR", -(alkylene or substituted alkylene)-S-S-(aryl or sub-

stituted aryl), wherein each R" is independently hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkoxy, substituted alkoxy, aryl, substituted aryl, heteroaryl, substituted heteroaryl, heterocycle, substituted heterocycle, alkaryl, substituted alkaryl, aralkyl, substituted aralkyl, or -C(O)OR';
or R_5 and any R_a optionally form a cycloalkyl or a heterocycloalkyl; and
each R' is independently H, alkyl, or substituted alkyl. Such non-natural amino acids may also be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide.

[0250] Such non-natural amino acids of Formula (XXXXXXV) may be formed by reduction of protected or masked amine moieties on the aromatic moiety of a non-natural amino acid. Such protected or masked amine moieties include, but are not limited to, imines, hydrazines, nitro, or azide substituents. The reducing agents used to reduce such protected or masked amine moieties include, but are not limited to, TCEP, Na_2S, $Na_2S_2O_4$, $LiAlH_4$, $NaBH_4$ or $NaBCNH_3$.

V. Non-Natural Amino Acid Linked Dolastatin Derivatives

[0251] In another aspect described herein are methods, strategies and techniques for incorporating at least one such dolastatin linker derivatives into a non-natural amino acid. Also included with this aspect are methods for producing, purifying, characterizing and using such dolastatin linker derivatives containing at least one such non-natural amino acid. Also included with this aspect are compositions of and methods for producing, purifying, characterizing and using oligonucleotides (including DNA and RNA) that can be used to produce, at least in part, a dolastatin linker derivative containing at least one non-natural amino acid. Also included with this aspect are compositions of and methods for producing, purifying, characterizing and using cells that can express such oligonucleotides that can be used to produce, at least in part, a dolastatin linker derivative containing at least one non-natural amino acid.

[0252] Thus, dolastatin linker derivatives comprising at least one non-natural amino acid or modified non-natural amino acid with a carbonyl, dicarbonyl, alkyne, cycloalkyne, azide, oxime or hydroxylamine group are provided and described herein. In certain embodiments, dolastatin linker derivatives with at least one non-natural amino acid or modified non-natural amino acid with a carbonyl, dicarbonyl, alkyne, cycloalkyne, azide, oxime or hydroxylamine group include at least one post-translational modification at some position on the polypeptide. In some embodiments the co-translational or post-translational modification occurs via the cellular machinery (e.g., glycosylation, acetylation, acylation, lipid-modification, palmitoylation, palmitate addition, phosphorylation, glycolipid-linkage modification, and the like), in many instances, such cellular-machinery-based co-translational or post-translational modifications occur at the naturally occurring amino acid sites on the polypeptide, however, in certain embodiments, the cellular-machinery-based co-translational or post-translational modifications occur on the non-natural amino acid site(s) on the polypeptide.

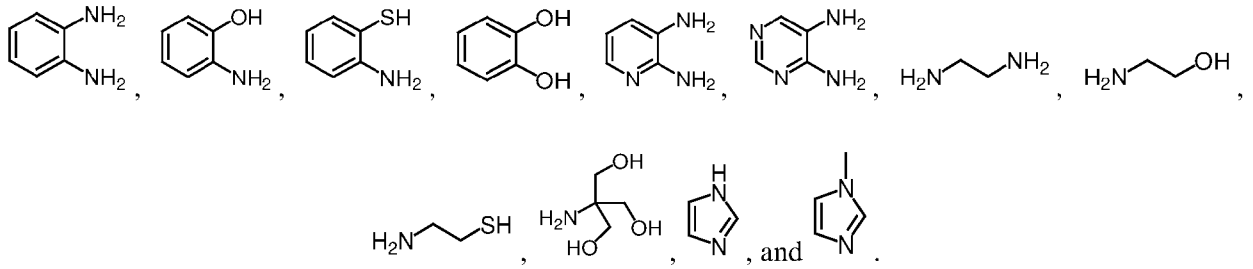
[0253] In other embodiments, the post-translational modification does not utilize the cellular machinery, but the functionality is instead provided by attachment of a molecule (a polymer; a water-soluble polymer; a derivative of polyethylene glycol; a second protein or polypeptide or polypeptide analog; an antibody or antibody fragment; and any combination thereof) comprising a second reactive group to the at least one non-natural amino acid comprising a first reactive group (including but not limited to, non-natural amino acid containing a ketone, aldehyde, acetal, hemiacetal, alkyne, cycloalkyne, azide, oxime, or hydroxylamine functional group) utilizing chemistry methodology described herein, or others suitable for the particular reactive groups. In certain embodiments, the co-translational or post-translational modification is made *in vivo* in a eukaryotic cell or in a non-eukaryotic cell. In certain embodiments, the post-translational modification is made *in vitro* not utilizing the cellular machinery. Also included with this aspect are methods for producing, purifying, characterizing and using such dolastatin linker derivatives containing at least one such co-translationally or post-translationally modified non-natural amino acids.

[0254] Also included within the scope of the methods, compositions, strategies and techniques described herein are reagents capable of reacting with a dolastatin linker derivative (containing a carbonyl or dicarbonyl group, oxime group, alkyne, cycloalkyne, azide, hydroxylamine group, or masked or protected forms thereof) that is part of a polypeptide so as to produce any of the aforementioned post-translational modifications. In certain embodiments, the resulting post-translationally modified dolastatin linker derivative will contain at least one oxime group; the resulting modified oxime-containing dolastatin linker derivative may undergo subsequent modification reactions. Also included with this aspect are methods for producing, purifying, characterizing and using such reagents that are capable of any such post-translational modifications of such dolastatin linker derivative(s).

[0255] In certain embodiments, the polypeptide or non-natural amino acid linked dolastatin derivative includes at least one co-translational or post-translational modification that is made *in vivo* by one host cell, where the post-translational modification is not normally made by another host cell type. In certain embodiments, the polypeptide includes at least one co-translational or post-translational modification that is made *in vivo* by a eukaryotic cell, where the co-translational or post-translational modification is not normally made by a non-eukaryotic cell. Examples of such co-translational or post-translational modifications include, but are not limited to, glycosylation, acetylation, acylation, lipid-modification, palmitoylation, palmitate addition, phosphorylation, glycolipid-linkage modification, and the like. In one embodiment, the

co-translational or post-translational modification comprises attachment of an oligosaccharide to an asparagine by a GlcNAc-asparagine linkage (including but not limited to, where the oligosaccharide comprises $(GlcNAc\text{-}Man)_2$-Man-GlcNAc-GlcNAc, and the like). In another embodiment, the co-translational or post-translational modification comprises attachment of an oligosaccharide (including but not limited to, Gal-GalNAc, Gal-GlcNAc, etc.) to a serine or threonine by a GalNAc-serine, a GalNAc-threonine, a GlcNAc-serine, or a GlcNAc-threonine linkage. In certain embodiments, a protein or polypeptide can comprise a secretion or localization sequence, an epitope tag, a FLAG tag, a polyhistidine tag, a GST fusion, and/or the like. Also included with this aspect are methods for producing, purifying, characterizing and using such polypeptides containing at least one such co-translational or post-translational modification. In other embodiments, the glycosylated non-natural amino acid polypeptide is produced in a non-glycosylated form. Such a non-glycosylated form of a glycosylated non-natural amino acid may be produced by methods that include chemical or enzymatic removal of oligosaccharide groups from an isolated or substantially purified or unpurified glycosylated non-natural amino acid polypeptide; production of the non-natural amino acid in a host that does not glycosylate such a non-natural amino acid polypeptide (such a host including, prokaryotes or eukaryotes engineered or mutated to not glycosylate such a polypeptide), the introduction of a glycosylation inhibitor into the cell culture medium in which such a non-natural amino acid polypeptide is being produced by a eukaryote that normally would glycosylate such a polypeptide, or a combination of any such methods. Also described herein are such non-glycosylated forms of normally-glycosylated non-natural amino acid polypeptides (by normally-glycosylated is meant a polypeptide that would be glycosylated when produced under conditions in which naturally-occurring polypeptides are glycosylated). Of course, such non-glycosylated forms of normally-glycosylated non-natural amino acid polypeptides (or indeed any polypeptide described herein) may be in an unpurified form, a substantially purified form, or in an isolated form.

[0256] In certain embodiments, the non-natural amino acid polypeptide includes at least one post-translational modification that is made in the presence of an accelerant, wherein the post-translational modification is stoichiometric, stoichiometric-like, or near-stoichiometric. In other embodiments the polypeptide is contacted with a reagent of Formula (XIX) in the presence of an accelerant. In other embodiments the accelerant is selected from the group consisting of:

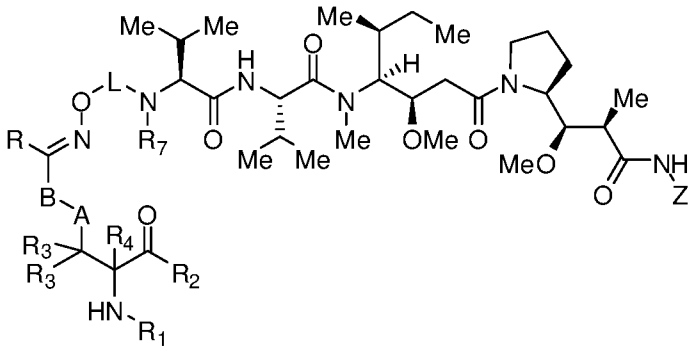


A. Chemical Synthesis of Non-Natural Amino Acid Linked Dolastatin Derivatives: Oxime-Containing Linked Dolastatin Derivatives

[0257] Non-natural amino acid dolastatin linked derivatives containing an oxime group allow for reaction with a variety of reagents that contain certain reactive carbonyl- or dicarbonyl- groups (including but not limited to, ketones, aldehydes, or other groups with similar reactivity) to form new non-natural amino acids comprising a new oxime group. Such an oxime exchange reaction allows for the further functionalization of dolastatin linked derivatives. Further, the original dolastatin linked derivative containing an oxime group may be useful in their own right as long as the oxime linkage is stable under conditions necessary to incorporate the amino acid into a polypeptide (e.g., the *in vivo, in vitro* and chemical synthetic methods described herein).

[0258] Thus, in certain embodiments described herein are non-natural amino acid dolastatin linked derivatives with sidechains comprising an oxime group, an oxime-like group (which has reactivity similar to an oxime group and is structurally similar to an oxime group), a masked oxime group (which can be readily converted into an oxime group), or a protected oxime group (which has reactivity similar to an oxime group upon deprotection).

[0259] Non-natural amino acid dolastatin linked derivatives of the invention include dolastatin linked derivatives having the structure of Formula (VIII):



(VIII)

;

wherein:

A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;

B is optional, and when present is a linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene, -O-, -O-(alkylene or substituted alkylene)-,-S-, -S-(alkylene or substituted alkylene)-, $-S(O)_k-$ where k is 1, 2, or 3, $-S(O)_k$(alkylene or substituted alkylene)-, -C(O)-, -C(O)-(alkylene or substituted alkylene)-, -C(S)-, -C(S)-(alkylene or substituted alkylene)-, -N(R')-, -NR'-(alkylene or substituted alkylene)-, -C(O)N(R')-, -CON(R')-(alkylene or substituted alkylene)-, -CSN(R')-, -CSN(R')-(alkylene or substituted alkylene)-, -N(R')CO-(alkylene or substituted alkylene)-, $-N(R')C(O)O-$, $-S(O)_kN(R')-$, $-N(R')C(O)N(R')-$, $-N(R')C(S)N(R')-$, $-N(R')S(O)_kN(R')-$, -N(R')-N=,-C(R')=N-, -C(R')=N-N(R')-, -C(R')=N-N=, $-C(R')_2-N=N-$, and $-C(R')_2-N(R')-N(R')-$, where each R' is independently H, alkyl, or substituted alkyl;

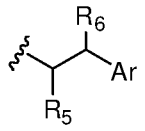
R is H, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;

R_1 is H, an amino protecting group, resin, at least one amino acid, polypeptide, or polynucleotide;

R_2 is OH, an ester protecting group, resin, at least one amino acid, polypeptide, or polynucleotide;

R_3 and R_4 are each independently H;

Z has the structure of:



;

R_5 is H, COR_8, C_1-C_6 alkyl, or thiazole;

R_8 is OH

R_6 is OH or H;

Ar is phenyl or pyridine;

R_7 is C_1-C_6 alkyl or hydrogen;

L is a linker selected from the group consisting of -alkylene-, $-(alkylene-O)_n-alkylene-$; and

n is an integer greater than or equal to one;

wherein alkyl means 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 and also includes heteroalkyl and haloalkyl;

wherein alkylene includes heteroalkylene;

wherein arylene means a divalent aryl radical, wherein aryl includes heteroaryl;

or solvate thereof.

[0260] In certain embodiments of compounds of Formula (VIII), R_5 is thiazole. In certain embodiments of compounds of Formula (VIII), R_6 is H. In certain embodiments of compounds of Formula (VIII), Ar is phenyl. In certain embodiments of compounds of Formula (VIII), R_7 is methyl. In certain embodiments of compounds of Formula (VIII), n is an integer

from 0 to 20. In certain embodiments of compounds of Formula (VIII), n is an integer from 0 to 10. In certain embodiments of compounds of Formula (VIII), n is an integer from 0 to 5.

[0261] In certain embodiments of compounds of Formula (VIII), R_5 is thiazole. In certain embodiments of compounds of Formula (VIII), R_5 is hydrogen. In certain embodiments of compounds of Formula (VIII), R_5 is methyl, ethyl, propyl, iso-propyl, butyl, iso-butyl, sec-butyl, tert-butyl, pentyl, or hexyl.

[0262] In certain embodiments of compounds of Formula (VIII), R_6 is H.

[0263] In certain embodiments of compounds of Formula (VIII), Ar is phenyl.

[0264] In certain embodiments of compounds of Formula (VIII), R_7 is methyl, ethyl, propyl, iso-propyl, butyl, sec-butyl iso-butyl, tert-butyl, pentyl, or hexyl. In certain embodiments of compounds of Formula (VIII) and (IX), R_7 is hydrogen.

[0265] In certain embodiments of compounds of Formula (VIII), each alkylene independently is $-CH_2-$,$-CH_2CH_2-$, $-CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2-$,$-CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$, $CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$,$-CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$, or $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$. In certain embodiments of compounds of Formula (VIII), alkylene is methylene, ethylene, propylene, butylenes, pentylene, hexylene, or heptylene.

[0266] In certain embodiments of compounds of Formula (VIII), each n independently is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100.

[0267] In certain embodiments of compounds of Formula (VIII), R_1 is a polypeptide. In certain embodiments of compounds of Formula (VIII), R_2 is a polypeptide. In certain embodiments of compounds of Formula (VIII), the polypeptide is an antibody. In certain embodiments of compounds of Formula (VIII), the antibody is trastuzumab.

[0268] In certain embodiments, compounds of Formula (VIII) are stable in aqueous solution for at least 1 month under mildly acidic conditions. In certain embodiments, compounds of Formula (VIII) are stable for at least 2 weeks under mildly acidic conditions. In certain embodiments, compound of Formula (VIII) are stable for at least 5 days under mildly acidic conditions. In certain embodiments, such acidic conditions are pH 2 to 8. Such non-natural amino acids may be in the form of a salt, or may be incorporated into a non-natural amino acid polypeptide, polymer, polysaccharide, or a polynucleotide and optionally post translationally modified.

[0269] Oxime-based non-natural amino acids may be synthesized by methods already described in the art, or by methods described herein, including: (a) reaction of a hydroxylamine-containing non-natural amino acid with a carbonyl- or dicarbonyl-containing reagent; (b) reaction of a carbonyl- or dicarbonyl-containing non-natural amino acid with a hydroxylamine-containing reagent; or (c) reaction of an oxime-containing non-natural amino acid with certain carbonyl- or dicarbonyl-containing reagents.

C. Chemical Synthesis of Non-Natural Amino Acid Linked Dolastatin Derivatives: Heteroaryl-Containing Linked Dolastatin Derivatives

[0270] In one aspect are non-natural amino acids for the chemical derivatization of dolastatin linked derivatives based upon the reactivity of a dicarbonyl group, including a group containing at least one ketone group, and/or at least one aldehyde groups, and/or at least one ester group, and/or at least one carboxylic acid, and/or at least one thioester group, and wherein the dicarbonyl group can be a 1,2-dicarbonyl group, a 1,3-dicarbonyl group, or a 1,4-dicarbonyl group. In further or additional aspects are non-natural amino acids for the chemical derivatization of dolastatin linked derivatives based upon the reactivity of a diamine group, including a hydrazine group, an amidine group, an imine group, a 1,1-diamine group, a 1,2-diamine group, a 1,3-diamine group, and a 1,4-diamine group. In further or additional embodiments, at least one of the aforementioned non-natural amino acids is incorporated into a dolastatin linked derivative, that is, such embodiments are non-natural amino acid linked dolastatin derivatives. In further or additional embodiments, the non-natural amino acids are functionalized on their sidechains such that their reaction with a derivatizing molecule generates a linkage, including a heterocyclic-based linkage, including a nitrogen-containing heterocycle, and/or an aldol-based linkage. In further or additional embodiments are non-natural amino acid polypeptides that can react with a derivatizing dolastatin linker to generate a non-natural amino acid linked dolastatin derivatives containing a linkage, including a heterocyclic-based linkage, including a nitrogen-containing heterocycle, and/or an aldol-based linkage. In further or additional embodiments, the non-natural amino acids are selected from amino acids having dicarbonyl and/or diamine sidechains. In further or additional embodiments, the non-natural amino acids comprise a masked sidechain, including a masked diamine group and/or a masked dicarbonyl group. In further or additional embodiments, the non-natural amino acids comprise a group selected from: keto-amine (i.e., a group containing both a ketone and an amine); keto-alkyne (i.e., a group containing both a ketone and an alkyne); and an ene-dione (i.e., a group containing a dicarbonyl group and an alkene).

[0271] In further or additional embodiments, the non-natural amino acids comprise dicarbonyl sidechains where the carbonyl is selected from a ketone, an aldehyde, a carboxylic acid, or an ester, including a thioester. In another embodiment are non-natural amino acids containing a functional group that is capable of forming a heterocycle, including a nitrogen-containing heterocycle, upon treatment with an appropriately functionalized reagent. In a further or additional embodiment, the non-natural amino acids resemble a natural amino acid in structure but contain one of the aforementioned functional groups. In another or further embodiment the non-natural amino acids resemble phenylalanine or tyrosine (aromatic amino acids); while in a separate embodiment, the non-natural amino acids resemble alanine and leucine (hydrophobic amino acids). In one embodiment, the non-natural amino acids have properties that are distinct from those of the natural amino acids. In one embodiment, such distinct properties are the chemical reactivity of the sidechain, in a further embodiment this distinct chemical reactivity permits the sidechain of the non-natural amino acid to undergo a reaction while being a unit of a polypeptide even though the sidechains of the naturally-occurring amino acid units in the same polypeptide do not undergo the aforementioned reaction. In a further embodiment, the sidechain of the non-natural amino acid has a chemistry orthogonal to those of the naturally-occurring amino acids. In a further embodiment, the sidechain of the non-natural amino acid comprises an electrophile-containing moiety; in a further embodiment, the electrophile-containing moiety on the sidechain of the non-natural amino acid can undergo nucleophilic attack to generate a heterocycle-derivatized protein, including a nitrogen-containing heterocycle-derivatized protein. In any of the aforementioned embodiments in this paragraph, the non-natural amino acid may exist as a separate molecule or may be incorporated into a polypeptide of any length; if the latter, then the polypeptide may further incorporate naturally-occurring or non-natural amino acids.

[0272] In another aspect are diamine-substituted molecules, wherein the diamine group is selected from a hydrazine, an amidine, an imine, a 1,1-diamine, a 1,2-diamine, a 1,3-diamine and a 1,4-diamine group, for the production of derivatized non-natural amino acid linked dolastatin derivatives based upon a heterocycle, including a nitrogen-containing heterocycle, linkage. In a further embodiment are diamine-substituted dolastatin derivatives used to derivatize dicarbonyl-containing non-natural amino acid polypeptides via the formation of a heterocycle, including a nitrogen-containing heterocycle, linkage between the derivatizing molecule and the dicarbonyl-containing non-natural amino acid polypeptide. In further embodiments the aforementioned dicarbonyl-containing non-natural amino acid polypeptides are diketone-containing non-natural amino acid polypeptides. In further or additional embodiments, the dicarbonyl-containing non-natural amino acids comprise sidechains where the carbonyl is selected from a ketone, an aldehyde, a carboxylic acid, or an ester, including a thioester. In further or additional embodiments, the diamine-substituted molecules comprise a group selected from a desired functionality. In a further embodiment, the sidechain of the non-natural amino acid has a chemistry orthogonal to those of the naturally-occurring amino acids that allows the non-natural amino acid to react selectively with the diamine-substituted molecules. In a further embodiment, the sidechain of the non-natural amino acid comprises an electrophile-containing moiety that reacts selectively with the diamine-containing molecule; in a further embodiment, the electrophile-containing moiety on the sidechain of the non-natural amino acid can undergo nucleophilic attack to generate a heterocycle-derivatized protein, including a nitrogen-containing heterocycle-derivatized protein. In a further aspect related to the embodiments described in this paragraph are the modified non-natural amino acid polypeptides that result from the reaction of the derivatizing molecule with the non-natural amino acid polypeptides. Further embodiments include any further modifications of the already modified non-natural amino acid polypeptides.

[0273] In another aspect are dicarbonyl-substituted molecules for the production of derivatized non-natural amino acid polypeptides based upon a heterocycle, including a nitrogen-containing heterocycle, linkage. In a further embodiment are dicarbonyl-substituted molecules used to derivatize diamine-containing non-natural amino acid polypeptides via the formation of a heterocycle, including a nitrogen-containing heterocycle group. In a further embodiment are dicarbonyl-substituted molecules that can form such heterocycle, including a nitrogen-containing heterocycle groups with a diamine-containing non-natural amino acid polypeptide in a pH range between about 4 and about 8. In a further embodiment are dicarbonyl-substituted molecules used to derivatize diamine-containing non-natural amino acid polypeptides via the formation of a heterocycle, including a nitrogen-containing heterocycle, linkage between the derivatizing molecule and the diamine-containing non-natural amino acid polypeptides. In a further embodiment the dicarbonyl-substituted molecules are diketone-substitued molecules, in other aspects ketoaldehyde-substituted molecules, in other aspects ketoacid-substituted molecules, in other aspects ketoester-substituted molecules, including ketothioester-substituted molecules. In further embodiments, the dicarbonyl-substituted molecules comprise a group selected from a desired functionality. In further or additional embodiments, the aldehyde-substituted molecules are aldehyde-substituted polyethylene glycol (PEG) molecules. In a further embodiment, the sidechain of the non-natural amino acid has a chemistry orthogonal to those of the naturally-occurring amino acids that allows the non-natural amino acid to react selectively with the carbonyl-substituted molecules. In a further embodiment, the sidechain of the non-natural amino acid comprises a moiety (e.g., diamine group) that reacts selectively with the dicarbonyl-containing molecule; in a further embodiment, the nucleophilic moiety on the sidechain of the non-natural amino acid can undergo electrophilic attack to generate a heterocyclic-derivatized protein, including a nitrogen-containing heterocycle-derivatized protein. In a further aspect related to the embodiments described in this paragraph are the modified non-natural amino acid polypeptides that result from the

reaction of the derivatizing molecule with the non-natural amino acid polypeptides. Further embodiments include any further modifications of the already modified non-natural amino acid polypeptides.

[0274] In one aspect are methods to derivatize proteins via the reaction of carbonyl and hydrazine reactants to generate a heterocycle-derivatized protein, including a nitrogen-containing heterocycle-derivatized dolastatin. Included within this aspect are methods for the derivatization of dolastatin linker derivatives based upon the condensation of carbonyl- and hydrazine-containing reactants to generate a heterocycle-derivatized dolastatin, including a nitrogen-containing heterocycle-derivatized dolastatin. In additional or further embodiments are methods to derivatize ketone-containing dolastatin derivatives or aldehyde-containing dolastatin derivatives with hydrazine-functionalized non-natural amino acids. In yet additional or further aspects, the hydrazine-substituted molecule can include proteins, other polymers, and small molecules.

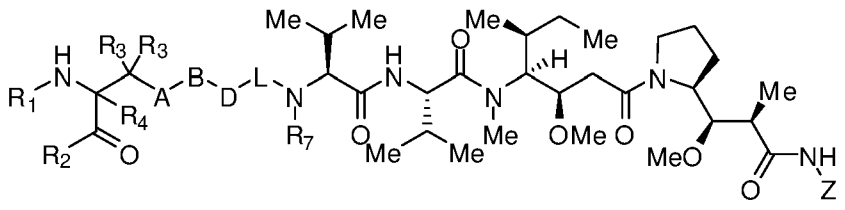
[0275] In another aspect are methods for the chemical synthesis of hydrazine-substituted molecules for the derivatization of carbonyl-substituted dolastatin derivatives. In one embodiment, the hydrazine-substituted molecule is a dolastatin linked derivative suitable for the derivatization of carbonyl-containing non-natural amino acid polypeptides, including by way of example only, ketone-, or aldehyde-containing non-natural amino acid polypeptides.

[0276] In one aspect are non-natural amino acids for the chemical derivatization of dolastatin analogs based upon a quinoxaline or phenazine linkage. In further or additional embodiments, the non-natural amino acids are functionalized on their sidechains such that their reaction with a derivatizing dolastatin linker generates a quinoxaline or phenazine linkage. In further or additional embodiments, the non-natural amino acids are selected from amino acids having 1,2-dicarbonyl or 1,2-aryldiamine sidechains. In further or additional embodiments, the non-natural amino acids are selected from amino acids having protected or masked 1,2-dicarbonyl or 1,2-aryldiamine sidechains. Further included are equivalents to 1,2-dicarbonyl sidechains, or protected or masked equivalents to 1,2-dicarbonyl sidechains.

[0277] In another aspect are derivatizing molecules for the production of derivatized non-natural amino acid polypeptides based upon quinoxaline or phenazine linkages. In one embodiment are 1,2-dicarbonyl substituted dolastatin linker derivatives used to derivatize 1,2-aryldiamine containing non-natural amino acid polypeptides to form quinoxaline or phenazine linkages. In another embodiment are 1,2-aryldiamine substituted dolastatin linker derivatives used to derivatize 1,2-dicarbonyl containing non-natural amino acid polypeptides to form quinoxaline or phenazine linkages. In a further aspect related to the above embodiments are the modified non-natural amino acid polypeptides that result from the reaction of the derivatizing dolastatin linker with the non-natural amino acid polypeptides. In one embodiment are 1,2-aryldiamine containing non-natural amino acid polypeptides derivatized with 1,2-dicarbonyl substituted dolastatin linker derivative to form quinoxaline or phenazine linkages. In another embodiment are 1,2-dicarbonyl containing non-natural amino acid polypeptides derivatized with 1,2-aryldiamine substituted dolastatin linker derivatives to form quinoxaline or phenazine linkages.

[0278] Provided herein in certain embodiments are derivatizing molecules for the production of toxic compounds comprising non-natural amino acid polypeptides based upon triazole linkages. In some embodiments, the reaction between the first and second reactive groups can proceed via a dipolarophile reaction. In certain embodiments, the first reactive group can be an azide and the second reactive group can be an alkyne. In further or alternative embodiments, the first reactive group can be an alkyne and the second reactive group can be an azide. In some embodiments, the Huisgen cycloaddition reaction (see, e.g., Huisgen, in 1,3-DIPOLAR CYCLOADDITION CHEMISTRY, (ed. Padwa, A., 1984), p. 1-176) provides for the incorporation of non-naturally encoded amino acids bearing azide and alkyne-containing side chains permits the resultant polypeptides to be modified with extremely high selectivity. In certain embodiments, both the azide and the alkyne functional groups are inert toward the twenty common amino acids found in naturally-occurring polypeptides. When brought into close proximity, however, the "spring-loaded" nature of the azide and alkyne groups is revealed and they react selectively and efficiently via Huisgen [3 2] cycloaddition reaction to generate the corresponding triazole. See, e.g., Chin et al., Science 301:964-7 (2003); Wang et al., J. Am. Chem. Soc., 125, 3192-3193 (2003); Chin et al., J. Am. Chem. Soc., 124:9026-9027 (2002). Cycloaddition reaction involving azide or alkyne-containing polypeptides can be carried out at room temperature under aqueous conditions by the addition of Cu(II) (e.g., in the form of a catalytic amount of $CuS0_4$) in the presence of a reducing agent for reducing Cu(II) to Cu(I), in situ, in catalytic amount. See, e.g., Wang et al., J. Am. Chem. Soc. 125, 3192-3193 (2003); Tomoe et al., J. Org. Chem. 67:3057-3064 (2002); Rostovtsev, Angew. Chem. Int. Ed. 41:2596-2599 (2002). Preferred reducing agents include ascorbate, metallic copper, quinine, hydroquinone, vitamin K, glutathione, cysteine, Fe_2, Co_2, and an applied electric potential.

[0279] Non-natural amino acid heteroaryl-linked dolastatin derivatives of the invention include dolastatin linked derivatives having the structure of Formula (XXXI):

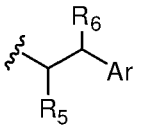


(XXXI)

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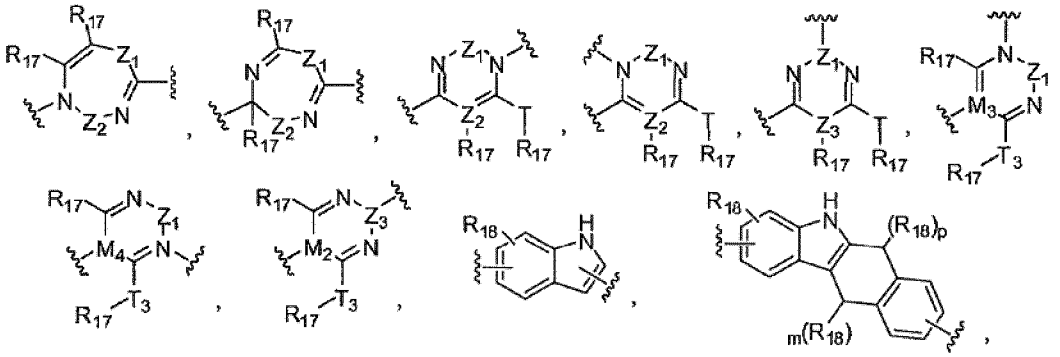
wherein:

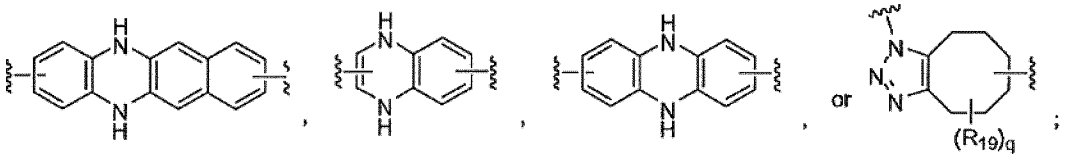
Z has the structure of:



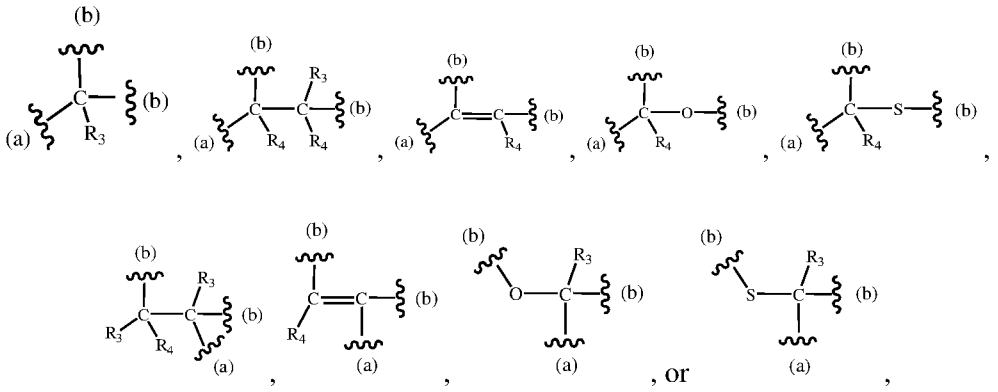
;

R_5 is H, CO_2H, C_1-C_6 alkyl, or thiazole;
R_6 is OH or H;
Ar is phenyl or pyridine;
A is optional, and when present is lower alkylene, substituted lower alkylene, lower cycloalkylene, substituted lower cycloalkylene, lower alkenylene, substituted lower alkenylene, alkynylene, lower heteroalkylene, substituted heteroalkylene, lower heterocycloalkylene, substituted lower heterocycloalkylene, arylene, substituted arylene, heteroarylene, substituted heteroarylene, alkarylene, substituted alkarylene, aralkylene, or substituted aralkylene;
B is optional, and when present is a linker selected from the group consisting of lower alkylene, substituted lower alkylene, lower alkenylene, substituted lower alkenylene, lower heteroalkylene, substituted lower heteroalkylene, -O-(alkylene or substituted alkylene)-, -S-(alkylene or substituted alkylene)-, -C(O)R"-, $-S(O)_k$(alkylene or substituted alkylene)-, where k is 1, 2, or 3, -C(O)-(alkylene or substituted alkylene)-, -C(S)-(alkylene or substituted alkylene)-, -NR"-(alkylene or substituted alkylene)-, -CON(R")-(alkylene or substituted alkylene)-, -CSN(R")-(alkylene or substituted alkylene)-, and -N(R")CO-(alkylene or substituted alkylene)-, where each R" is independently H, alkyl, or substituted alkyl ;
R_1 is H, an amino protecting group, resin, at least one amino acid, polypeptide, or polynucleotide;
R_2 is OH, an ester protecting group, resin, at least one amino acid, polypeptide, or polynucleotide;
R_3 and R_4 are each independently H, halogen, lower alkyl, or substituted lower alkyl;
R_7 is C_1-C_6 alkyl or hydrogen;
L is-alkylene- or -(alkylene-O)n-alkylene-; and
n is an integer greater than or equal to one;
D has the structure of:

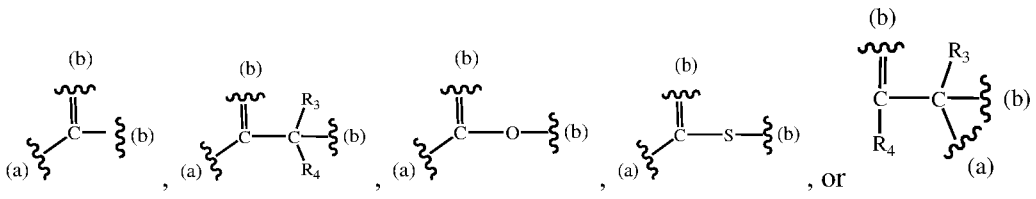




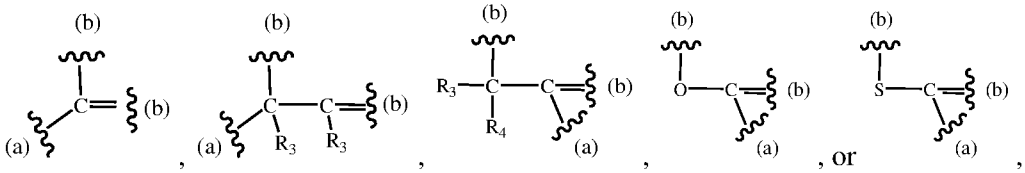
each R_{17} is independently selected from the group consisting of H, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, alkoxy, substituted alkoxy, alkylalkoxy, substituted alkylalkoxy, polyalkylene oxide, substituted polyalkylene oxide, aryl, substituted aryl, heteroaryl, substituted heteroaryl, alkaryl, substituted alkaryl, aralkyl, substituted aralkyl, -(alkylene or substituted alkylene)-$ON(R'')_2$, -(alkylene or substituted alkylene)-C(O)SR", -(alkylene or substituted alkylene)-S-S-(aryl or substituted aryl), -C(O)R", $-C(O)_2R''$, or $-C(O)N(R'')_2$, wherein each R" is independently hydrogen, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkoxy, substituted alkoxy, aryl, substituted aryl, heteroaryl, alkaryl, substituted alkaryl, aralkyl, or substituted aralkyl;
each Z_1 is a bond, $CR_{17}R_{17}$, O, S, NR', $CR_{17}R_{17}$-$CR_{17}R_{17}$, $CR_{17}R_{17}$-O, O-$CR_{17}R_{17}$, $CR_{17}R_{17}$-S, S-$CR_{17}R_{17}$, $CR_{17}R_{17}$-NR', or NR'-$CR_{17}R_{17}$; each R' is H, alkyl, or substituted alkyl;
each Z_2 is selected from the group consisting of a bond, -C(O)-, -C(S)-, optionally substituted C_1-C_3 alkylene, optionally substituted C_1-C_3 alkenylene, and optionally substituted heteroalkyl;
each Z_3 are independently selected from the group consisting of a bond, optionally substituted C_1-C_4 alkylene, optionally substituted C_1-C_4 alkenylene, optionally substituted heteroalkyl, -O-, -S-, -C(O)-, -C(S)-, and -N(R')-;
each T_3 is a bond, C(R"')(R"'), O, or S; with the proviso that when T_3 is O or S, R"' cannot be halogen;
each R"' is H, halogen, alkyl, substituted alkyl, cycloalkyl, or substituted cycloalkyl;
m and p are 0, 1, 2, or 3, provided that at least one of m or p is not 0;
M_2 is



where (a) indicates bonding to the B group and (b) indicates bonding to respective positions within the heterocycle group;
M_3 is



where (a) indicates bonding to the B group and (b) indicates bonding to respective positions within the heterocycle group;
M_4 is



where (a) indicates bonding to the B group and (b) indicates bonding to respective positions within the heterocycle group;

each R_{19} is independently selected from the group consisting of C_1-C_6 alkyl, C_1-C_6 alkoxy, ester, ether, thioether, aminoalkyl, halogen, alkyl ester, aryl ester, amide, aryl amide, alkyl halide, alkyl amine, alkyl sulfonic acid, alkyl nitro, thioester, sulfonyl ester, halosulfonyl, nitrile, alkyl nitrile, and nitro;

q is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11; and

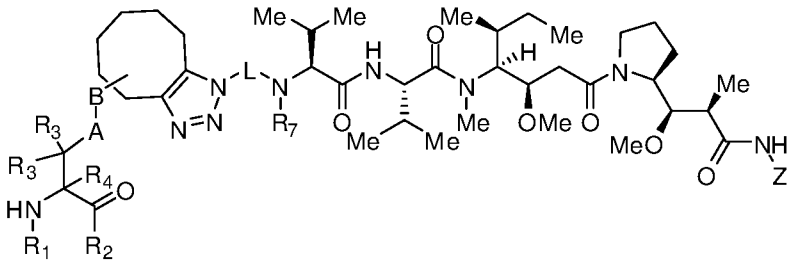
each R_{16} is independently selected from the group consisting of hydrogen, halogen, alkyl, NO_2, CN, and substituted alkyl;

wherein alkyl means 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 and also includes heteroalkyl and haloalkyl;

wherein alkylene includes heteroalkylene;

wherein arylene means a divalent aryl radical, wherein aryl includes heteroaryl.

[0280] In some embodiments, the compound of Formula (XXXI) include compounds having the structure of Formula (XXXI-A):



(XXXI-A)

[0281] In certain embodiments of compounds of Formula (XXXI), R_5 is thiazole or carboxylic acid. In certain embodiments of compounds of Formula (XXXI), R_6 is H. In certain embodiments of compounds of Formula (XXXI), Ar is phenyl. In certain embodiments of compounds of Formula (XXXI), R_7 is methyl. In certain embodiments of compounds of Formula (XXXI), n is an integer from 0 to 20. In certain embodiments of compounds of Formula (XXXI), n is an integer from 0 to 10. In certain embodiments of compounds of Formula (XXXI), n is an integer from 0 to 5.

[0282] In certain embodiments of compounds of Formula (XXXI), R_5 is thiazole or carboxylic acid. In certain embodiments of compounds of Formula (XXXI), R_5 is hydrogen. In certain embodiments of compounds of Formula (XXXI), R_5 is methyl, ethyl, propyl, iso-propyl, butyl, iso-butyl, sec-butyl, tert-butyl, pentyl, or hexyl.

[0283] In certain embodiments of compounds of Formula (XXXI), R_6 is H. In some embodiments of compounds of Formula (XXXI), R_6 is hydroxy.

[0284] In certain embodiments of compounds of Formula (XXXI), Ar is phenyl.

[0285] In certain embodiments of compounds of Formula (XXXI), R_7 is methyl, ethyl, propyl, iso-propyl, butyl, sec-butyl iso-butyl, tert-butyl, pentyl, or hexyl. In certain embodiments of compounds of Formula (XXXI), R_7 is hydrogen.

[0286] In certain embodiments of compounds of Formula (XXXI), each alkylene independently is $-CH_2-$, $--CH_2CH_2-$, $--CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$, or $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2-$. In certain embodiments of compounds of Formula (XXXI), alkylene is methylene, ethylene, propylene, butylenes, pentylene, hexylene, or heptylene.

[0287] In certain embodiments of compounds of Formula (XXXI), n is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100.

[0288] In certain embodiments of compounds of Formula (XXXI), R1 is a polypeptide. In certain embodiments of compounds of Formula (XXXI), R2 is a polypeptide. In certain embodiments of compounds of Formula (XXXI), the polypeptide is an antibody. In certain embodiments of compounds of Formula (XXXI), the antibody is trastuzumab.

[0289] The formation of such non-natural amino acid heterocycle-linked dolastatin derivatives having the structure of Formula (XXXI) includes, but is not limited to, (i) reactions of diamine-containing non-natural amino acids with dicarbonyl-containing dolastatin linked derivatives or reactions of diamine-containing non-natural amino acids with ketoalkyne-containing dolastatin linked derivatives, (ii) reactions of dicarbonyl-containing non-natural amino acids with either diamine-containing dolastatin linked derivatives or reactions of dicarbonyl-containing non-natural amino acids with ketoamine-containing dolastatin linked derivatives, (iii) reactions of ketoalkyne-containing non-natural amino acids with diamine-containing dolastatin linked derivatives, or (iv) reactions of ketoamine-containing non-natural amino acids with dicarbonyl-containing v.

[0290] Modification of dolastatin linked derivatives described herein with such reactions have any or all of the following advantages. First, diamines undergo condensation with dicarbonyl-containing compounds in a pH range of about 5 to about 8 (and in further embodiments in a pH range of about 4 to about 10, in other embodiments in a pH range of about 3 to about 8, in other embodiments in a pH range of about 4 to about 9, and in further embodiments a pH range of about 4 to about 9, in other embodiments a pH of about 4, and in yet another embodiment a pH of about 8) to generate heterocycle, including a nitrogen-containing heterocycle, linkages. Under these conditions, the sidechains of the naturally occurring amino acids are unreactive. Second, such selective chemistry makes possible the site-specific derivatization of recombinant proteins: derivatized proteins can now be prepared as defined homogeneous products. Third, the mild conditions needed to effect the reaction of the diamines described herein with the dicarbonyl-containing polypeptides described herein generally do not irreversibly destroy the tertiary structure of the polypeptide (excepting, of course, where the purpose of the reaction is to destroy such tertiary structure). Fourth, the reaction occurs rapidly at room temperature, which allows the use of many types of polypeptides or reagents that would be unstable at higher temperatures. Fifth, the reaction occurs readily is aqueous conditions, again allowing use of polypeptides and reagents incompatible (to any extent) with non-aqueous solutions. Six, the reaction occurs readily even when the ratio of polypeptide or amino acid to reagent is stoichiometric, near stoichiometric, or stoichiometric-like, so that it is unnecessary to add excess reagent or polypeptide to obtain a useful amount of reaction product. Seventh, the resulting heterocycle can be produced regioselectively and/or regiospecifically, depending upon the design of the diamine and dicarbonyl portions of the reactants. Finally, the condensation of diamines with dicarbonyl-containing molecules generates heterocycle, including a nitrogen-containing heterocycle, linkages which are stable under biological conditions.

VI. Location of non-natural amino acids in dolastatin linker derivatives

[0291] The methods and compositions described herein include incorporation of one or more non-natural amino acids into a dolastatin linker derivative. One or more non-natural amino acids may be incorporated at one or more particular positions which do not disrupt activity of the dolastatin linker derivative. This can be achieved by making "conservative" substitutions, including but not limited to, substituting hydrophobic amino acids with non-natural or natural hydrophobic amino acids, bulky amino acids with non-natural or natural bulky amino acids, hydrophilic amino acids with non-natural or natural hydrophilic amino acids) and/or inserting the non-natural amino acid in a location that is not required for activity.

[0292] A variety of biochemical and structural approaches can be employed to select the desired sites for substitution with a non-natural amino acid within the dolastatin linker derivative. In some embodiments, the non-natural amino acid is linked at the C-terminus of the dolastatin derivative. In other embodiments, the non-natural amino acid is linked at the N-terminus of the dolastatin derivative. Any position of the dolastatin linker derivative is suitable for selection to incorporate a non-natural amino acid, and selection may be based on rational design or by random selection for any or no particular desired purpose. Selection of desired sites may be based on producing a non-natural amino acid polypeptide (which may be further modified or remain unmodified) having any desired property or activity, including but not limited to a receptor binding modulators, receptor activity modulators, modulators of binding to binder partners, binding partner activity modulators, binding partner conformation modulators, dimer or multimer formation, no change to activity or property compared to the native molecule, or manipulating any physical or chemical property of the polypeptide such as solubility, aggregation, or stability. Alternatively, the sites identified as critical to biological activity may also be good candidates for substitution with a non-natural amino acid, again depending on the desired activity sought for the polypeptide. Another alternative would be to simply make serial substitutions in each position on the polypeptide chain with a non-natural amino acid and observe the effect on the activities of the polypeptide. Any means, technique, or method for selecting a position for substitution with a non-natural amino acid into any polypeptide is suitable for use in the methods, techniques and compositions described herein.

[0293] The structure and activity of naturally-occurring mutants of a polypeptide that contain deletions can also be examined to determine regions of the protein that are likely to be tolerant of substitution with a non-natural amino acid. Once residues that are likely to be intolerant to substitution with non-natural amino acids have been eliminated, the

impact of proposed substitutions at each of the remaining positions can be examined using methods including, but not limited to, the three-dimensional structure of the relevant polypeptide, and any associated ligands or binding proteins. X-ray crystallographic and NMR structures of many polypeptides are available in the Protein Data Bank (PDB, www.rcsb.org), a centralized database containing three-dimensional structural data of large molecules of proteins and nucleic acids, one can be used to identify amino acid positions that can be substituted with non-natural amino acids.. In addition, models may be made investigating the secondary and tertiary structure of polypeptides, if three-dimensional structural data is not available. Thus, the identity of amino acid positions that can be substituted with non-natural amino acids can be readily obtained.

[0294] Exemplary sites of incorporation of a non-natural amino acid include, but are not limited to, those that are excluded from potential receptor binding regions, or regions for binding to binding proteins or ligands may be fully or partially solvent exposed, have minimal or no hydrogen-bonding interactions with nearby residues, may be minimally exposed to nearby reactive residues, and/or may be in regions that are highly flexible as predicted by the three-dimensional crystal structure of a particular polypeptide with its associated receptor, ligand or binding proteins.

[0295] A wide variety of non-natural amino acids can be substituted for, or incorporated into, a given position in a polypeptide. By way of example, a particular non-natural amino acid may be selected for incorporation based on an examination of the three dimensional crystal structure of a polypeptide with its associated ligand, receptor and/or binding proteins, a preference for conservative substitutions

[0296] In one embodiment, the methods described herein include incorporating into the dolastatin linker derivative, where the dolastatin linker derivative comprises a first reactive group; and contacting the dolastatin linker derivative with a molecule (including but not limited to a second protein or polypeptide or polypeptide analog; an antibody or antibody fragment; and any combination thereof) that comprises a second reactive group. In certain embodiments, the first reactive group is a hydroxylamine moiety and the second reactive group is a carbonyl or dicarbonyl moiety, whereby an oxime linkage is formed. In certain embodiments, the first reactive group is a carbonyl or dicarbonyl moiety and the second reactive group is a hydroxylamine moiety, whereby an oxime linkage is formed. In certain embodiments, the first reactive group is a carbonyl or dicarbonyl moiety and the second reactive group is an oxime moiety, whereby an oxime exchange reaction occurs. In certain embodiments, the first reactive group is an oxime moiety and the second reactive group is carbonyl or dicarbonyl moiety, whereby an oxime exchange reaction occurs.

[0297] In some cases, the dolastatin linker derivative incorporation(s) will be combined with other additions, substitutions, or deletions within the polypeptide to affect other chemical, physical, pharmacologic and/or biological traits. In some cases, the other additions, substitutions or deletions may increase the stability (including but not limited to, resistance to proteolytic degradation) of the polypeptide or increase affinity of the polypeptide for its appropriate receptor, ligand and/or binding proteins. In some cases, the other additions, substitutions or deletions may increase the solubility (including but not limited to, when expressed in E. coli or other host cells) of the polypeptide. In some embodiments sites are selected for substitution with a naturally encoded or non-natural amino acid in addition to another site for incorporation of a non-natural amino acid for the purpose of increasing the polypeptide solubility following expression in E. coli, or other recombinant host cells. In some embodiments, the polypeptides comprise another addition, substitution, or deletion that modulates affinity for the associated ligand, binding proteins, and/or receptor, modulates (including but not limited to, increases or decreases) receptor dimerization, stabilizes receptor dimers, modulates circulating half-life, modulates release or bioavailability, facilitates purification, or improves or alters a particular route of administration. Similarly, the non-natural amino acid polypeptide can comprise chemical or enzyme cleavage sequences, protease cleavage sequences, reactive groups, antibody-binding domains (including but not limited to, FLAG or poly-His) or other affinity based sequences (including but not limited to, FLAG, poly-His, GST, etc.) or linked molecules (including but not limited to, biotin) that improve detection (including but not limited to, GFP), purification, transport thru tissues or cell membranes, prodrug release or activation, size reduction, or other traits of the polypeptide.

VII. HER2 Gene as Exemplar

[0298] The methods, compositions, strategies and techniques described herein are not limited to a particular type, class or family of polypeptides or proteins. Indeed, virtually any polypeptides may be designed or modified to include at least one "modified or unmodified" non-natural amino acids containing dolastatin linker derivative described herein. By way of example only, the polypeptide can be homologous to a therapeutic protein selected from the group consisting of: alpha-1 antitrypsin, angiostatin, antihemolytic factor, antibody, antibody fragment, monoclonal antibody (e.g., bevacizumab, cetuximab, panitumumab, infliximab, adalimumab, basiliximab, daclizumab, omalizumab, ustekinumab, etanercept, gemtuzumab, alemtuzumab, rituximab, trastuzumab, nimotuzumab, palivizumab, and abciximab), apolipoprotein, apoprotein, atrial natriuretic factor, atrial natriuretic polypeptide, atrial peptide, C-X-C chemokine, T39765, NAP-2, ENA-78, gro-a, gro-b, gro-c, IP-10, GCP-2, NAP-4, SDF-1, PF4, MIG, calcitonin, c-kit ligand, cytokine, CC chemokine, monocyte chemoattractant protein-1, monocyte chemoattractant protein-2, monocyte chemoattractant protein-3, monocyte inflammatory protein-1 alpha, monocyte inflammatory protein-i beta, RANTES, 1309, R83915, R91733, HCC1, T58847,

D31065, T64262, CD40, CD40 ligand, c-kit ligand, collagen, colony stimulating factor (CSF), complement factor 5a, complement inhibitor, complement receptor 1, cytokine, epithelial neutrophil activating peptide-78, MIP-16, MCP-1, epidermal growth factor (EGF), epithelial neutrophil activating peptide, erythropoietin (EPO), exfoliating toxin, Factor IX, Factor VII, Factor VIII, Factor X, fibroblast growth factor (FGF), fibrinogen, fibronectin, four-helical bundle protein, G-CSF, glp-1, GM-CSF, glucocerebrosidase, gonadotropin, growth factor, growth factor receptor, grf, hedgehog protein, hemoglobin, hepatocyte growth factor (hGF), hirudin, human growth hormone (hGH), human serum albumin, ICAM-1, ICAM-1 receptor, LFA-1, LFA-1 receptor, insulin, insulin-like growth factor (IGF), IGF-I, IGF-II, interferon (IFN), IFN-alpha, IFN-beta, IFN-gamma, interleukin (IL), IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, keratinocyte growth factor (KGF), lactoferrin, leukemia inhibitory factor, luciferase, neurturin, neutrophil inhibitory factor (NIF), oncostatin M, osteogenic protein, oncogene product, paracitonin, parathyroid hormone, PD-ECSF, PDGF, peptide hormone, pleiotropin, protein A, protein G, pth, pyrogenic exotoxin A, pyrogenic exotoxin B, pyrogenic exotoxin C, pyy, relaxin, renin, SCF, small biosynthetic protein, soluble complement receptor I, soluble I-CAM 1, soluble interleukin receptor, soluble TNF receptor, somatomedin, somatostatin, somatotropin, streptokinase, superantigens, staphylococcal enterotoxin, SEA, SEB, SEC1, SEC2, SEC3, SED, SEE, steroid hormone receptor, superoxide dismutase, toxic shock syndrome toxin, thymosin alpha 1, tissue plasminogen activator, tumor growth factor (TGF), tumor necrosis factor, tumor necrosis factor alpha, tumor necrosis factor beta, tumor necrosis factor receptor (TNFR), VLA-4 protein, VCAM-1 protein, vascular endothelial growth factor (VEGF), urokinase, mos, ras, raf, met, p53, tat, fos, myc, jun, myb, rel, estrogen receptor, progesterone receptor, testosterone receptor, aldosterone receptor, LDL receptor, and corticosterone.

[0299] Also described is a method for treating solid tumor which overexpresses HER-2 selected from the group consisting of breast cancer, small cell lung carcinoma, ovarian cancer, prostate cancer, gastric carcinoma, cervical cancer, esophageal carcinoma, and colon cancer. The solid tumor may be breast cancer. The solid tumor may be ovarian cancer.

[0300] Thus, the following description of trastuzumab is provided for illustrative purposes and by way of example only, and not as a limit on the scope of the methods, compositions, strategies and techniques described herein. Further, reference to trastuzumab in this application is intended to use the generic term as an example of any antibody. Thus, it is understood that the modifications and chemistries described herein with reference to trastuzumab can be equally applied to any antibody or monoclonal antibody, including those specifically listed herein.

[0301] Trastuzumab is a humanized monoclonal antibody that binds to the domain IV of the extracellular segment of the HER2/neu receptor. The HER2 gene (also known as HER2/neu and ErbB2 gene) is amplified in 20-30% of early-stage breast cancers, which makes it overexpressed. Also, in cancer, HER2 may send signals without mitogens arriving and binding to any receptor, making it overactive.

[0302] HER2 extends through the cell membrane, and carries signals from outside the cell to the inside. In healthy people, signaling compounds called mitogens arrive at the cell membrane, and bind to the outside part of other members of the HER family of receptors. Those bound receptors then link (dimerize) with HER2, activating it. HER2 then sends a signal to the inside of the cell. The signal passes through different biochemical pathways. This includes the PI3K/Akt pathway and the MAPK pathway. These signals promote invasion, survival and growth of blood vessels (angiogenesis) of cells.

[0303] Cells treated with trastuzumab undergo arrest during the G1 phase of the cell cycle so there is reduced proliferation. It has been suggested that trastuzumab induces some of its effect by downregulation of HER2/neu leading to disruption of receptor dimerization and signaling through the downstream PI3K cascade. P27Kip1 is then not phosphorylated and is able to enter the nucleus and inhibit cdk2 activity, causing cell cycle arrest. Also, trastuzumab suppresses angiogenesis by both induction of antiangiogenic factors and repression of proangiogenic factors. It is thought that a contribution to the unregulated growth observed in cancer could be due to proteolytic cleavage of HER2/neu that results in the release of the extracellular domain. Trastuzumab has been shown to inhibit HER2/neu ectodomain cleavage in breast cancer cells.

VIII. Cellular uptake of non-natural amino acids

[0304] Non-natural amino acid uptake by a eukaryotic cell is one issue that is typically considered when designing and selecting non-natural amino acids, including but not limited to, for incorporation into a protein. For example, the high charge density of α-amino acids suggests that these compounds are unlikely to be cell permeable. Natural amino acids are taken up into the eukaryotic cell via a collection of protein-based transport systems. A rapid screen can be done which assesses which non-natural amino acids, if any, are taken up by cells (examples 15 & 16 herein illustrate non-limiting examples of tests which can be done on non-natural amino acids). *See, e.g.,* the toxicity assays in, e.g., the U.S. Patent Publication No. 2004/198637 entitled "Protein Arrays,", and Liu, D.R. & Schultz, P.G. (1999) Progress toward the evolution of an organism with an expanded genetic code. PNAS United States 96:4780-4785. Although uptake is easily analyzed with various assays, an alternative to designing non-natural amino acids that are amenable to cellular uptake pathways is to provide biosynthetic pathways to create amino acids *in vivo*.

[0305] Typically, the non-natural amino acid produced via cellular uptake as described herein is produced in a con-

centration sufficient for efficient protein biosynthesis, including but not limited to, a natural cellular amount, but not to such a degree as to affect the concentration of the other amino acids or exhaust cellular resources. Typical concentrations produced in this manner are about 10 mM to about 0.05 mM

IX. Biosynthesis of Non-Natural Amino Acids

[0306] Many biosynthetic pathways already exist in cells for the production of amino acids and other compounds. While a biosynthetic method for a particular non-natural amino acid may not exist in nature, including but not limited to, in a cell, the methods and compositions described herein provide such methods. For example, biosynthetic pathways for non-natural amino acids can be generated in host cell by adding new enzymes or modifying existing host cell pathways. Additional new enzymes include naturally occurring enzymes or artificially evolved enzymes. For example, the biosynthesis of *p*-aminophenylalanine (as presented in an example in WO 2002/085923 entitled "In vivo incorporation of unnatural amino acids") relies on the addition of a combination of known enzymes from other organisms. The genes for these enzymes can be introduced into a eukaryotic cell by transforming the cell with a plasmid comprising the genes. The genes, when expressed in the cell, provide an enzymatic pathway to synthesize the desired compound. Examples of the types of enzymes that are optionally added are provided herein. Additional enzymes sequences are found, for example, in Genbank. Artificially evolved enzymes can be added into a cell in the same manner. In this manner, the cellular machinery and resources of a cell are manipulated to produce non-natural amino acids.

[0307] A variety of methods are available for producing novel enzymes for use in biosynthetic pathways or for evolution of existing pathways. For example, recursive recombination, including but not limited to, as developed by Maxygen, Inc. (available on the world wide web at www.maxygen.com), can be used to develop novel enzymes and pathways. *See, e.g.,* Stemmer (1994), Rapid evolution of a protein in vitro by DNA shuffling, Nature _370(4):389-391; and, Stemmer, (1994), DNA shuffling by random fragmentation and reassembly: In vitro recombination for molecular evolution, Proc. Natl. Acad. Sci. USA., 91:10747-10751. Similarly DesignPath™, developed by Genencor (available on the world wide web at genencor.com) is optionally used for metabolic pathway engineering, including but not limited to, to engineer a pathway to create a non-natural amino acid in a cell. This technology reconstructs existing pathways in host organisms using a combination of new genes, including but not limited to those identified through functional genomics, molecular evolution and design. Diversa Corporation (available on the world wide web at diversa.com) also provides technology for rapidly screening libraries of genes and gene pathways, including but not limited to, to create new pathways for biosynthetically producing non-natural amino acids.

[0308] Typically, the non-natural amino acid produced with an engineered biosynthetic pathway as described herein is produced in a concentration sufficient for efficient protein biosynthesis, including but not limited to, a natural cellular amount, but not to such a degree as to affect the concentration of the other amino acids or exhaust cellular resources. Typical concentrations produced *in vivo* in this manner are about 10 mM to about 0.05 mM. Once a cell is transformed with a plasmid comprising the genes used to produce enzymes desired for a specific pathway and a non-natural amino acid is generated, *in vivo* selections are optionally used to further optimize the production of the non-natural amino acid for both ribosomal protein synthesis and cell growth.

X. Additional Synthetic Methodology

[0309] The non-natural amino acids described herein may be synthesized using methodologies described in the art or using the techniques described herein or by a combination thereof. As an aid, the following table provides various starting electrophiles and nucleophiles which may be combined to create a desired functional group. The information provided is meant to be illustrative and not limiting to the synthetic techniques described herein.

Table 1: Examples of Covalent Linkages and Precursors Thereof

Covalent Linkage Product	Electrophile	Nucleophile
Carboxamides	Activated esters	amines/anilines
Carboxamides	acyl azides	amines/anilines
Carboxamides	acyl halides	amines/anilines
Esters	acyl halides	alcohols/phenols
Esters	acyl nitriles	alcohols/phenols
Carboxamides	acyl nitriles	amines/anilines
Imines	Aldehydes	amines/anilines

(continued)

Covalent Linkage Product	Electrophile	Nucleophile
Hydrazones	aldehydes or ketones	Hydrazines
Oximes	aldehydes or ketones	Hydroxylamines
Alkyl amines	alkyl halides	amines/anilines
Esters	alkyl halides	carboxylic acids
Thioethers	alkyl halides	Thiols
Ethers	alkyl halides	alcohols/phenols
Thioethers	alkyl sulfonates	Thiols
Esters	alkyl sulfonates	carboxylic acids
Ethers	alkyl sulfonates	alcohols/phenols
Esters	Anhydrides	alcohols/phenols
Carboxamides	Anhydrides	amines/ anilines
Thiophenols	aryl halides	Thiols
Aryl amines	aryl halides	Amines
Thioethers	Azindines	Thiols
Boronate esters	Boronates	Glycols
Carboxamides	carboxylic acids	amines/ anilines
Esters	carboxylic acids	Alcohols
hydrazines	Hydrazides	carboxylic acids
N-acylureas or Anhydrides	carbodiimides	carboxylic acids
Esters	diazoalkanes	carboxylic acids
Thioethers	Epoxides	Thiols
Thioethers	haloacetamides	Thiols
Ammotriazines	halotriazines	amines/ anilines
Triazinyl ethers	halotriazines	alcohols/phenols
Amidines	imido esters	amines/ anilines
Ureas	Isocyanates	amines/ anilines
Urethanes	Isocyanates	alcohols/phenols
Thioureas	isothiocyanates	amines/ anilines
Thioethers	Maleimides	Thiols
Phosphite esters	phosphoramidites	Alcohols
Silyl ethers	silyl halides	Alcohols
Alkyl amines	sulfonate esters	amines/anilines
Thioethers	sulfonate esters	Thiols
Esters	sulfonate esters	carboxylic acids
Ethers	sulfonate esters	Alcohols
Sulfonamides	sulfonyl halides	amines/anilines
Sulfonate esters	sulfonyl halides	phenols/alcohols

[0310] In general, carbon electrophiles are susceptible to attack by complementary nucleophiles, including carbon nucleophiles, wherein an attacking nucleophile brings an electron pair to the carbon electrophile in order to form a new bond between the nucleophile and the carbon electrophile.

[0311] Non-limiting examples of carbon nucleophiles include, but are not limited to alkyl, alkenyl, aryl and alkynyl Grignard, organolithium, organozinc, alkyl-, alkenyl , aryl- and alkynyl-tin reagents (organostannanes), alkyl-, alkenyl-, aryl- and alkynyl-borane reagents (organoboranes and organoboronates); these carbon nucleophiles have the advantage of being kinetically stable in water or polar organic solvents. Other non-limiting examples of carbon nucleophiles include phosphorus ylids, enol and enolate reagents; these carbon nucleophiles have the advantage of being relatively easy to generate from precursors well known to those skilled in the art of synthetic organic chemistry. Carbon nucleophiles, when used in conjunction with carbon electrophiles, engender new carbon-carbon bonds between the carbon nucleophile and carbon electrophile.

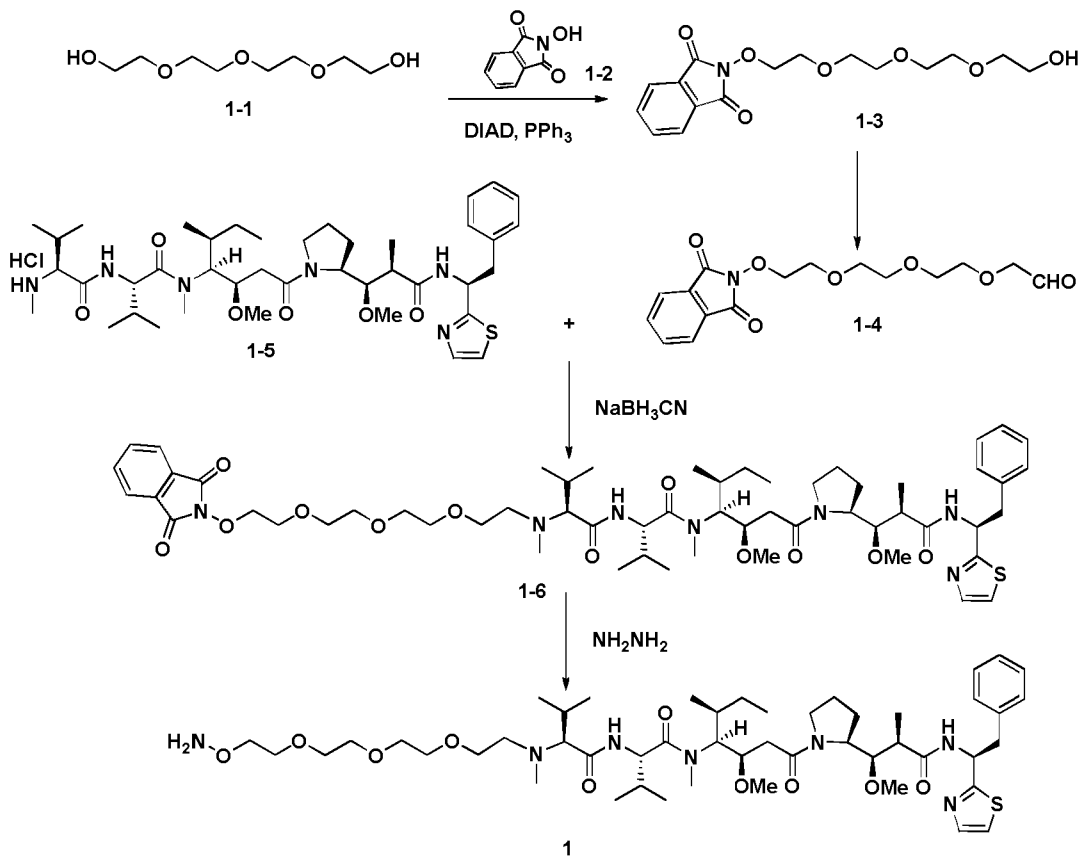
[0312] Non-limiting examples of non-carbon nucleophiles suitable for coupling to carbon electrophiles include but are not limited to primary and secondary amines, thiols, thiolates, and thioethers, alcohols, alkoxides, azides, semicarbazides, and the like. These non-carbon nucleophiles, when used in conjunction with carbon electrophiles, typically generate heteroatom linkages (C-X-C), wherein X is a hetereoatom, including, but not limited to, oxygen, sulfur, or nitrogen.

EXAMPLES

Example 1: Synthesis of Compound 1

[0313]

Scheme 1



[0314] **Compound 1-3:** Tetra (ethylene glycol) **1-1** (10g, 51.5mmol), N-hydroxyphthalimide **1-2** (8.4g, 51.15mmol) and triphenylphosphine (17.6g, 67mmol) were dissolved in 300 mL of tetrahydrofuran followed by addition of DIAD (12.8 mL, 61.78 mmol) at 0°C. The resulting solution was stirred at room temperature overnight, and then concentrated to dryness. The residue was purified by flash column chromatography to give 5.47g (31%) of compound **1-3.**

[0315] **Compound 1-4:** To a solution of compound **1-3** (200mg, 0.59mmol) in 15mL dichloromethane was added Dess-Martin Periodinane (300mg, 0.71 mmol). The reaction mixture was stirred at ambient temperature overnight. The

reaction was quenched with the solution of sodium bisulfite in 15 mL of saturated sodium bicarbonate. The mixture was separated. The organic layer was washed with saturated sodium bicarbonate, brine, dried over sodium sulfate, filtered and concentrated in vacuo. The residue was purified by flash column chromatography to give 150mg(75%) of compound **1-4.**

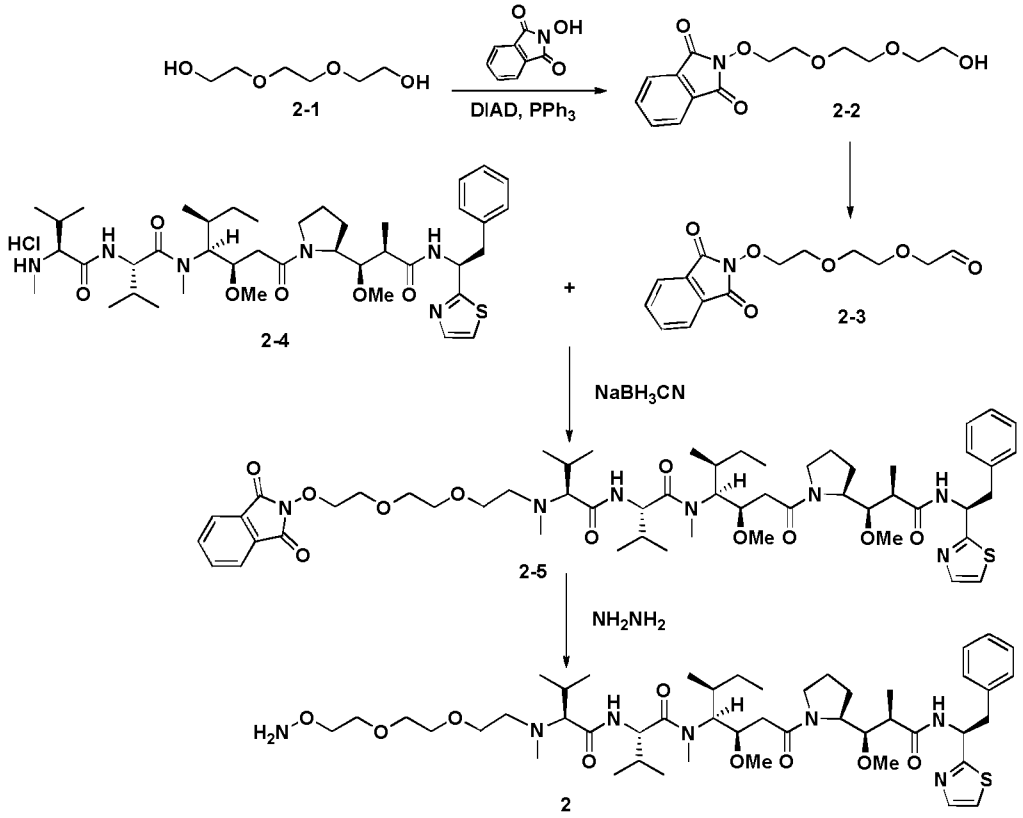
[0316] **Compound 1-6:** To a solution of monomethyldolastatin hydrochloride salt **1-5** (50mg, 0.062 mmol) in 1mL of DMF was added compound **1-4** (63mg, 0.186mmol) and 70μL of acetic acid, followed by addition of 8 mg of sodium cyanoborohydride. The resulting mixture was stirred at ambient temperature for 2 hours. The reaction mixture was diluted with water and purified by HPLC to give 60mg (80%) of compound **1-6.** MS (ESI) m/z 547 [M+2H], 1092 [M+H]. 1d.

[0317] **Compound 1:** Compound **1-6** (60mg, 0.05mmol) was dissolved in 1 mL of DMF. 32 μL of hydrazine was added. The resulting solution was stirred at ambient temperature for 1 hour. The reaction was quenched with IN hydrochloride solution. The reaction mixture was purified by HPLC to give 33 mg (55%) of compound 1. MS (ESI) m/z 482 [M+2H], 962 [M+H].

Example 2: Synthesis of Compound 2

[0318]

Scheme 2

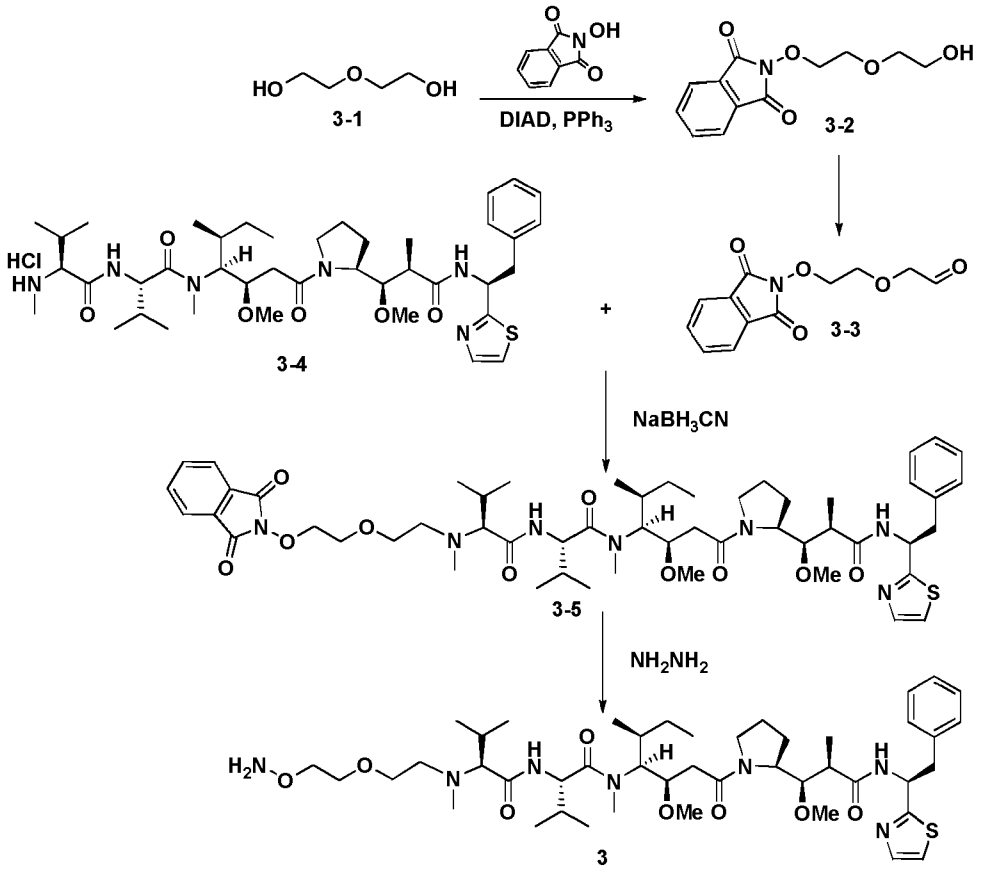


[0319] Compound 2 was synthesized via a similar synthetic route as described in Example 1. MS (ESI) m/z 460 [M+2H], 918 [M+H].

Example 3: Synthesis of Compound 3

[0320]

Scheme 3

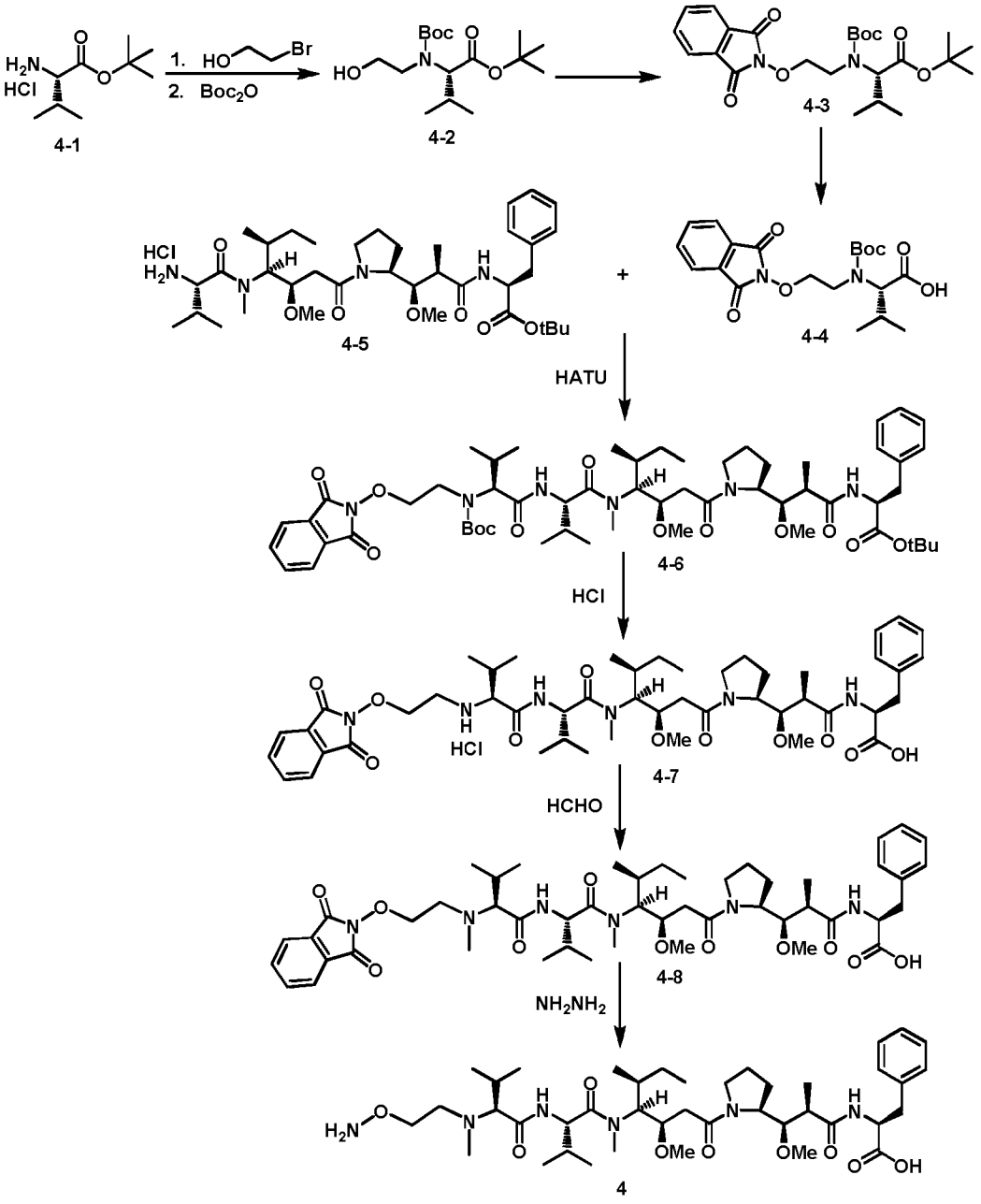


[0321] Compound 3 was synthesized via similar synthetic route to Example 1. MS (ESI) m/z 438 [M+2H], 974 [M+H].

Example 4: Synthesis of Compound 4

[0322] **Compound 4-2:** To a solution of Val (OtBu)-OH.HCl **4-1** (1 g, 4.77 mmol) and bromoethanol (304.7 μL, 4.3 mmol) in 10 mL of DMF was added 1.68 ml of DIEA. The reaction mixture was stirred at room temperature for 2 days. 4.8 mmol of Boc_2O was added to the reaction mixture, followed by 0.84 mL of DIEA. The reaction mixture was stirred at room temperature for 2 days. The reaction mixture was concentrated in vacuo and extracted with ethyl acetate, and washed with water, brine, dried over sodium sulfate and concentrated in vacuo. The residue was purified by flash column chromatography to give 0.66 g of compound 4-2.

Scheme 4



[0323] **Compound 4-3:** To a solution of compound **4-2** (500 mg, 1.58 mmol), N-hydroxyphthalimide (261 mg, 1.6 mmol) and triphenylphosphine (538 mg, 2.05 mmol) in 15 mL THF was added DIAD (394 μL, 1.9 mmol) at at 0°C. The resulting solution was stirred at room temperature overnight, and then concentrated in vacuo. The residue was purified by flash column chromatography to give 0.68 g of compound **4-3.**

[0324] **Compound 4-4:** Compound **4-3** was dissolved in 15 mL 4N HCl/Dioxane. The reaction mixture was stirred at room temperature for 2 days and concentrated in vacuo. The residue was dissolved in DMF and treated with Boc_2O (230 μL, 1 mmol) and DIEA (352 μL, 2 mmol). The reaction mixture was stirred at room temperature for 2 days. The reaction mixture was purified by HPLC to give 100 mg of compound **4-4.**

[0325] **Compound 4-5:** To a solution of compound Boc-Val-Dil-methylDap-OH in DMF is added phe(OtBu)-OH.HCl, HATU and N-methylmorpholine. The reaction mixture is stirred at room temperature for 4 hours. The reaction mixture is concentrated in vacuo and extracted with ethyl acetate (100 mLX1, 50 mL X2). The organic layer is combined and washed with brine, dried over sodium sulfate and concentrated in vacuo. The residue is purified by flash chromatography. The resulting compound is treated with HCl/EtOAC to give compound **4-5.**

[0326] **Compound 4-6:** To a solution of compound **4-5** in DMF is added compound **4-4,** HATU and DIEA. The reaction

mixture is stirred at room temperature for 4 hours. The reaction mixture is concentrated in vacuo and extracted with ethyl acetate (100 mLX1, 50 mL X2). The organic layer is combined and washed with brine, dried over sodium sulfate and concentrated in vacuo. The residue is purified by flash chromatography to give compound **4-6.**

[0327] **Compound 4-7:** Compound **4-6** is dissolved in 15 mL 4N HCl/Dioxane. The reaction mixture is stirred at room temperature for 2 hours and concentrated in vacuo to give compound **4-7.**

[0328] **Compound 4-8:** To a solution of compound **4-7** in 1 mL of DMF is added formylaldehyde and acetic acid, followed by addition of sodium cyanoborohydride. The resulting mixture is stirred at ambient temperature for 2 hours. The reaction mixture is diluted with water and purified by HPLC to give compound **4-8.**

[0329] **Compound 4:** Compound **4-8** is dissolved in 1 mL of DMF. Hydrazine is added. The resulting solution is stirred at ambient temperature for 1 hour. The reaction is quenched with IN hydrochloride solution. The reaction mixture is purified by HPLC to give Compound **4.**

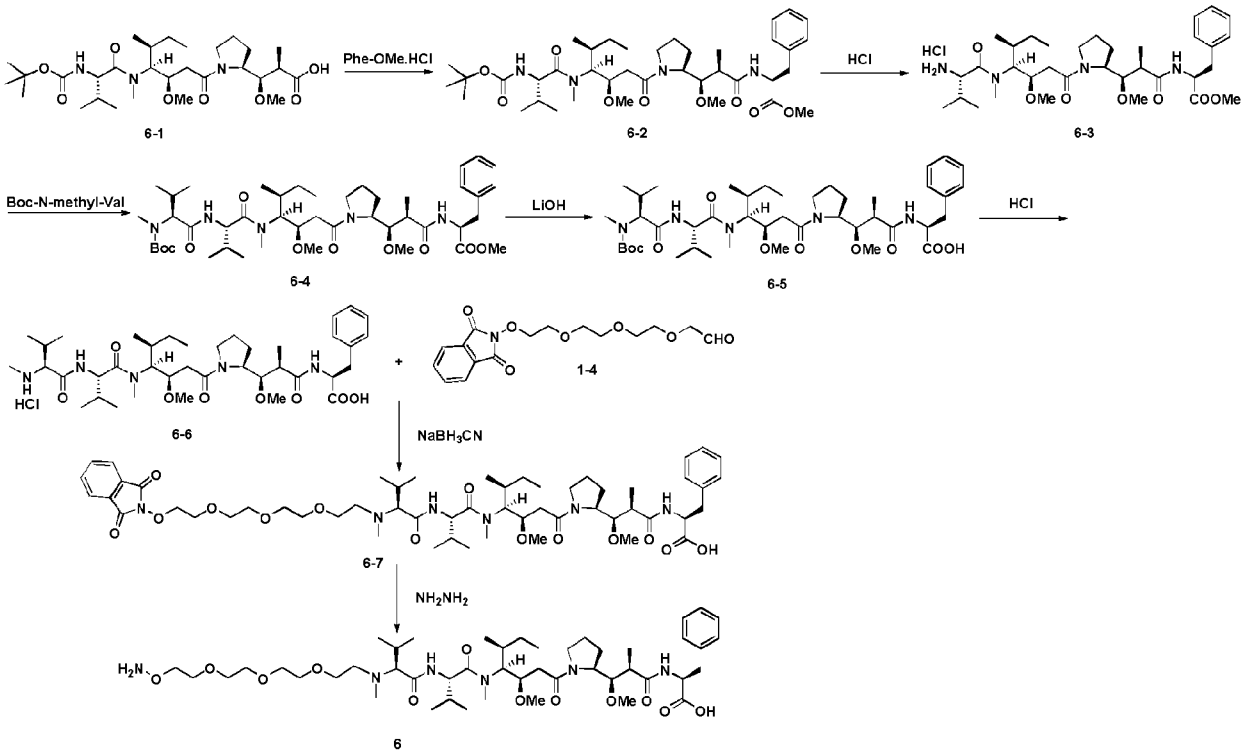
Example 5: Synthesis of Compound 5

[0330] Compound **4-7** is dissolved in 1 mL of DMF. Hydrazine is added. The resulting solution is stirred at ambient temperature for 1 hour. The reaction is quenched with IN hydrochloride solution. The reaction mixture is purified by HPLC to give Compound **5.**

Example 6: Synthesis of Compound 6

[0331]

Scheme 6



[0332] **Compound 6-2:** To a solution of compound **6-1** (500 mg, 0.875 mmol) in 3 mL of DMF was added 283 mg of phenylalanine hydrochloride, 433mg of HATU and 581 μL of N-methylmorpholine. The reaction mixture was stirred at room temperature for 4 hours. The reaction mixture was concentrated in vacuo and extracted with ethyl acetate (100 mLX1, 50 mL X2). The organic layer was combined and washed with brine, dried over sodium sulfate and concentrated in vacuo. The residue was purified by flash chromatography to give 560 mg (76%) of compound **6-2.**

[0333] **Compound 6-3:** Compound **6-2** was dissolved in 15 mL 4N HCl/Dioxane. The reaction mixture was stirred at room temperature for 2 hours and concentrated in vacuo to give 511 mg of compound **6-3.**

[0334] **Compound 6-4:** To a solution of compound **6-3** (368 mg, 0.55 mmol) in 3 mL of DMF was added 255 mg of Boc-N-methyl valine, 314mg of HATU and 303 μL of N-methylmorpholine. The reaction mixture was stirred at room temperature for 4 hours. The reaction mixture was concentrated in vacuo and extracted with ethyl acetate (100 mLX1,

50 mL X2). The organic layer was combined and washed with brine, dried over sodium sulfate and concentrated in vacuo. The residue was purified by flash chromatography to give 370 mg (79%) of compound **6-4.**

[0335] **Compound 6-5:** To a solution of compound **6-4** (170mg) in 10 mL MeOH was added 5eq of IN LiOH. The reaction mixture was stirred at room temperature for 2 hours. The reaction mixture was acidified by 1NHCl and extracted with ethyl acetate washed with brine, dried over sodium sulfate and concentrated in vacuo to give 150 mg (90%) of compound **6-5.**

[0336] **Compound 6-6:** Compound **6-5** was dissolved in 4N HCl/Dioxane. The reaction mixture was stirred at room temperature for 2 hours and concentrated in vacuo and purified by HPLC to give 150mg of compound **6-6.**

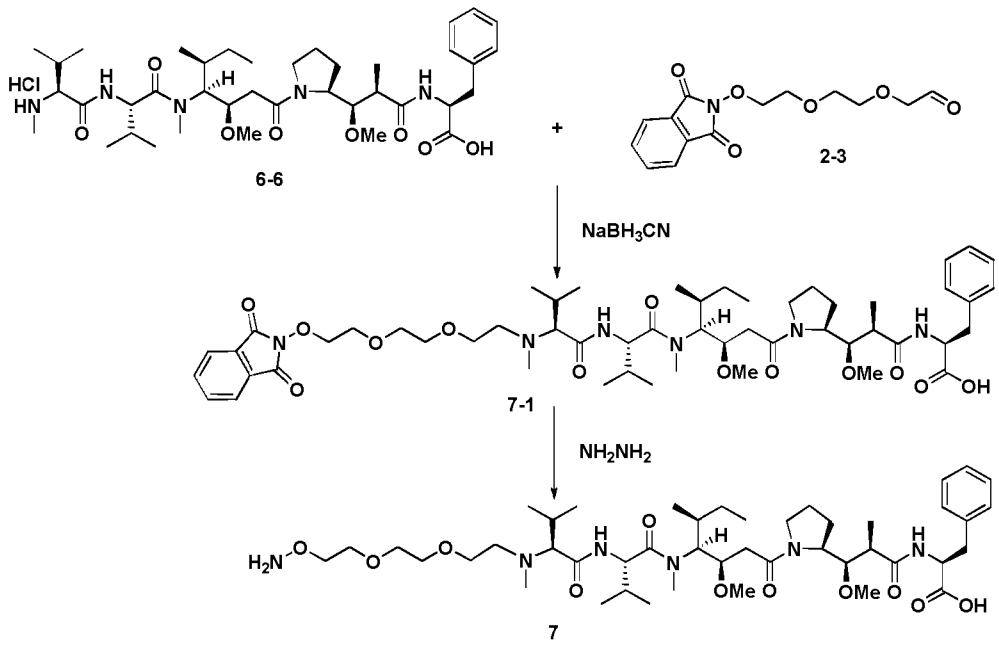
[0337] **Compound 6-7:** To a solution of compound **6-6** (50mg, 0.062 mmol) in 1mL of DMF was added compound **1-4** (63mg, 0.186mmol) and 70μL of acetic acid, followed by addition of 8 mg of sodium cyanoborohydride. The resulting mixture was stirred at ambient temperature for 2 hours. The reaction mixture was diluted with water and purified by HPLC to give 60mg (80%) of compound **6-7.**

[0338] **Compound 6:** Compound **6-7** (60mg, 0.05mmol) was dissolved in 1 mL of DMF. 32 μL of hydrazine was added. The resulting solution was stirred at ambient temperature for 1 hour. The reaction was quenched with IN hydrochloride solution. The reaction mixture was purified by HPLC to give 33 mg (55%) of Compound **6.**

Example 7: Synthesis of Compound 7

[0339]

Scheme 7

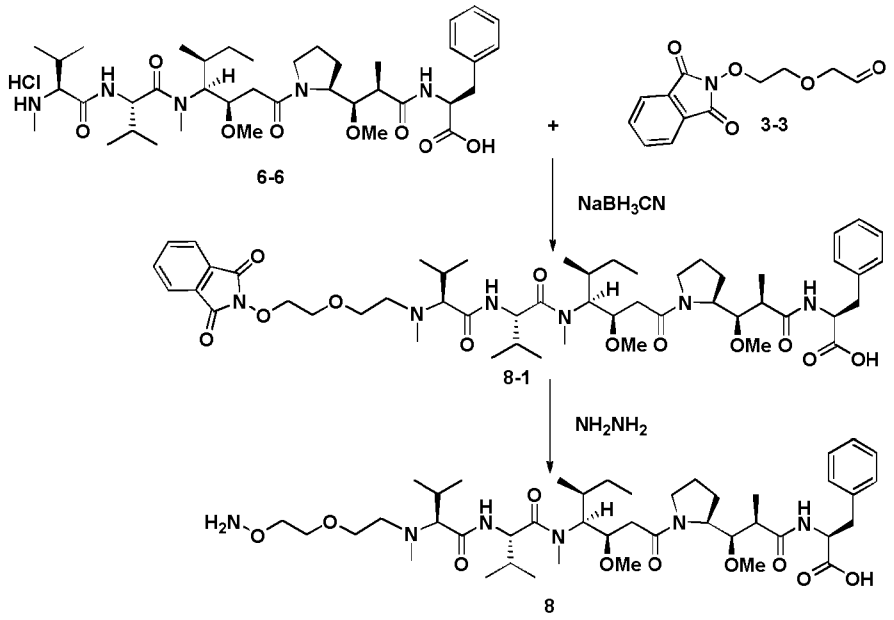


[0340] Compound 7 was synthesized via similar synthetic route to Compound 1. MS (ESI) m/z 440 [M+2H], 879 [M+H].

Example 8: Synthesis of Compound 8

[0341]

Scheme 8

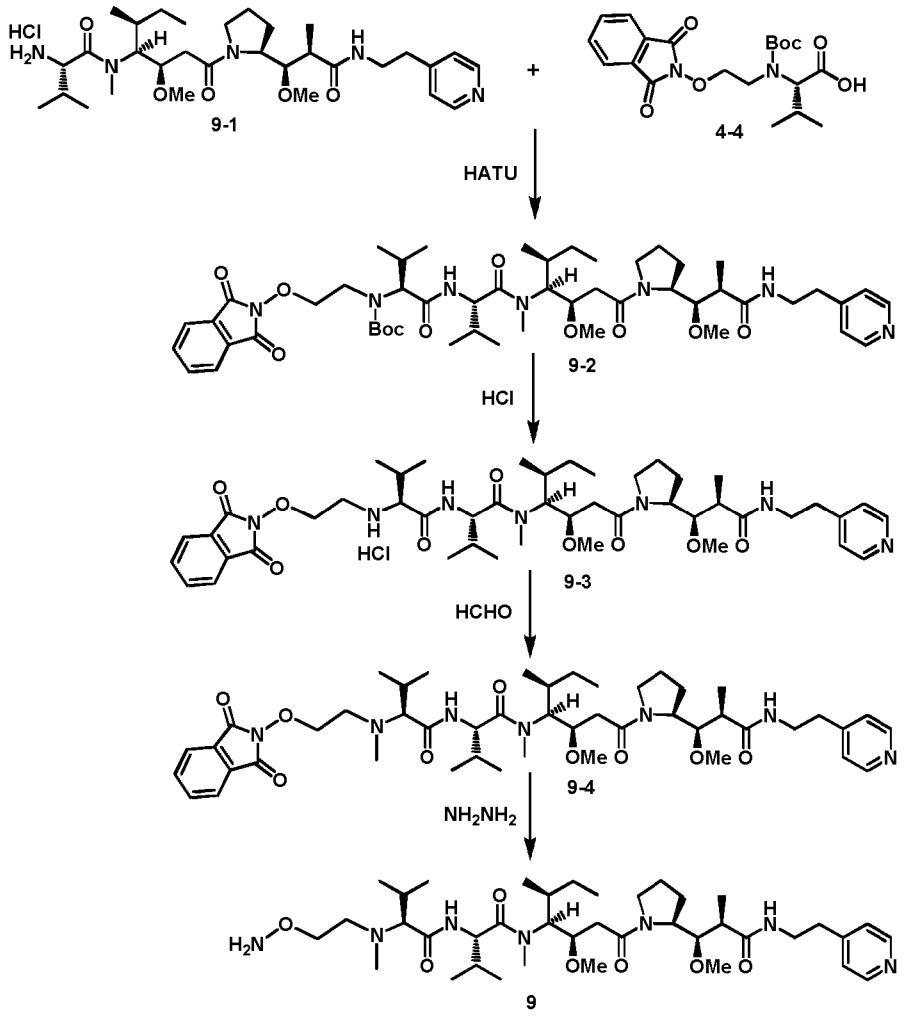


[0342] Compound 8 was synthesized via similar synthetic route to Compound 1. MS (ESI) m/z 418 [M+2H], 835 [M+H].

Example 9: Synthesis of Compound 9

[0343]

Scheme 9



[0344] Compound 9-1: To a solution of compound Boc-Val-Dil-methylDap-OH in DMF is added 4-(2-Aminoethyl) pyridine, HATU and N-methylmorpholine. The reaction mixture is stirred at room temperature for 4 hours. The reaction mixture is concentrated in vacuo and extracted with ethyl acetate (100 mLX1, 50 mL X2). The organic layer is combined and washed with brine, dried over sodium sulfate and concentrated in vacuo. The residue is purified by flash chromatography. The resulting compound is treated with HCl/EtOAC to give compound **9-1.**

[0345] **Compound 9-2:To** a solution of compound **9-1** in DMF is added compound **4-4,** HATU and DIEA. The reaction mixture is stirred at room temperature for 4 hours. The reaction mixture is concentrated in vacuo and extracted with ethyl acetate (100 mLX1, 50 mL X2). The organic layer is combined and washed with brine, dried over sodium sulfate and concentrated in vacuo. The residue is purified by flash chromatography to give compound **9-2.**

[0346] **Compound 9-3**:Compound **9-2** is dissolved in 15 mL 4N HCl/Dioxane. The reaction mixture is stirred at room temperature for 2 hours and concentrated in vacuo to give compound **9-3.**

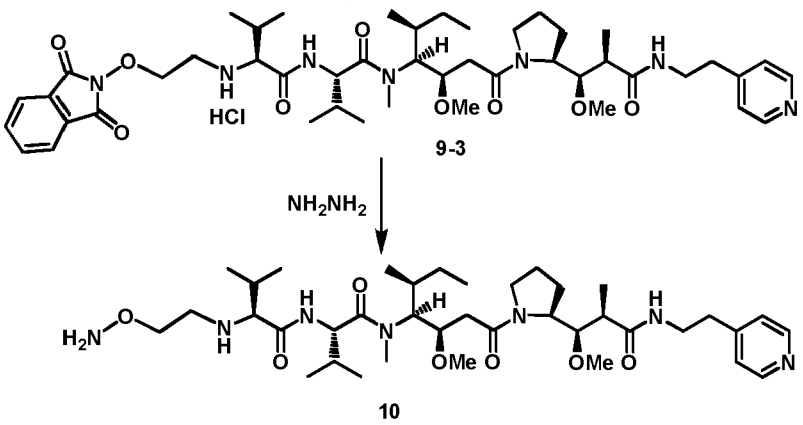
[0347] **Compound 9-4:** To a solution of compound **9-3** in 1mL of DMF is added formylaldehyde and acetic acid, followed by addition of sodium cyanoborohydride. The resulting mixture is stirred at ambient temperature for 2 hours. The reaction mixture is diluted with water and purified by HPLC to give compound **9-4.**

[0348] **Compound 9:** Compound **9-4** is dissolved in 1 mL of DMF. Hydrazine is added. The resulting solution is stirred at ambient temperature for 1 hour. The reaction is quenched with IN hydrochloride solution. The reaction mixture is purified by HPLC to give compound **9.**

Example 10: Synthesis of Compound 10

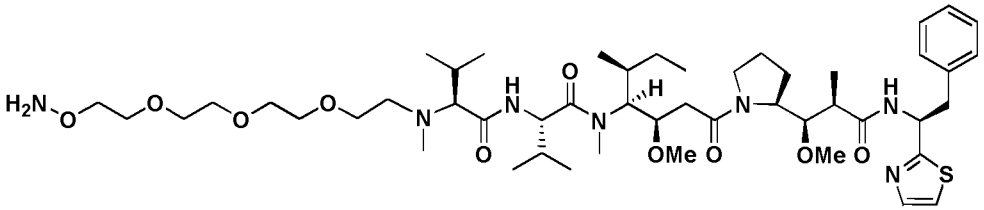
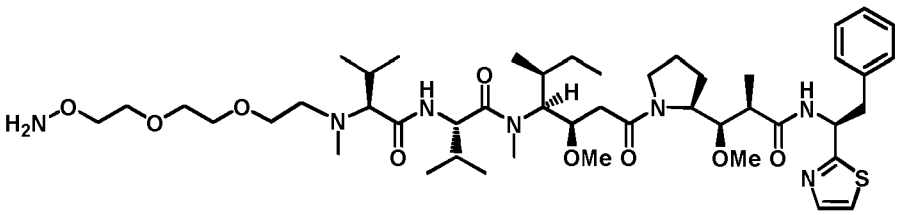
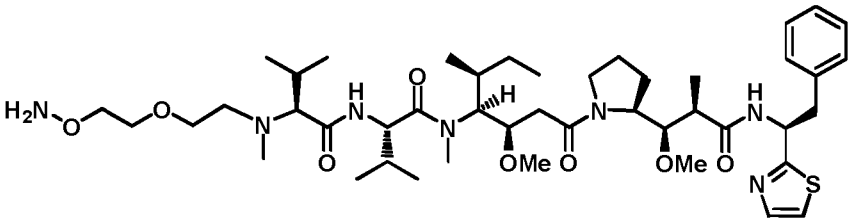
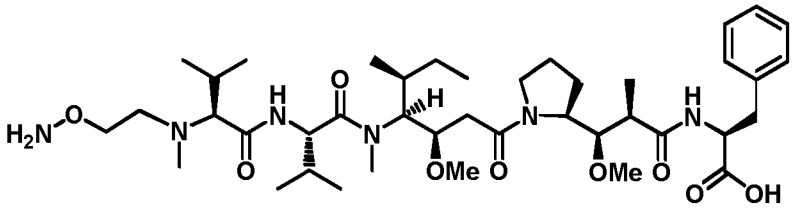
[0349]

Scheme 10

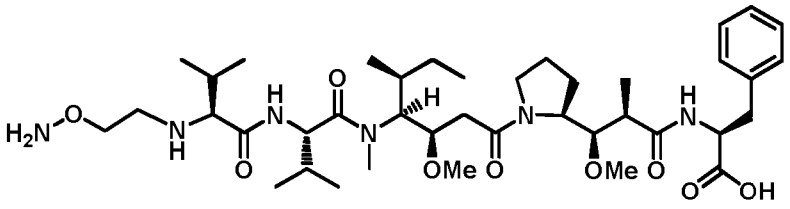
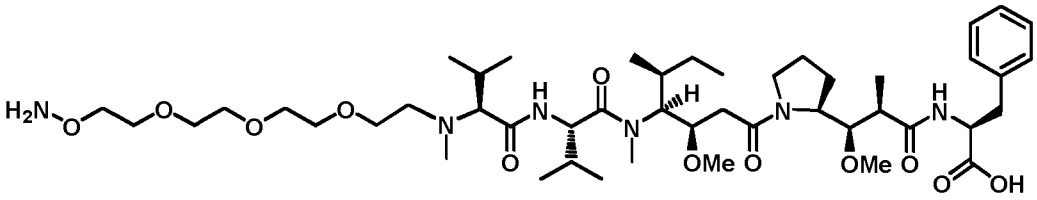
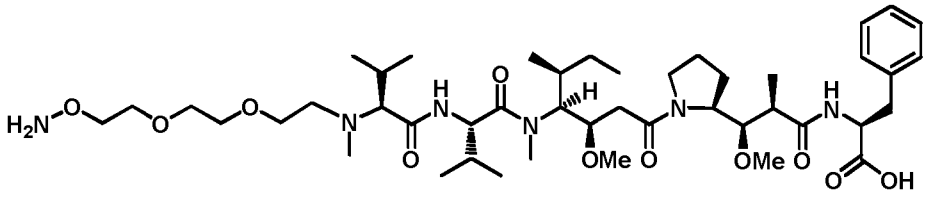
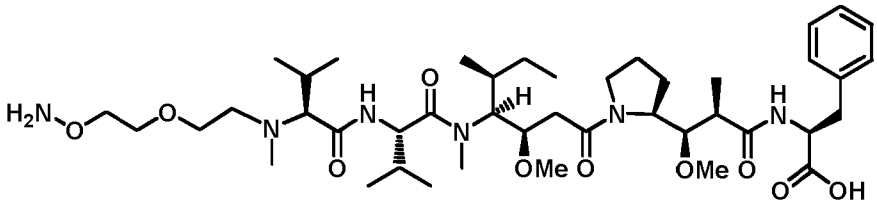
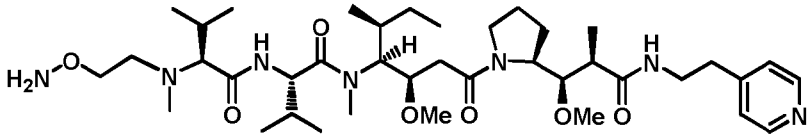
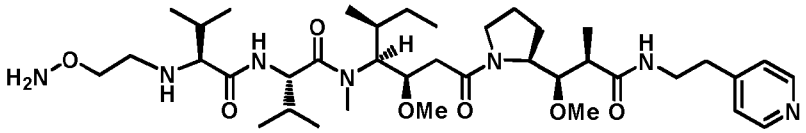


[0350] Compound 10: Compound **9-3** is dissolved in 1 mL of DMF. Hydrazine is added. The resulting solution is stirred at ambient temperature for 1 hour. The reaction is quenched with IN hydrochloride solution. The reaction mixture is purified by HPLC to give Example **10.**

Table 1. Structures of Compounds 1-10

Example	Structure
1	
2	
3	
4	

(continued)

Example	Structure
5	
6	
7	
8	
9	
10	

Example 11: Analysis of HER-tox binding to HER2 receptor

[0351] Her2-Fc was immobilized on CM5 chip to a density of ~ 280 RU. Flow rate was adjusted to 50 ul/min with HBS-EP as buffer. HerTox variants were injected for 3 min with 15 dissociation phase. 30sec pulse of 20mM HCl was used for regeneration. The Bivalent Analyte model was utilize to fit the data (Figure 1). Analysis of the data indicates that HerTox HA121-NC2D: Kd ~ 60 pM (chi2=4) and HerTox HA121-NC1D: Kd ~ 300 pM (chi2=14).

Example 12: Transient Transfection

[0352] CHO-S culture is seeded at 0.75x10^6/mL approximately 16 hours pre-transfection in FreeStyle Cho medium. Cells are ready to transfect the next day when the cell count has reached 1.4 - 1.6x10^6/mL. When cells reach target count, 400 mM pAF stock is added to a 1.4mM final culture concentration. PEI/DNA complex is prepared as described: DNA (1.42 ug/1x10^6 cells) is dissolved in RPMI (5% (v/v) of total culture volume), DNA/RPMI mixture is incubated at room temperature for 2 minutes, PEI stock (1 mg/mL) is added to DNA solution at a 3:1 ratio (mL PEI/ug DNA), and the

mixture is incubated at room temperature for 5 min. The culture is gently added to the mixture and swirled. The flasks are transferred to a 32°C incubator. At day 6 post-transfection , a western blot analysis is performed. At day 7 post-transfection, the supernatant is harvested.

Example 13: Anti-Her2 variant expression test

[0353] 30 ml shaker cultures, CHO-S in FreeStyle medium; 56 ug DNA in PEI reagent were used. 1.5 mM pAF was also used. At day 6, the supernatant was harvested. Titer was determined by Fc ELISA. (Figures 2 and 3)

Example 14: In vitro Inhibition of Proliferation Assay

[0354] At day 1, the cells were seeded. The media was aspirated and the T-225 culture flasks of cells was rinsed with 30mL PBS -/. PBS was aspirated and 6 mL of 0.25% Trypsin-EDTA was added to each flask. The flasks were incubated at 37C, 5%CO2 for 2-5 minutes. Adherent cells were dislodged by hitting flask and trypsin was neutralized by adding 14mL culture medium. The cell suspension was mixed and transered to a 50 mL conical tube. The cells were spun down at 1200 rpm, 5 min, room temperature. The resultant cell pellet was resuspended in 12 mL of culture medium. The cells were counted in a hemacytometer. Cells were seeded at appropriate cell densities into 96-well flat-bottom, clear plates and incubated overnight at 37°C, 5% CO_2 to allow cells to attach. Plating volume was 80uL/well. 80uL/well of culture medium was utilized as the "no cell" control.

[0355] Cell plating density examples include:

BT474 (high Her2) - 20,000 cells/well in F12k/DMEM (50/50), 10% FBS, P/S
MDA-MB-468 (Her2 negative) - 6,000 cells/well in F12k/DMEM (50/50), 10% FBS, P/S
HCC1954 - 5,000 cells/well in RPMI 1640, 10% FBS, P/S
SKOV-3 - 6,000 cells/well in RPMI 1640, 10% FBS, P/S
HT29 - 20,000 cells/well in McCoy's 5A, 10% FBS, P/S

[0356] At day 2, the test samples were added to the cells. The test samples were diluted in culture medium to a 9x stock concentration in Column 2 of a round-bottom 96-well plate. 3x serial dilutions were made from Column 2 to Column 11 with a multi-channel pipettor. 10uL/well of the above samples was added in duplicate to the appropriate wells of the seeded plate. The total volume in the wells was about 90uL/well. 10uL/well of culture medium was added to avoid edge effects for sample wells. Media Control wells were included in the inner wells where no sample was added (just 10uL/well of culture medium) to be used in proliferation calculations. The plates were incubated at 37°C, 5%CO_2 for 72 hrs.

[0357] At day 5, proliferation readout occurred. To detect cell proliferation, 10uL/well of WST-8 reagent was added (Cell Counting Kit-8, cat# CK04-20, Dojindo Labs) to all wells. The plate was incubated for 4 hrs at 37°C, 5%CO_2. The plates were measured for absorbance at OD 450nm. Calculation of proliferation inhibition of test samples: subtract the "no cell control" OD450 value from all wells' OD450 values; calculate average of the Media Control (untreated) wells; calculate each sample's % Media Control value using the formula: (OD450 sample/OD450 avg % Media Control) *100; calculate the average, standard deviation, and %CV of each sample's duplicate % Media Control values; plot each samples average % Media Control value against sample concentration; calculate IC50 values using 4-parameter logistic fit regression analysis to determine potency of test samples.

[0358] Figure 4 and Figure 5 illustrate results from the in vitro proliferation assay with dolastatin linker derivatives and breast cancer line HCC1954, HER2 +++. Figures 6 and 7 illustrate results from the in vitro proliferation assay with dolastatin linker derivatives and ovarian cancer line SKOV-3, HER2 +++. Figures 8 and 9 illustrate results from the in vitro proliferation assay with dolastatin linker derivatives and breast cancer line MDS-MB-468, HER2 negative.

TABLE 2. HER-Tox Proliferation Assay Summary: IC_{50} Values [nM] after 72hr drug treatment

	Data Set I								
Experiment Date	**9/4/10**	**9/4/10**	**9/4/10**	**9/4/10**	**9/4/10**	**9/4/10**	**9/13/10**	**9/13/10**	**9/13/10**
HER2 exp. (literature/in house)	+++/ +++	+++/+++	?/+++	+++/++++	+++/+++	+++/++++	+++/ +++	+++/+++	?
In vivo sensitivity to	+	+	-	?	-	?	+	-	?

Herceptin									
Sample	**BT474**	**HCC1954**	**LS513**	**NCI-N87**	**SKOV-3**	**ZR-75-30**	**BT474**	**SKOV-3**	**MDA-MB-175**
Dolastatin	**0.1**	**0.06**	**0.2**	**>30**	**0.2**	**>30**	**0.1**	**0.1**	**no fit**
NC-D1	**2.7**	**0.9**	**11**	**>100**	**3.7**	**>100**	**2.9**	**2.1**	**no fit**
NC-D2	**2.4**	**1.5**	**5.2**	**>100**	**3.4**	**>100**	**2.8**	**2.3**	**no fit**
PHC-D2									
Herceptin	**>300**	**>300**	**>300**	**>300**	**>300**	**>300**	**>300**	**>300**	**>300**
Mab HA121-NC-D1	**0.2**	**0.1**	**>10**	**>10**	**(0.04)***	**>10**	**0.2**	**no fit**	**0.3**
Mab HA121-NC-D2	**1.0**	**0.3**	**>10**	**>10**	**0.3**	**>10**	**0.4**	**no fit**	**0.7**
Mab HA121-PHC-D2									
Fab K136pAF	**>10**	**>10**	**>10**	**>10**	**>10**	**>10**			
Fab K136-NC-D1	**1.8**	**0.5**	**>10**	**>10**	**2.5**	**>10**			

	Data Set II				
Experiment Date	9/24/10	9/24/10	10/8/10	10/8/10	10/8/10
HER2 exp. (literature/in house)	++/?	+++/ +++	+++/+++	+++/+++	-/?
In vivo sensitivity to Herceptin	unlikely	+	+	-	?
Sample	**HT29**	**BT474**	**HCC1954**	**SKOV-3**	**MDA-MB-468**
Dolastatin	4	2.5	0.04	0.2	<0.01
NC-D1	4	(45)*	<0.01	<0.1	<0.1
NC-D2	4	3	<0.01	(0.1)*	0.3
PHC-D2	12	7	2	8	2
Herceptin	>300	>300	>300	>300	>300
Mab HA121-NC-D1	>10	1	0.2	1.3	>30
Mab HA121-NC-D2	>10	0.8	0.03	(1.3)*	>30

Mab HA121-PHC-D2	5*	2	0.1	0.3	(5.8)*
Fab K136pAF					
Fab K136-NC-D1					

TABLE 3. In vitro Cellular Data

					Small Molecule, IC50, nM				ADC, IC50, nM		
Cancer	**Cell Line**	**KRAS**	**BRAF**	**EGFR expression**	**MMD**	**NC-D-1**	**C-D-1**	**NC-D-2**	**C225-NC-D-1**	**C225-HC-D-1**	**C225-NC-D-2**
Skin	A431	wt	wt	+++	0.1	8.22	16.54		0.09	0.12	0.19
Colon	Colo 205	wt	mut	+	0.25	6.81	40.03		>100	>100	
	HCT-116	mut	wt	++	0.13	2.14	24.86	5.7	51.73	>100	62.8
	HT-29	wt	mut	++	0.1	4.3		1.7	36.9		16.7
	SW620	mut	wt	-	0.14	5		3.2	121		56.8
	HCT-15	mut	wt	++	2.65	31.03	>100	>100	>300	>300	
Lung	A549*	mut	wt	++	0.19	6.44	39.82	39.82	>100	>100	
	H2122	mut	wt	+	0.11	12.71	31.76	31.76	>100	>100	
	H460	mut	wt	+	0.48	10.4	95.1	95.1	>300	>300	
Prostate	DU145	wt	wt	++	0.24	4.8	20.51	20.51	>100	>100	

Example 15: In vivo anti-tumor efficacy of Her2-ADCs in HCC1954 (human breast carcinoma) xenograft animal model

[0359] HCC1954 (human breast carcinoma) cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI + 10% FBS, 37°C, 5% CO_2 until 80% confluent. Cells were harvested by trypsinization and suspended in PBS at 1 x 10^8 cells/mL.

[0360] Female, SCID-beige mice, 5-8 weeks old, were obtained from Charles River Laboratories. HCC1954 cells (human, breast carcinoma, ATCC, # CRL-2338) were mixed 1:1 with Matrigel (BD Biosciences, Bedford MA) and injected subcutaneously into the mice. When tumors reached an average size of 100-200 mm^3, mice were sorted into groups of 9-10 mice each. Caliper measurements were taken twice weekly until the end of the study. To estimate tumor volume, two orthogonal diameters were measured with calipers and the values entered into the formula, (L x W x W)/2=V, (where W=the shortest diameter, L=the longest diameter and V=volume), to obtain an estimated volume. The tumor volume was converted to tumor weight in the Excel data file by assuming 1 mm^3 = 1 mg. Endpoint was based on a study design of tumor growth inhibition (TGI). When the mean tumor volume of the control group reached approx. 1,000 mm^3 all mice were euthanized or day 28, whichever came first.

[0361] Mice were given a single IV injection (tail vein) on day 1 of dosing. Test article was dissolved at 4 mg/mL, 2 mg/mL and 0.66 mg/mL and administered at a dose volume of 5 mL/kg to deliver 20, 10 and 3.3 mg/kg. Test articles were: Herceptin® clinical grade (Trastuzumab), Her2-HS122-NCD1 (Ab:Drug ratio = 1:2, non-cleavable linker), and Her2-HS122/LK145-HCD1 (Ab:Drug ratio = 1:4, cleavable linker) See Figure 16.

Example 16: In vivo Studies of Her2-Dolastatin linked Derivative

[0362] HCC1954 cells were utilized for this study with 10^7 cells/mouse in Matrigel, SC in the right flank. Mice were SCID-bg female 4-8 weeks. Grouping was performed at day 5 after cell implantation (tumors ~100 mm^3): sorted into 11 groups of 10 mice each. A single IV dose was given on day 1 of dosing with each compound at 3 dose levels, 20 mg/kg, 10 mg/kg and 3.3 mg/kg. Tumor volume was monitored until the endpoint was reached (1,000 mm^3 or 60 days). (Figure 10) Paclitaxel 25 mg/kg, IV, qod x 5 was employed as the control chemotherapy. The vehicle = 50 mM histamine, 0.1 M NaCl, 5% trehalose, pH 6. Herceptin® clinical grade (Trastuzumab), Her2-HS122-NCD1 (non-cleavable linker) and Her2-HS122/LK145-HCD1 (cleavable linker) were tested. (Figures 11 and 12)

Table 4. Calculation of T/C (Treated / Control) for the HCC1954 study at day 28

Group	n	Treatment Regimen 1				Median Tumor Volume (mm3)	T/C
		Agent	mg/kg	Route	Schedule		
1#	10	vehicle	-	iv	qd x 1	486	--
2	10	trastuzumab	3.3	iv	qd x 1	405	0.833
3	10	trastuzumab	10	iv	qd x 1	446	0.918
4	10	trastuzumab	20	iv	qd x 1	385	0.792
5	10	Her-HS122-NC1D-002	3.3	iv	qd x 1	40	0.082
6	10	Her-HS122-NC1D-002	10	iv	qd x 1	14	0.029
7	10	Her-HS122-NC1D-002	20	iv	qd x 1	18	0.037
8	10	Her-HS122/LK145-HC1D-001	3.3	iv	qd x 1	40	0.082
9	10	Her-HS122/LK145-HC1D-001	10	iv	qd x 1	25	0.051
10	10	Her-HS122/LK145-HC1D-001	20	iv	qd x 1	18	0.037
11	10	paclitaxel	25	iv	qod x 5	18	0.037

Example 17: Pharmacokinetic studies

[0363] Assay was performed that detected antibody binding to ErbB2 receptor. (Figure 13) Assay was performed that

detected at least two dolastatins linked to an antibody (Figure 14).

[0364] Figure 15.

Example 18: Treatment for Breast Cancer

Human Clinical Trial of the Safety and/or Efficacy of Trastuzumab-Linked Dolastatin Derivative for Breast Cancer Therapy

[0365] Objective: To compare the safety and pharmacokinetics of administered composition comprising trastuzumab-linked dolastatin derivative.

[0366] Study Design: This study will be a Phase I, single-center, open-label, randomized dose escalation study followed by a Phase II study in breast cancer patients. Patients should not have had exposure to trastuzumab-linked dolastatin derivative prior to the study entry. Patients must not have received treatment for their cancer within 2 weeks of beginning the trial. Treatments include the use of chemotherapy, hematopoietic growth factors, and biologic therapy such as monoclonal antibodies. Patients must have recovered from all toxicities (to grade 0 or 1) associated with previous treatment. All subjects are evaluated for safety and all blood collections for pharmacokinetic analysis are collected as scheduled. All studies are performed with institutional ethics committee approval and patient consent.

[0367] Phase I: Patients receive i.v. trastuzumab-linked dolastatin derivative on days 1, 8, and 15 of each 28-day cycle. Doses of trastuzumab-linked dolastatin derivative may be held or modified for toxicity based on assessments as outlined below. Treatment repeats every 28 days in the absence of unacceptable toxicity. Cohorts of 3-6 patients receive escalating doses of trastuzumab-linked dolastatin derivative until the maximum tolerated dose (MTD) for trastuzumab-linked dolastatin derivative is determined. The MTD is defined as the dose preceding that at which 2 of 3 or 2 of 6 patients experience dose-limiting toxicity. Dose limiting toxicities are determined according to the definitions and standards set by the National Cancer Institute (NCI) Common Terminology for Adverse Events (CTCAE) Version 3.0 (August 9, 2006).

[0368] Phase II: Patients receive trastuzumab-linked dolastatin derivative as in phase I at the MTD determined in phase I. Treatment repeats every 4 weeks for 2-6 courses in the absence of disease progression or unacceptable toxicity. After completion of 2 courses of study therapy, patients who achieve a complete or partial response may receive an additional 4 courses. Patients who maintain stable disease for more than 2 months after completion of 6 courses of study therapy may receive an additional 6 courses at the time of disease progression, provided they meet original eligibility criteria.

[0369] Blood Sampling Serial blood is drawn by direct vein puncture before and after administration of trastuzumab-linked dolastatin derivative. Venous blood samples (5 mL) for determination of serum concentrations are obtained at about 10 minutes prior to dosing and at approximately the following times after dosing: days 1, 8, and 15. Each serum sample is divided into two aliquots. All serum samples are stored at -20°C. Serum samples are shipped on dry ice.

[0370] Pharmacokinetics: Patients undergo plasma/serum sample collection for pharmacokinetic evaluation before beginning treatment and at days 1, 8, and 15. Pharmacokinetic parameters are calculated by model independent methods on a Digital Equipment Corporation VAX 8600 computer system using the latest version of the BIOAVL software. The following pharmacokinetics parameters are determined: peak serum concentration (C_{max}); time to peak serum concentration (tmax); area under the concentration-time curve (AUC) from time zero to the last blood sampling time (AUC_{0-72}) calculated with the use of the linear trapezoidal rule; and terminal elimination half-life ($t_{1/2}$), computed from the elimination rate constant. The elimination rate constant is estimated by linear regression of consecutive data points in the terminal linear region of the log-linear concentration-time plot. The mean, standard deviation (SD), and coefficient of variation (CV) of the pharmacokinetic parameters are calculated for each treatment. The ratio of the parameter means (preserved formulation/non-preserved formulation) is calculated.

[0371] Patient Response to combination therapy: Patient response is assessed via imaging with X-ray, CT scans, and MRI, and imaging is performed prior to beginning the study and at the end of the first cycle, with additional imaging performed every four weeks or at the end of subsequent cycles. Imaging modalities are chosen based upon the cancer type and feasibility/availability, and the same imaging modality is utilized for similar cancer types as well as throughout each patient's study course. Response rates are determined using the RECIST criteria. (Therasse et al, J. Natl. Cancer Inst. 2000 Feb 2; 92(3):205-16; http://ctep.cancer.gov/forms/TherasseRECISTJNCI.pdf). Patients also undergo cancer/tumor biopsy to assess changes in progenitor cancer cell phenotype and clonogenic growth by flow cytometry, Western blotting, and IHC, and for changes in cytogenetics by FISH. After completion of study treatment, patients are followed periodically for 4 weeks.

Example 19: Treatment for Breast Cancer

Human Clinical Trial of the Safety and Efficacy of Trastuzumab-Linked Dolastatin Derivative for Breast Cancer Therapy

[0372] Objective: Compare the efficacy and toxicity of trastuzumab-linked dolastatin derivative alone followed at dis-

ease progression by combination trastuzumab and paclitaxel vs first-line combination trastuzumab and paclitaxel in women with HER2-overexpressing metastatic breast cancer.

[0373] Study Design: This study is a randomized, multicenter study. Patients are stratified according to degree of HER2/neu-overexpression (2+ vs 3+), prior anthracycline-containing adjuvant treatment (no prior treatment vs prior treatment without radiotherapy to left chest wall vs prior treatment with radiotherapy to left chest wall), estrogen-receptor status (positive vs negative vs unknown), prior therapy (first-line vs second/third-line), and center. Patients are randomized to one of two treatment arms. Arm I: Patients receive trastuzumab-linked dolastatin derivative IV over 30-90 minutes weekly. At time of disease progression, patients receive combination trastuzumab-linked dolastatin derivative IV and paclitaxel IV as in arm II. Arm II: Patients receive trastuzumab-linked dolastatin derivative IV over 30-90 minutes weekly. Paclitaxel is administered IV over 1 hour weekly for 3 weeks followed by 1 week of rest.

[0374] Treatment continues in both arms in the absence of disease progression or unacceptable toxicity. Quality of life is assessed at baseline and day 1 of courses 2, 3, 4, 5, 6, 8, 10, and 12. Patients are followed at 1, 3, and 6 months and then every 6 months thereafter.

Example 20: Treatment for Bladder Cancer

Human Clinical Trial of the Safety and Efficacy of a Dolastatin Derivative described herein for Bladder Cancer Therapy

[0375] Objective: Determine the acute toxicity of paclitaxel and radiotherapy with or without trastuzumab-linked dolastatin derivative in patients who have undergone prior transurethral bladder resection for muscle-invasive transitional cell carcinoma of the bladder.

[0376] Disease Characteristics: Histologically or cytologically is confirmed primary transitional cell carcinoma (TCC) of the bladder; histologic evidence of muscularis propria invasion; meets 1 of the following stage criteria: stage T2-4a; NX, N0, or N1; and M0 disease or clinical stage T1, grade 3/3 disease AND requires definitive local therapy; tumor involvement of the prostatic urethra allowed provided the following criteria are met: tumor is visibly completely resected; no evidence of stromal invasion of the prostate, no evidence of distant metastases by chest x-ray or CT scan AND abdominal/pelvic CT scan; has undergone transurethral bladder resection (as thorough as is judged safely possible) within the past 3-8 weeks, including bimanual examination with tumor mapping; sufficient tumor tissue available for HER2/neu analysis; not a candidate for radical cystectomy.

[0377] Study Design: This study is a non-randomized, multicenter study. Patients are assigned to 1 of 2 treatment groups according to HER2/neu status (HER2/neu 2+ or 3+ staining [group 1] vs HER2/neu 0 or 1+ staining [group 2]).

[0378] Group 1: Patients receive paclitaxel IV over 1 hour on days 1, 8, 15, 22, 29, 36, and 43 and trastuzumab-linked dolastatin derivative IV over 90 minutes on day 1 and then over 30 minutes on days 8, 15, 22, 29, 36, and 43. Patients also undergo radiotherapy once daily on days 1-5, 8-12, 15-19, 22-26, 29-33, 36-40, 43-47, and 50. Treatment continues in the absence of disease progression or unacceptable toxicity.

[0379] Group 2: Patients receive paclitaxel and undergo radiotherapy as in group 1. After completion of study treatment, patients are followed at 4-5 weeks, every 3 months for 1 year, every 4 months for 1 year, every 6 months for 3 years, and then annually thereafter.

Example 21: Treatment for Ovarian Cancer

Human Clinical Trial of the Safety and Efficacy of Dolastatin Derivative described herein for Ovarian Cancer Therapy

[0380] Objective: Evaluate the safety and efficacy of a four week once weekly IV dosage of composition comprising a dolastatin derivative described herein in women with HER2-overexpressing ovarian cancer.

[0381] Study Design: This study is a non-randomized, open-label, 11 week, multicenter study. This study will evaluate the safety profile of four once weekly IV dosage, the MTD, PK and immunogenicity of trastuzumab-linked dolastatin derivative. Patients are assigned to a single group. Patients receive one dose of trastuzumab-linked dolastatin derivative once a week for 4 weeks. Trastuzumab-linked dolastatin derivative will be administered by IV infusion on Study Days 1, 8, 15, and 22. Urine samples will be taken on days 1 and 22.

[0382] Blood Sampling Serial blood is drawn by direct vein puncture before and after administration of trastuzumab-linked dolastatin derivative. Venous blood samples (5 mL) for determination of serum concentrations are obtained at about 10 minutes prior to dosing and at approximately the following times after dosing: days 1, 2, 4, 5, 8, 15, 22, 36, 43 and 50. Each serum sample is divided into two aliquots. All serum samples are stored at -20°C. Serum samples are shipped on dry ice.

[0383] Treatment continues in the absence of disease progression or unacceptable toxicity. Quality of life is assessed at baseline and day 1 of courses 2, 3, 4, 5, 6, 8, 10, and 12. Patients are followed on days 29. 36, 43, and 50. Patients will be asked about adverse events. Patients will have an imaging scan and ECG to evaluate tumor siz and heart function

(day 43). At the termination of the study patients will have a physical exam day 50). Patients with evidence of disease regression may receive continued therapy until evidence of progression of disease is documented.

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

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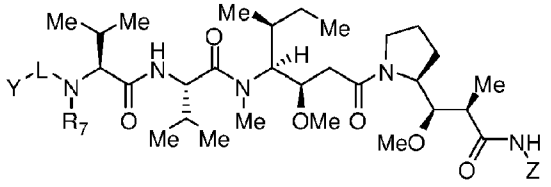
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Patentkrav

1. Forbindelse omfattende formel (I):

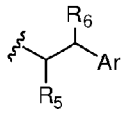


(I)

;

hvor:

Z har strukturen af:



;

R_5 er H, COR_8, C_1-C_6alkyl eller thiazol;

R_8 er OH;

R_6 er OH eller H;

Ar er phenyl eller pyridin;

R_7 er C_1-C_6-alkyl eller hydrogen;

Y er valgt fra gruppen bestående af en hydroxylamin, azid, alkyn og cycloalkyn;

L er -alkylen- eller -(alkylen-O)$_n$-alkylen-; og

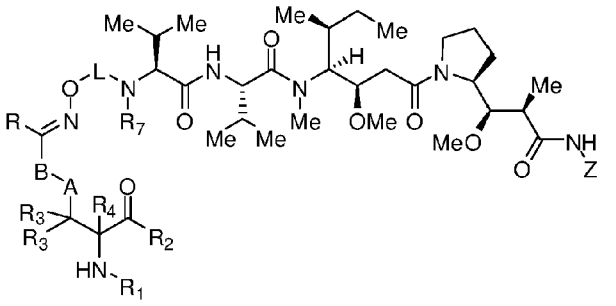
n er et heltal større end eller lig med en;

hvor alkyl betegner et ligekædet eller forgrenet, eller cyklisk carbonhydridradikal, eller kombination deraf, som kan være fuldstændigt mættet, mono- eller polyumættet og kan inkludere di- og multivalente radikaler og også inkludere heteroalkyl og halogenalkyl;

hvor alkylen inkluderer heteroalkylen;

eller et salt deraf.

2. Forbindelse omfattende formel (VIII):



(VIII)

;

hvor:

A er valgtfrit, og når det er til stede, er lavere alkylen, substitueret lavere alkylen, lavere cycloalkylen, substitueret lavere cycloalkylen, lavere alkenylen, substitueret lavere alkenylen, alkynylen, lavere heteroalkylen, substitueret heteroalkylen, lavere heterocycloalkylen, substitueret lavere heterocycloalkylen, arylen, substitueret arylen, heteroarylen, substitueret heteroarylen, alkarylen, substitueret alkarylen, aralkylen eller substitueret aralkylen;

B er valgfrit, og når det er til stede, er en linker valgt fra gruppen bestående af lavere alkylen, substitueret lavere alkylen, lavere alkenylen, substitueret lavere alkenylen, lavere heteroalkylen, substitueret lavere heteroalkylen, -O-, -O-(alkylen eller substitueret alkylen)-, -S-, -S-(alkylen eller substitueret alkylen)-, $-S(O)_k-$, hvor k er 1, 2 eller 3, $-S(O)_k$(alkylen eller substitueret alkylen)-, -C(O)-, -C(O)-(alkylen eller substitueret alkylen)-, -C(S)-, -C(S)-(alkylen eller substitueret alkylen)-, -N(R')-, -NR'-(alkylen eller substitueret alkylen)-, -C(O)N(R')-, -CON(R')-(alkylen eller substitueret alkylen)-, -CSN(R')-, -CSN(R')-(alkylen eller substitueret alkylen)-, -N(R')CO-(alkylen eller substitueret alkylen)-, -N(R')C(O)O-, $-S(O)_kN(R')-$, -N(R')C(O)N(R')-, -N(R')C(S)N(R')-, $-N(R')S(O)_kN(R')-$, -N(R')-N=, -C(R')=N-, -C(R')=N-N(R')-, -C(R')=N-N=, $-C(R')_2-N=N-$, og $-C(R')_2-N(R')-N(R')-$, hvor hvert R' er uafhængigt H, alkyl eller substitueret alkyl;

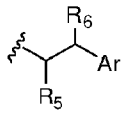
R er H, alkyl, substitueret alkyl, cycloalkyl eller substitueret cycloalkyl;

R_1 er H, en aminobeskyttelsesgruppe, et resin, mindst en aminosyre, et polypeptid eller et polynukleotid;

R_2 er OH, en esterbeskyttelsesgruppe, et resin, mindst en aminosyre, et polypeptid eller et polynukleotid;

R_3 og R_4 er hver uafhængigt H;

Z har strukturen af:



;

R_5 er H, COR_8, C_1-C_6alkyl eller thiazol;

R_8 er OH;

R_6 er OH eller H;

Ar er phenyl eller pyridin;

R_7 er C_1-C_6alkyl eller hydrogen; og

L er -alkylen- eller -(alkylen-O)$_n$-alkylen-; og

n er et heltal som er større end eller lig med en;

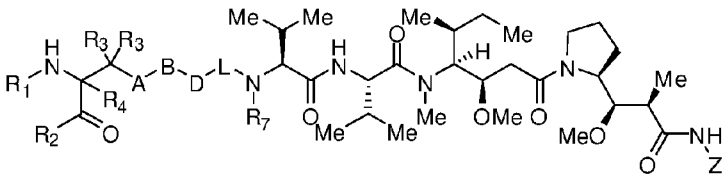
hvor alkyl betegner et ligekædet eller forgrenet, eller cyklisk carbonhydridradikal, eller kombination deraf, som kan være fuldtændigt mættet, mono- eller polyumættet og kan inkludere di- og multivalente radikaler og også inkluderer heteroalkyl og halogenalkyl;

hvor alkylen inkluderer heteroalkylen;

hvor arylen betegner et divalent arylradikal, hvor aryl inkluderer heteroaryl;

eller solvat deraf.

3. Forbindelse omfattende formel (XXXI):

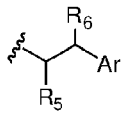


(XXXI)

;

hvor:

Z har strukturen af:



;

R_5 er H, CO_2H, C_1-C_6alkyl eller thiazol;

R_6 er OH eller H;

Ar er phenyl eller pyridin;

A er valgfrit, og når det er tilstede, er lavere alkylen, substitueret lavere alkylen, lavere cycloalkylen, substitueret lavere cycloalkylen, lavere alkenylen, substitueret lavere alkenylen, alkynylen, lavere heteroalkylen,

substitueret heteroalkylen, lavere heterocycloalkylen, substitueret lavere heterocycloalkylen, arylen, substitueret arylen, heteroarylen, substitueret heteroarylen, alkarylen, substitueret alkarylen, aralkylen eller substitueret aralkylen;

B er valgfrit, og når det er til stede, er en linker valgt fra gruppen bestående af lavere alkylen, substitueret lavere alkylen, lavere alkenylen, substitueret lavere alkenylen, lavere heteroalkylen, substitueret lavere heteroalkylen, -O-(alkylen eller substitueret alkylen)-, -S-(alkylen eller substitueret alkylen)-, -C(O)R"-, -S(O)$_k$(alkylen eller substitueret alkylen)-, hvor k er 1, 2 eller 3, -C(O)-(alkylen eller substitueret alkylen)-, -C(S)-(alkylen eller substitueret alkylen)-, -NR"-(alkylen eller substitueret alkylen)-, -CON(R")-(alkylen eller substitueret alkylen)-, -CSN(R")-(alkylen eller substitueret alkylen)-, og -N(R")CO-(alkylen eller substitueret alkylen)-, hvor hvert R" er uafhængigt H, alkyl eller substitueret alkyl;

R_1 er H, en aminobeskyttelsesgruppe, et resin, mindst en aminosyre, et polypeptid eller et polynukleotid;

R_2 er OH, en esterbeskyttelsesgruppe, et resin, mindst en aminosyre, et polypeptid eller et polynukleotid;

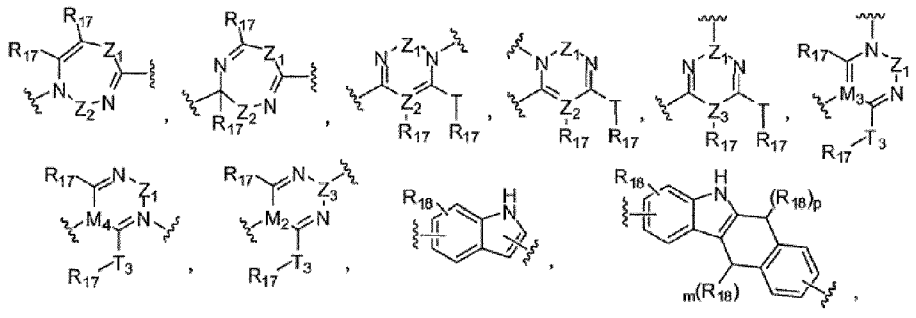
R_3 og R_4 er hver uafhængigt H, halogen, lavere alkyl eller substitueret lavere alkyl;

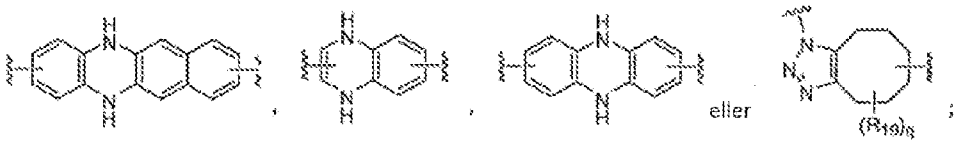
R_7 er C_1-C_6alkyl eller hydrogen;

L er -alkylen- eller -(alkylen-O)$_n$-alkylen-;

n er et heltal som er større end eller lig med en;

D har strukturen af:





hvert R_{17} er uafhængigt valgt fra gruppen bestående af H, alkyl, substitueret alkyl, alkenyl, substitueret alkenyl, alkynyl, substitueret alkynyl, alkoxy, substitueret alkoxy, alkylalkoxy, substitueret alkylalkoxy, polyalkylenoxid, substitueret polyalkylenoxid, aryl, substitueret aryl, heteroaryl, substitueret heteroaryl, alkaryl, substitueret alkaryl, aralkyl, substitueret aralkyl, -(alkylen eller substitueret alkylen)-ON(R")$_2$, -(alkylen eller substitueret alkylen)-C(O)SR", -(alkylen eller substitueret alkylen)-S-S-(aryl eller substitueret aryl), -C(O)R", -C(O)$_2$R" eller -C(O)N(R")$_2$, hvor hvert R" er uafhængigt hydrogen, alkyl, substitueret alkyl, alkenyl, substitueret alkenyl, alkoxy, substitueret alkoxy, aryl, substitueret aryl, heteroaryl, alkaryl, substitueret alkaryl, aralkyl eller substitueret aralkyl;

hvert Z_1 er en binding, $CR_{17}R_{17}$, O, S, NR', $CR_{17}R_{17}$-$CR_{17}R_{17}$, $CR_{17}R_{17}$-O, O-$CR_{17}R_{17}$, $CR_{17}R_{17}$-S, S-$CR_{17}R_{17}$, $CR_{17}R_{17}$-NR' eller NR'-$CR_{17}R_{17}$;

hvert R' er H, alkyl eller substitueret alkyl;

hvert Z_2 er valgt fra gruppen bestående af en binding, -C(O)-, -C(S)-, eventuelt substitueret C_1-C_3-alkylen, eventuelt substitueret C_1-C_3-alkenylen, og eventuelt substitueret heteroalkyl;

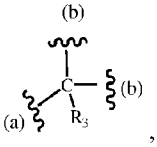
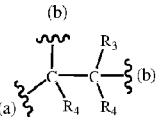
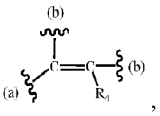
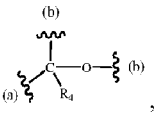
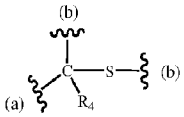
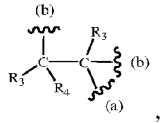
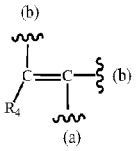
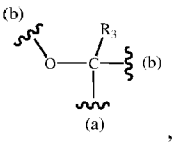
hvert Z_3 er uafhængigt valgt fra gruppen bestående af en binding, eventuelt substitueret C_1-C_4-alkylen, eventuelt substitueret C_1-C_4-alkenylen, eventuelt substitueret heteroalkyl, -O-, -S-, -C(O)-, -C(S)- og -N(R')-;

hvert T_3 er en binding, C(R"')(R"'), O eller S; med det forbehold at når T_3 er O eller S, kan R'" ikke være halogen;

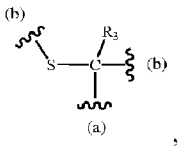
hvert R"' er H, halogen, alkyl, substitueret alkyl, cycloalkyl eller substitueret cycloalkyl;

m og p er 0, 1, 2 eller 3, forudsat at mindst en af m eller p ikke er 0;

M_2 er

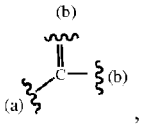
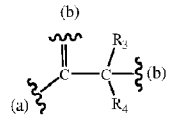
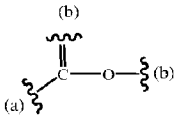
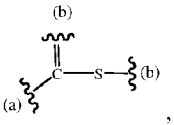









eller

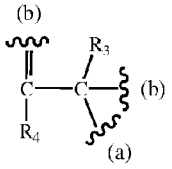


hvor (a) indikerer binding til B-gruppen, og (b) indikerer binding til respektive positioner inden i heterocyclegruppen;

M_3 er

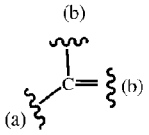
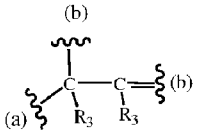
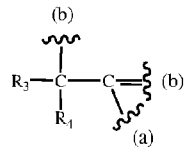
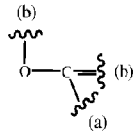





eller

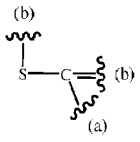


hvor (a) indikerer binding til B-gruppen, og (b) indikerer binding til respektive positioner inden i heterocyclegruppen;

M_4 er

eller



hvor (a) indikerer binding til B-gruppen, og (b) indikerer binding til respektive positioner inden i heterocyclegruppen;
hvert R_{19} er uafhængigt valgt fra gruppen bestående af C_1-C_6-alkyl, C_1-C_6-alkoxy, ester, ether, thioether, aminoalkyl, halogen, alkylester, arylester, amid, arylamid, alkylhalogenid, alkylamin, alkylsulfonsyre, alkylnitro, thioester, sulfonylester, halogensulfonyl, nitril, alkylnitril og nitro;
q er 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 eller 11; og
hvert R_{16} er uafhængigt valgt fra gruppen bestående af hydrogen, halogen, alkyl, NO_2, CN og substitueret alkyl;
hvor alkyl betegner et ligekædet eller forgrenet, eller cyklisk carbonhydrid-radikal, eller kombination deraf, som kan være fuldstændigt mættet, mono- eller polyumættet og kan inkludere di- og multivalente radikaler og også inkluderer heteroalkyl og halogenalkyl;
hvor alkylen inkluderer heteroalkylen;
hvor arylen betegner et divalent arylradikal, hvor aryl inkluderer heteroaryl.

4. Forbindelsen ifølge krav 1, hvor R_5 er thiazol.

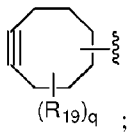
5. Forbindelsen ifølge krav 1, hvor R_6 er H.

6. Forbindelsen ifølge krav 1, hvor Ar er phenyl.

7. Forbindelsen ifølge krav 1, hvor R_7 er methyl.

8. Forbindelsen ifølge krav 1, hvor n er et heltal fra 0 til 20,
for eksempel hvor n er et heltal fra 0 til 10,
for eksempel hvor n er et heltal fra 0 til 5.

9. Forbindelsen ifølge krav 1, hvor Y er cyclooctyn,
for eksempel hvor cyclooctynen har strukturen af:



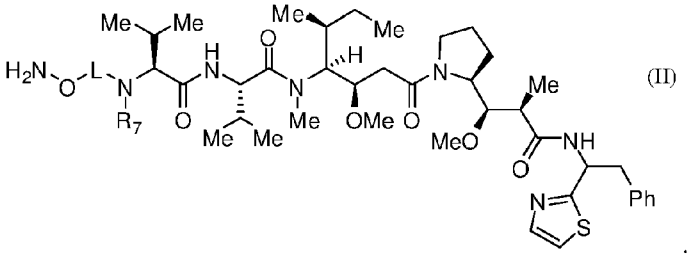
;

hvert R_{19} er uafhængigt valgt fra gruppen bestående af C_1-C_6-alkyl, C_1-C_6-alkoxy, ester, ether, thioether, aminoalkyl, halogen, alkylester, arylester, amid, arylamid, alkylhalogenid, alkylamin, alkylsulfonsyre, alkylnitro, thioester, sulfonylester, halogensulfonyl, nitril, alkylnitril og nitro; og

q er 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 eller 11;

eller hvor Y er azid.

10. Forbindelsen ifølge krav 1, omfattende formel (II):



(II)

eventuelt hvor L er -(alkylen-O)$_n$-alkylen-,

for eksempel hvor hvert alkylen er -CH_2CH_2-, n er lig med 3, og R_7 er methyl;

eller eventuelt hvor L er -alkylen,

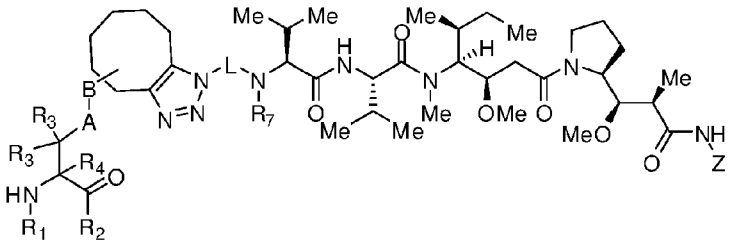
for eksempel hvor hvert alkylen er -CH_2CH_2-, og R_7 er methyl eller hydrogen.

11. Forbindelsen ifølge krav 2 eller 3, hvor R_1 er et polypeptid, og/eller R_2 er et polypeptid.

12. Forbindelsen ifølge krav 11, hvor polypeptidet er et antistof.

13. Forbindelsen ifølge krav 12, hvor antistoffet er trastuzumab.

14. Forbindelsen ifølge krav 3, omfattende formel (XXXI-A):



(XXXI-A)

15. Farmaceutisk sammensætning omfattende en forbindelse ifølge et hvilket som helst af kravene 1-14 og en farmaceutisk acceptabel bærer, et hjælpestof eller bindemiddel.

16. Forbindelsen ifølge krav 1, hvor Y er hydroxylamin, L er -(alkylen-O)$_n$-alkylen-, hver alkylen er -CH_2CH_2-, n er lig med 3, R_7 er methyl, R_5 er COR_8, R_8 er OH, R_6 er H, og Ar er phenyl.

DRAWINGS

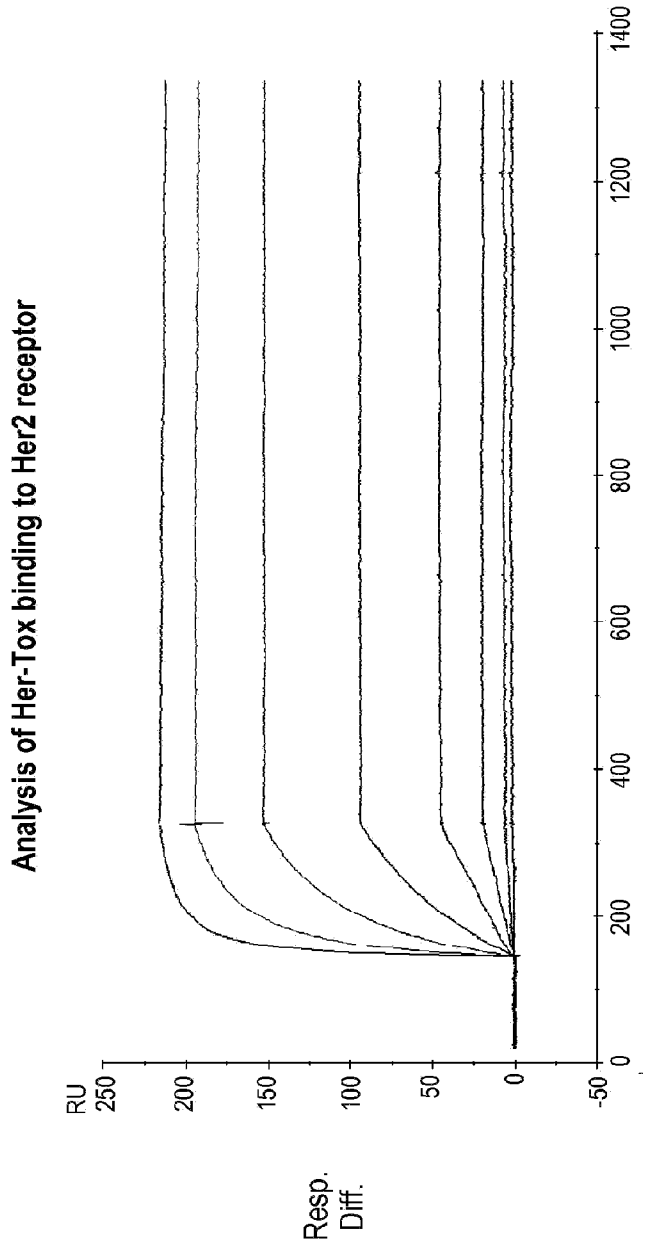


FIG. 1

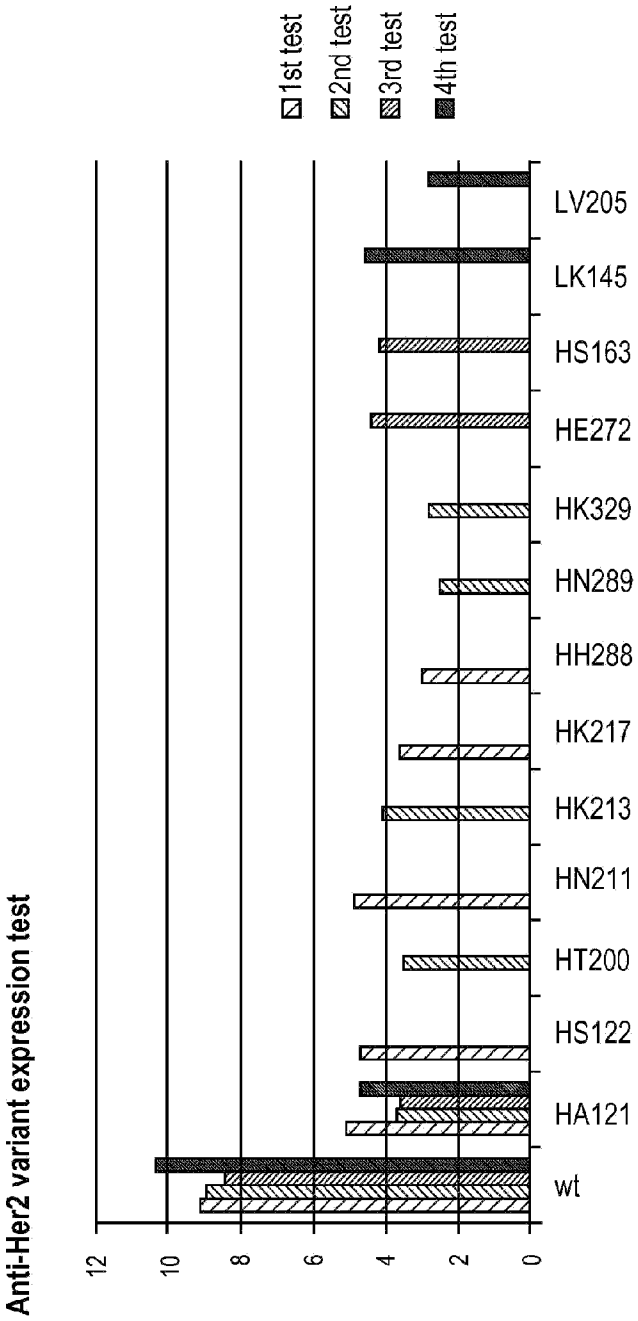

1st test
2nd test
3rd test
4th test
LV205
LK145
HS163
HE272
HK329
HN289
HH288
HK217
HK213
HN211
HT200
HS122
HA121
wt
12
10
8
6
4
2
0
Anti-Her2 variant expression test

FIG. 2

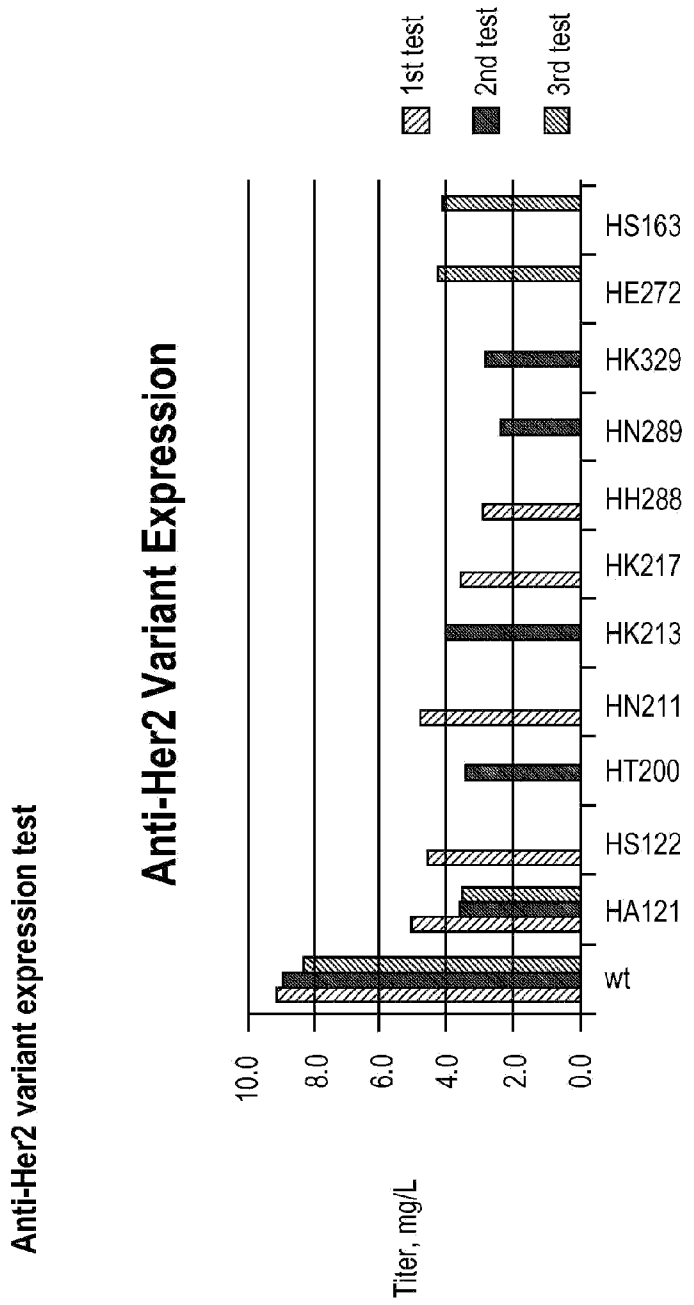

Anti-Her2 variant expression test
Anti-Her2 Variant Expression
1st test
2nd test
3rd test
HS163
HE272
HK329
HN289
HH288
HK217
HK213
HN211
HT200
HS122
HA121
wt
10.0
8.0
6.0
4.0
2.0
0.0
Titer, mg/L

FIG. 3

Proliferation Assay with HCC 1954 Breast Cancer Line and Dolastatin Linker Derivative

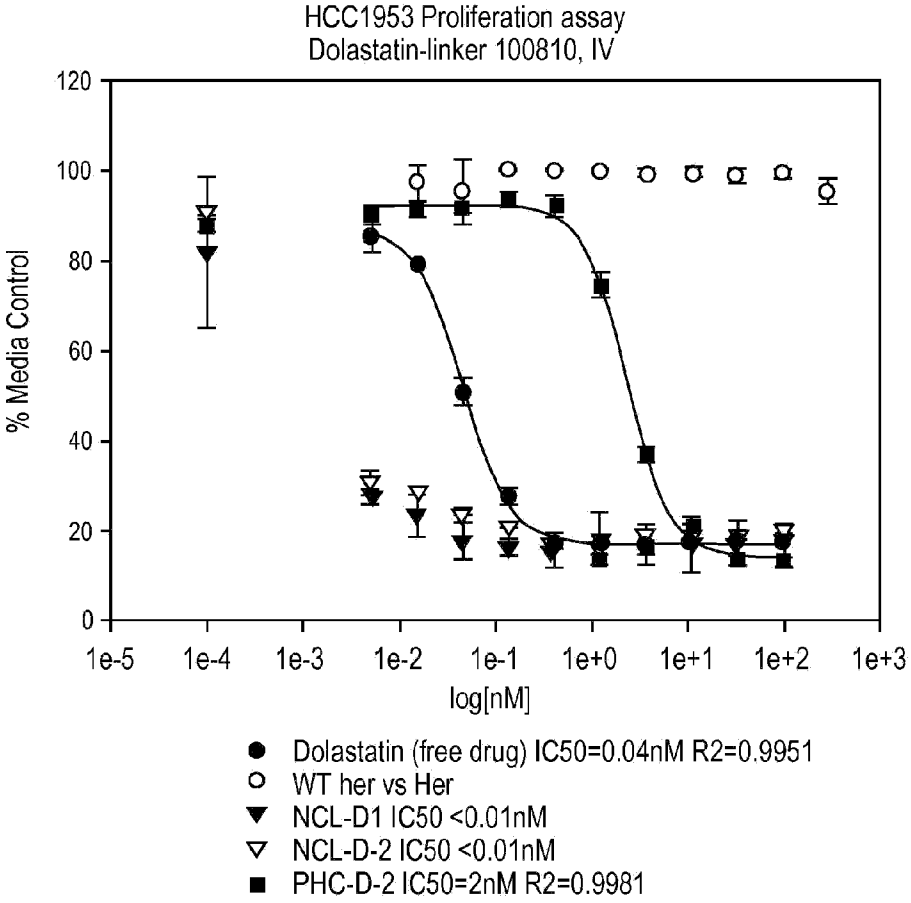


FIG. 4

Proliferation Assay with HCC 1954 Breast Cancer Line and Her-tox conjugates

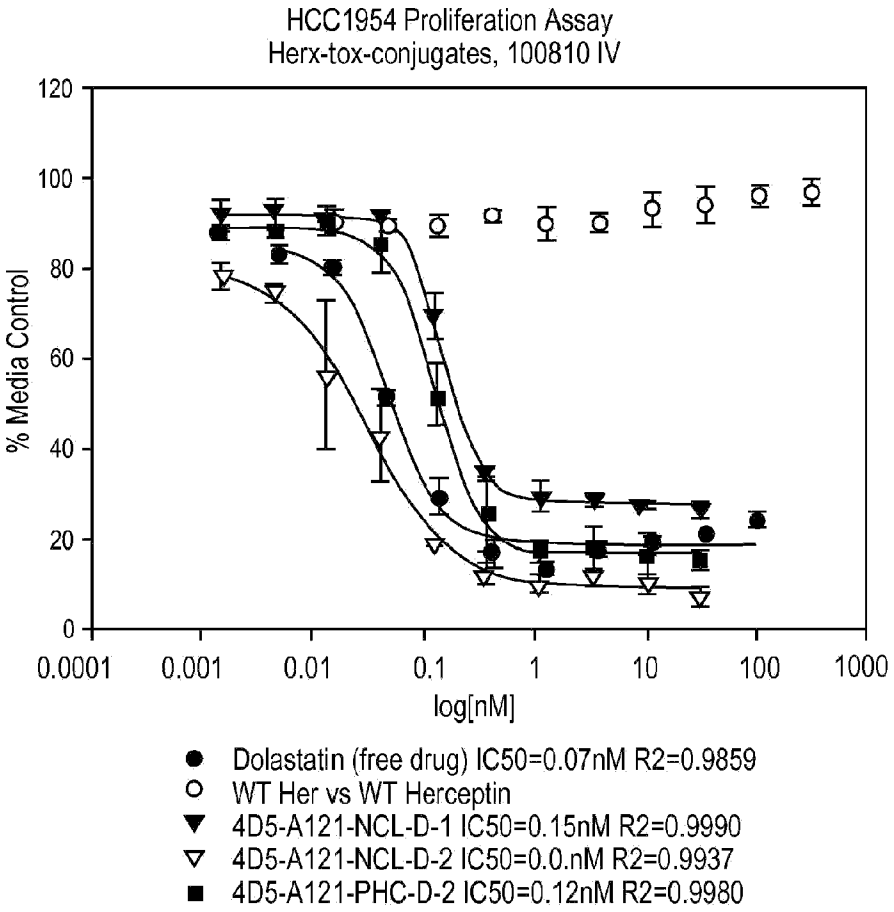


FIG. 5

Proliferation Assay with SKOV-3 Ovarian Cancer Line and Dolastatin Linker Derivatives

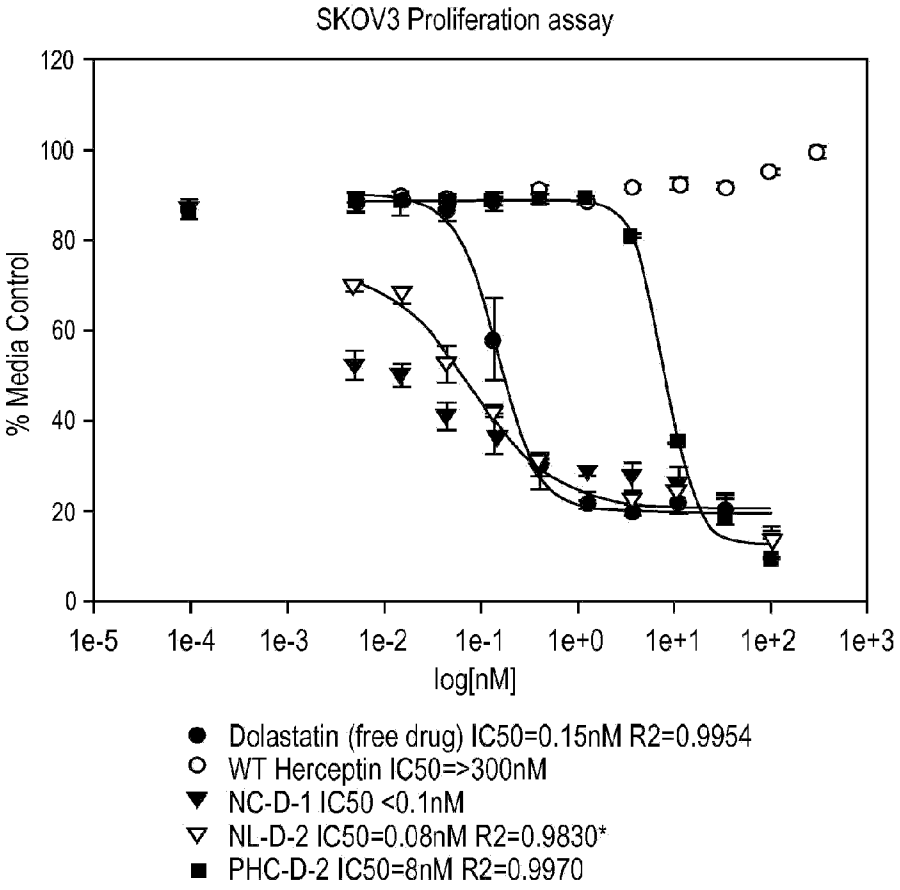


FIG. 6

Proliferation Assay with SKOV-3 Ovarian Cancer Line and Her-tox conjugates

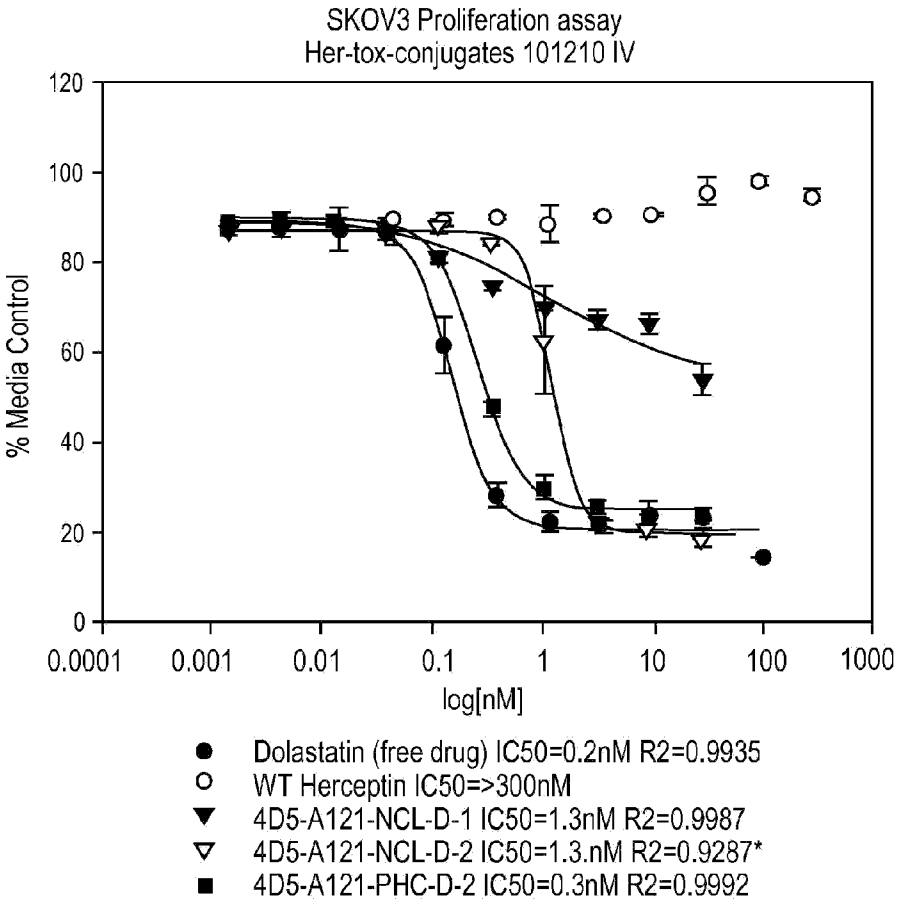


FIG. 7

Proliferation Assay with MDA-MB-468 Breast Cancer Line and Dolastatin Linker Derivatives

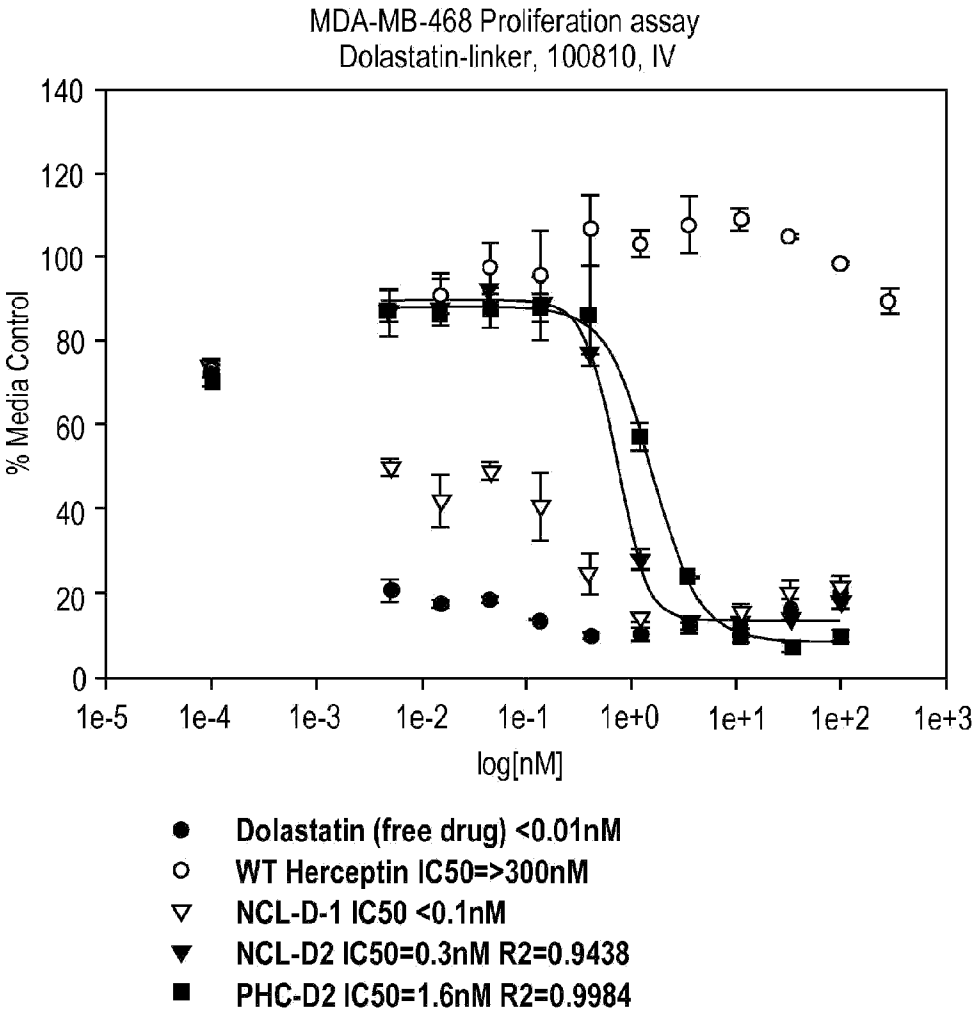


FIG. 8

Proliferation Assay with MDA-MB-468 Breast Cancer Line and Her-tox Conjugates

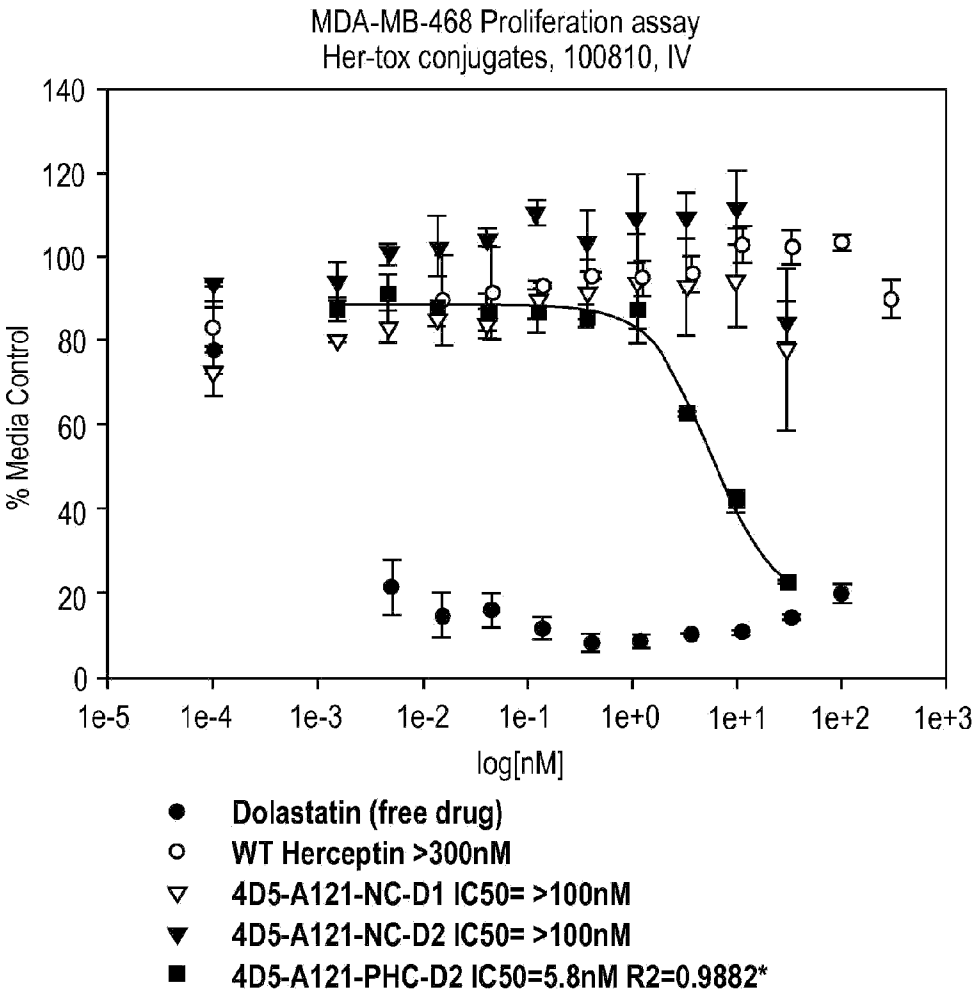


FIG. 9

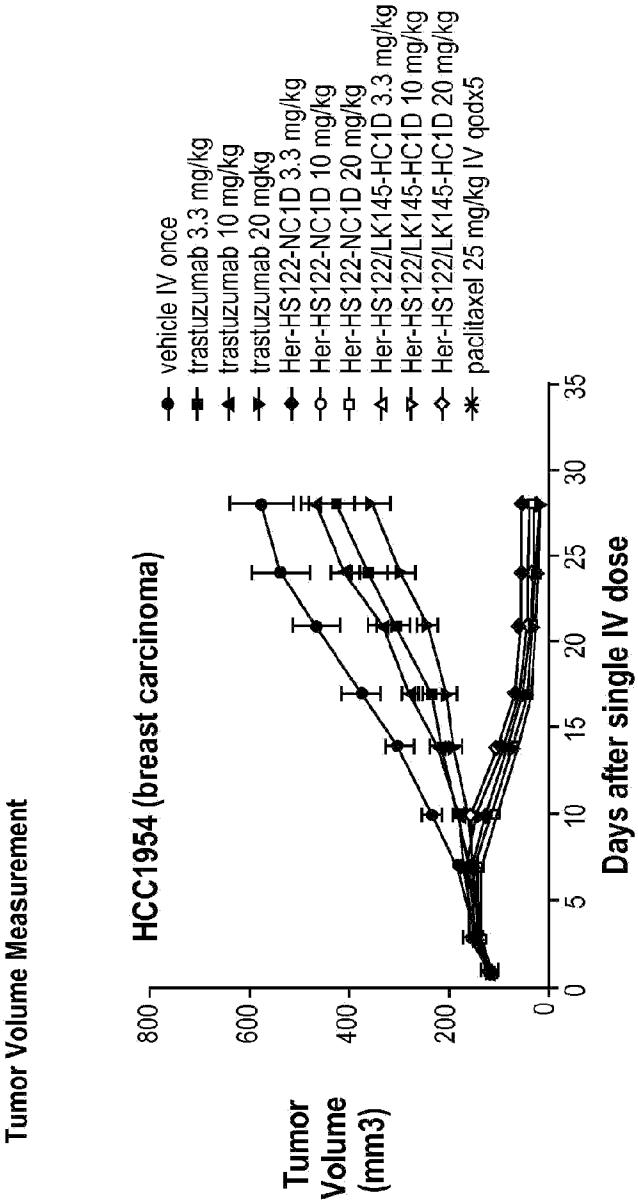
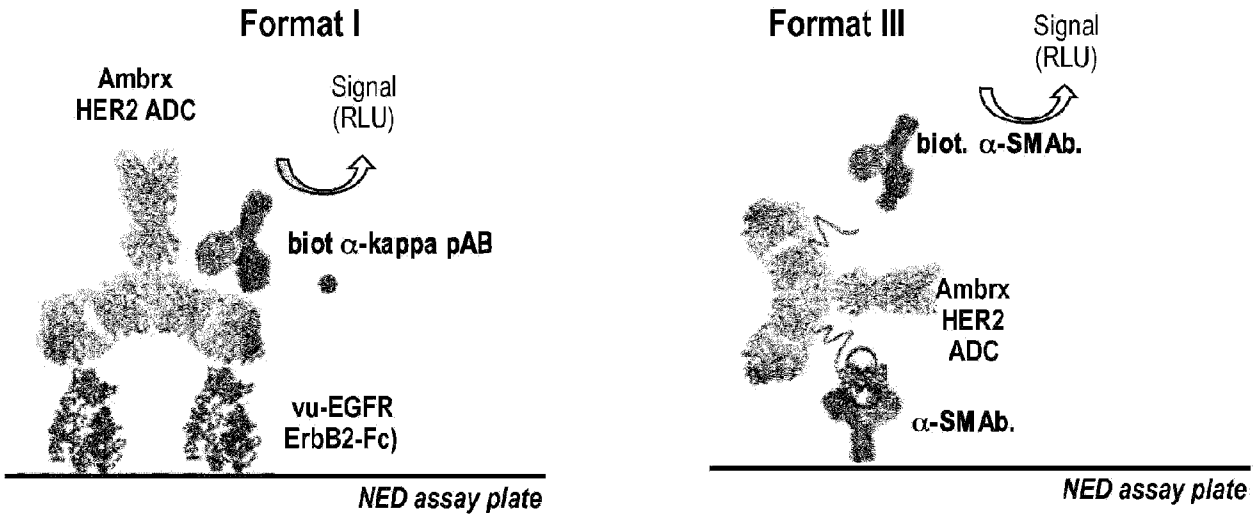

Tumor Volume Measurement
HCC1954 (breast carcinoma)
vehicle IV once
trastuzumab 3.3 mg/kg
trastuzumab 10 mg/kg
trastuzumab 20 mgkg
Her-HS122-NC1D 3.3 mg/kg
Her-HS122-NC1D 10 mg/kg
Her-HS122-NC1D 20 mg/kg
Her-HS122/LK145-HC1D 3.3 mg/kg
Her-HS122/LK145-HC1D 10 mg/kg
Her-HS122/LK145-HC1D 20 mg/kg
paclitaxel 25 mg/kg IV qodx5
Tumor Volume (mm3)
800
600
400
200
0
0
5
10
15
20
25
30
35
Days after single IV dose

FIG. 10

Assay Formats Used to Measure HER2-linked Dolastatin Derivative Concentration in SD Rat Serum

Small Molecule(SM)- Dolastatin



Measures (**non-selectively**) total levels of HER-2 antibody in animal matrix (non-selective)

Application:
- PK profile for HER2 +/- SM
  - *Non conjugated Ab.*
  - *Conjugated Ab.*

Measures (**selectively**) levels of intact HER2-ADC with two or more payloads per drug

Application:
- PK profile for all Ambrx ADC *(+SM)*
- Assessment of linker stability
- Conformatory assay for Format I

FIG. 11

DK/EP 3170821 T3

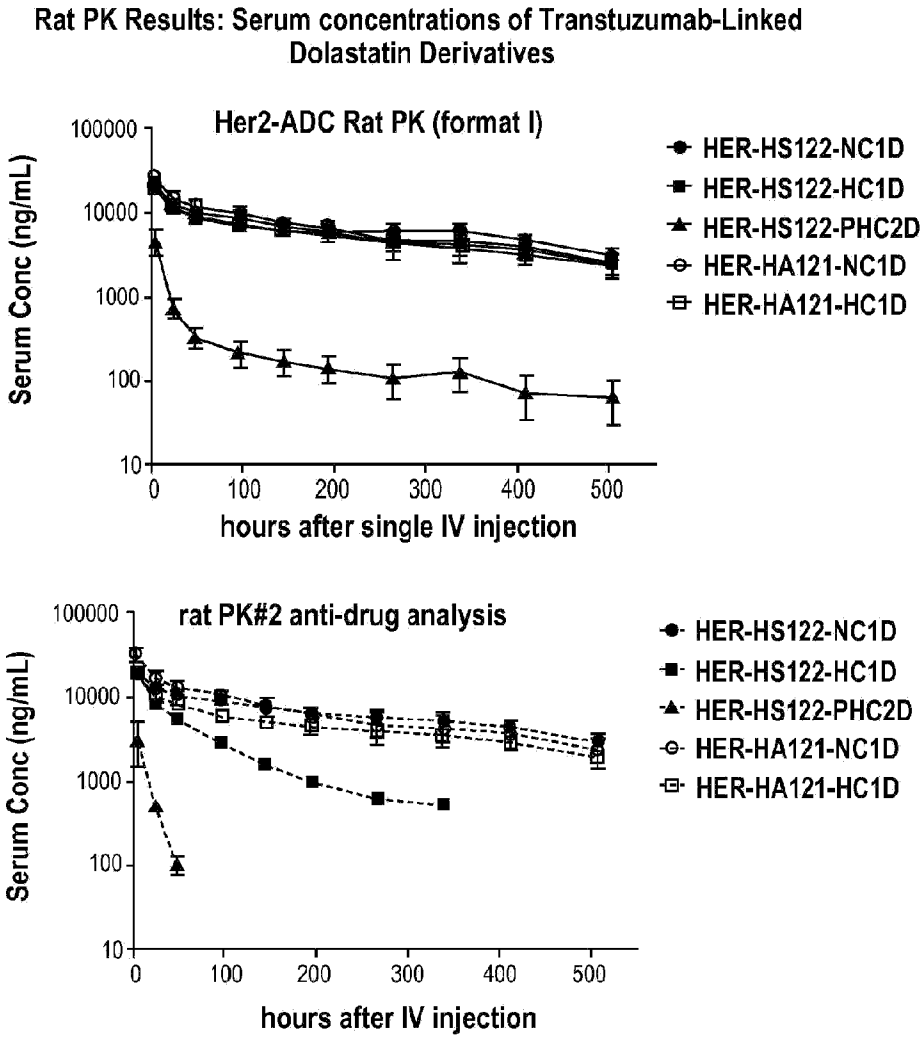

Rat PK Results: Serum concentrations of Transtuzumab-Linked Dolastatin Derivatives
Her2-ADC Rat PK (format I)
Serum Conc (ng/mL)
100000
10000
1000
100
10
0
100
200
300
400
500
hours after single IV injection
HER-HS122-NC1D
HER-HS122-HC1D
HER-HS122-PHC2D
HER-HA121-NC1D
HER-HA121-HC1D
rat PK#2 anti-drug analysis
Serum Conc (ng/mL)
hours after IV injection
HER-HS122-NC1D
HER-HS122-HC1D
HER-HS122-PHC2D
HER-HA121-NC1D
HER-HA121-HC1D

FIG. 12

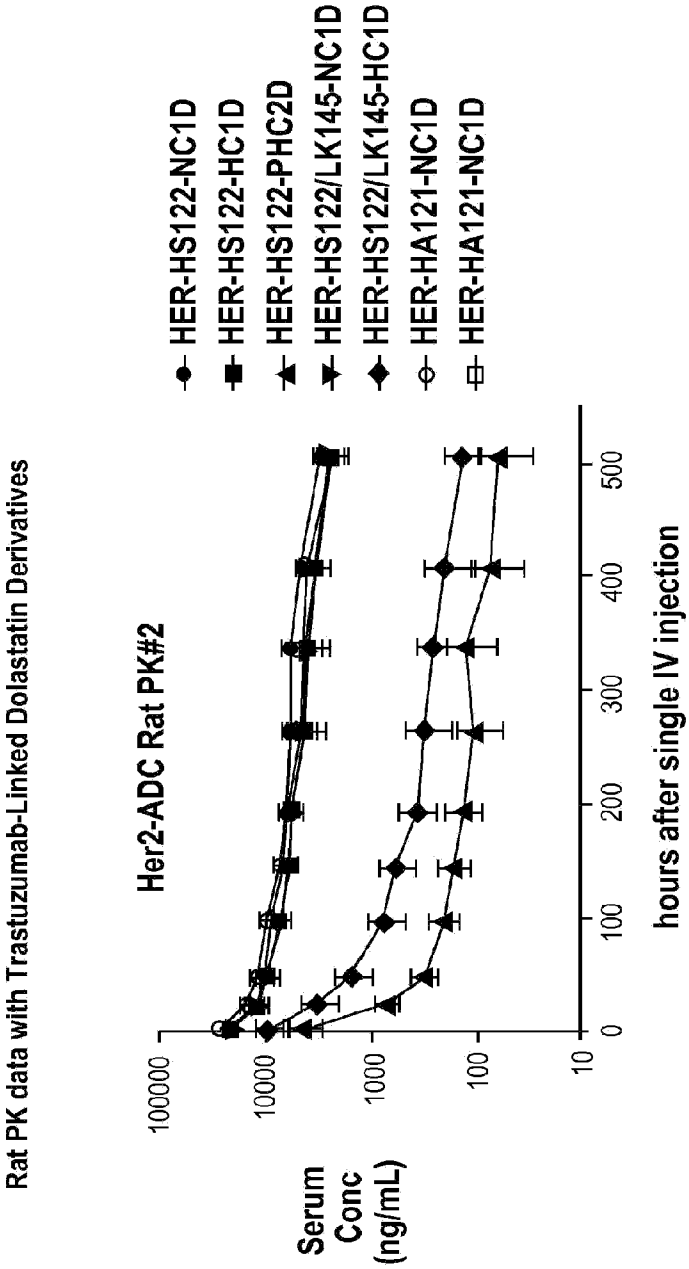

Rat PK data with Trastuzumab-Linked Dolastatin Derivatives
Her2-ADC Rat PK#2
HER-HS122-NC1D
HER-HS122-HC1D
HER-HS122-PHC2D
HER-HS122/LK145-NC1D
HER-HS122/LK145-HC1D
HER-HA121-NC1D
HER-HA121-NC1D
Serum Conc (ng/mL)
100000
10000
1000
100
10
0
100
200
300
400
500
hours after single IV injection

FIG. 13

In vivo stability measurements detecting at least two dolastatin derivatives linked to transtuzumab

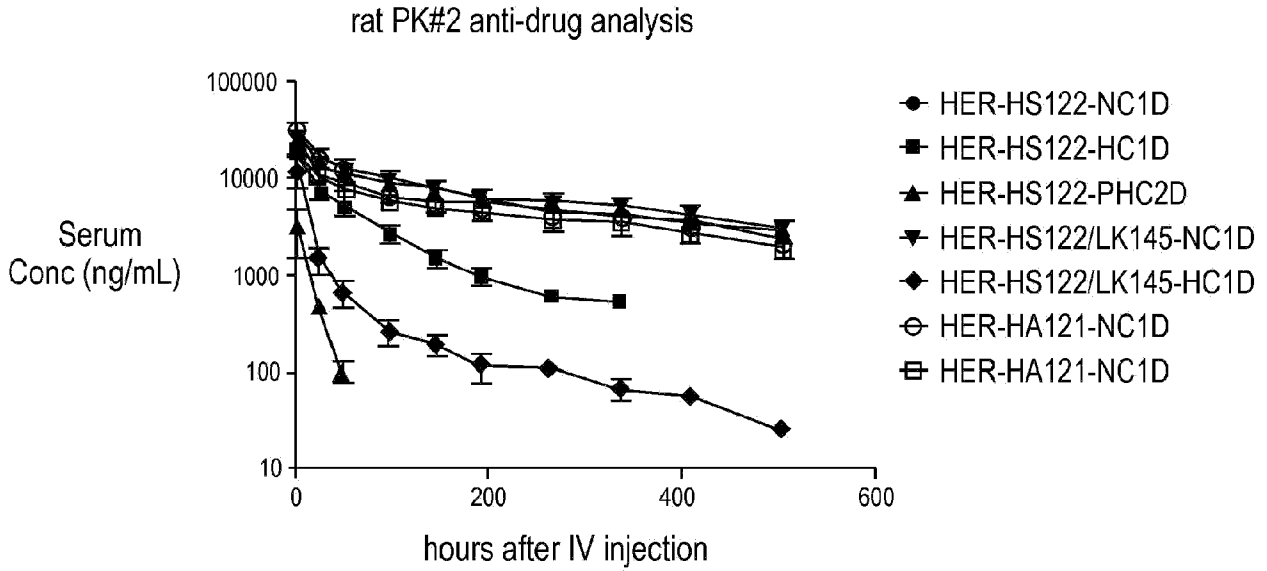


FIG. 14

DK/EP 3170821 T3

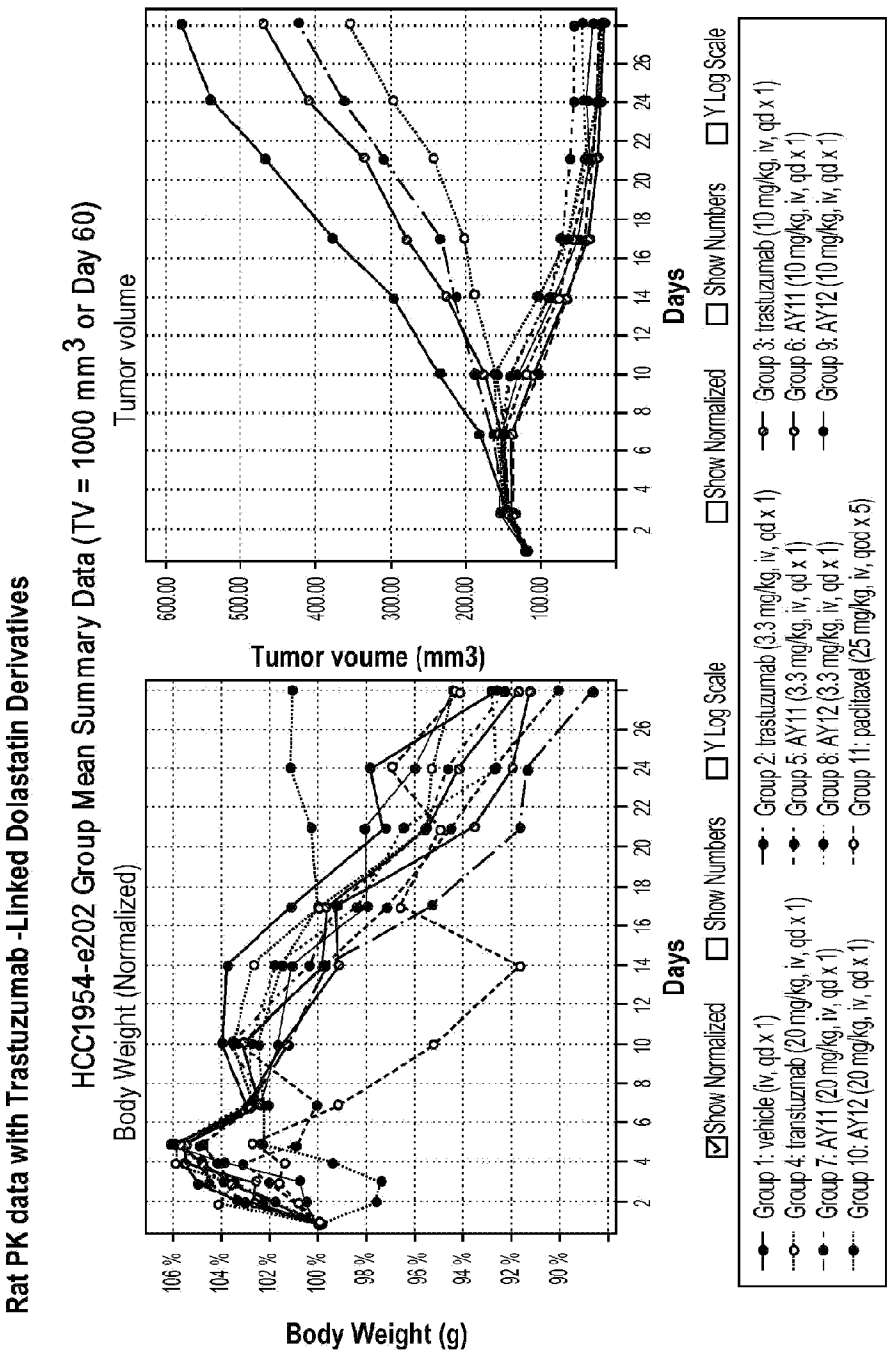

Rat PK data with Trastuzumab -Linked Dolastatin Derivatives
HCC1954-e202 Group Mean Summary Data (TV = 1000 mm3 or Day 60)
Body Weight (Normalized)
Body Weight (g)
Tumor volume
Tumor voume (mm3)
Days
Show Normalized
Show Numbers
Y Log Scale
Group 1: vehicle (iv, qd x 1)
Group 2: trastuzumab (3.3 mg/kg, iv, qd x 1)
Group 3: trastuzumab (10 mg/kg, iv, qd x 1)
Group 4: trastuzumab (20 mg/kg, iv, qd x 1)
Group 5: AY11 (3.3 mg/kg, iv, qd x 1)
Group 6: AY11 (10 mg/kg, iv, qd x 1)
Group 7: AY11 (20 mg/kg, iv, qd x 1)
Group 8: AY12 (3.3 mg/kg, iv, qd x 1)
Group 9: AY12 (10 mg/kg, iv, qd x 1)
Group 10: AY12 (20 mg/kg, iv, qd x 1)
Group 11: paclitaxel (25 mg/kg, iv, qod x 5)

FIG. 15

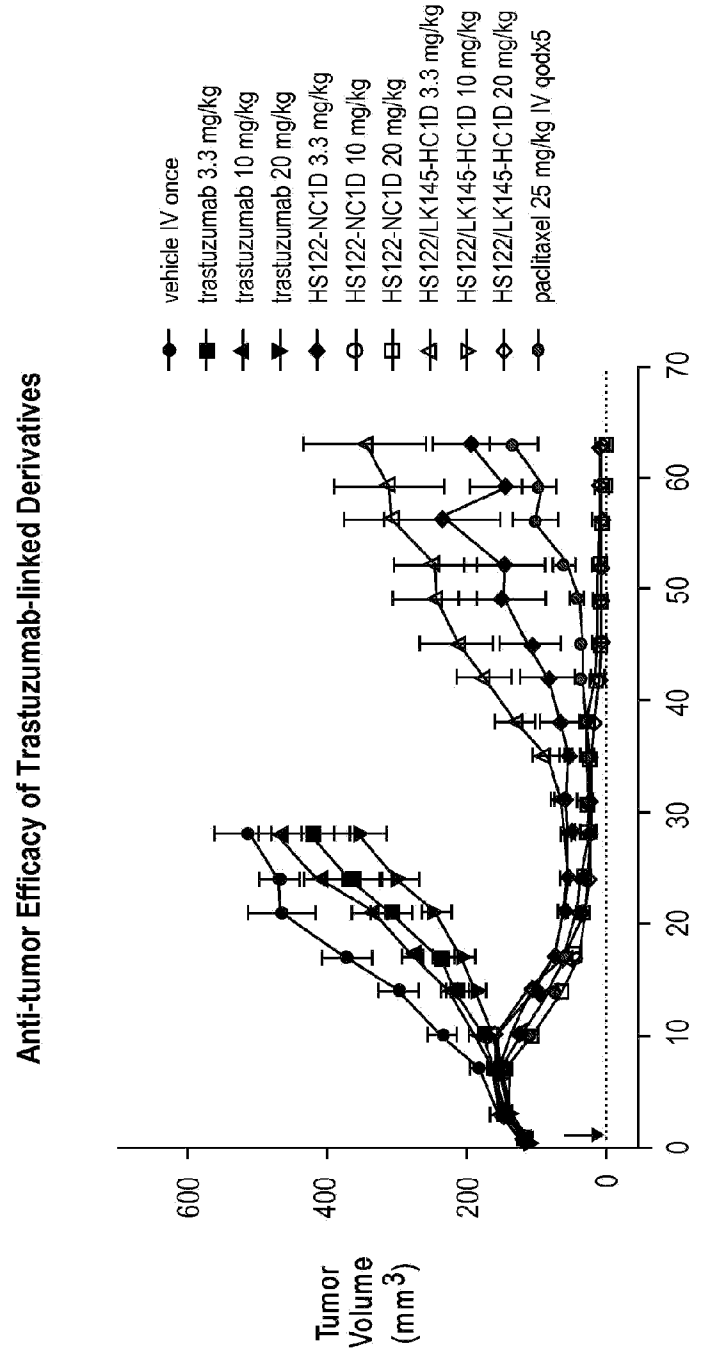

Anti-tumor Efficacy of Trastuzumab-linked Derivatives
vehicle IV once
trastuzumab 3.3 mg/kg
trastuzumab 10 mg/kg
trastuzumab 20 mg/kg
HS122-NC1D 3.3 mg/kg
HS122-NC1D 10 mg/kg
HS122-NC1D 20 mg/kg
HS122/LK145-HC1D 3.3 mg/kg
HS122/LK145-HC1D 10 mg/kg
HS122/LK145-HC1D 20 mg/kg
paclitaxel 25 mg/kg IV qodx5
Tumor Volume (mm3)
600
400
200
0
0
10
20
30
40
50
60
70
Days after single IV dose at day 1

FIG. 16

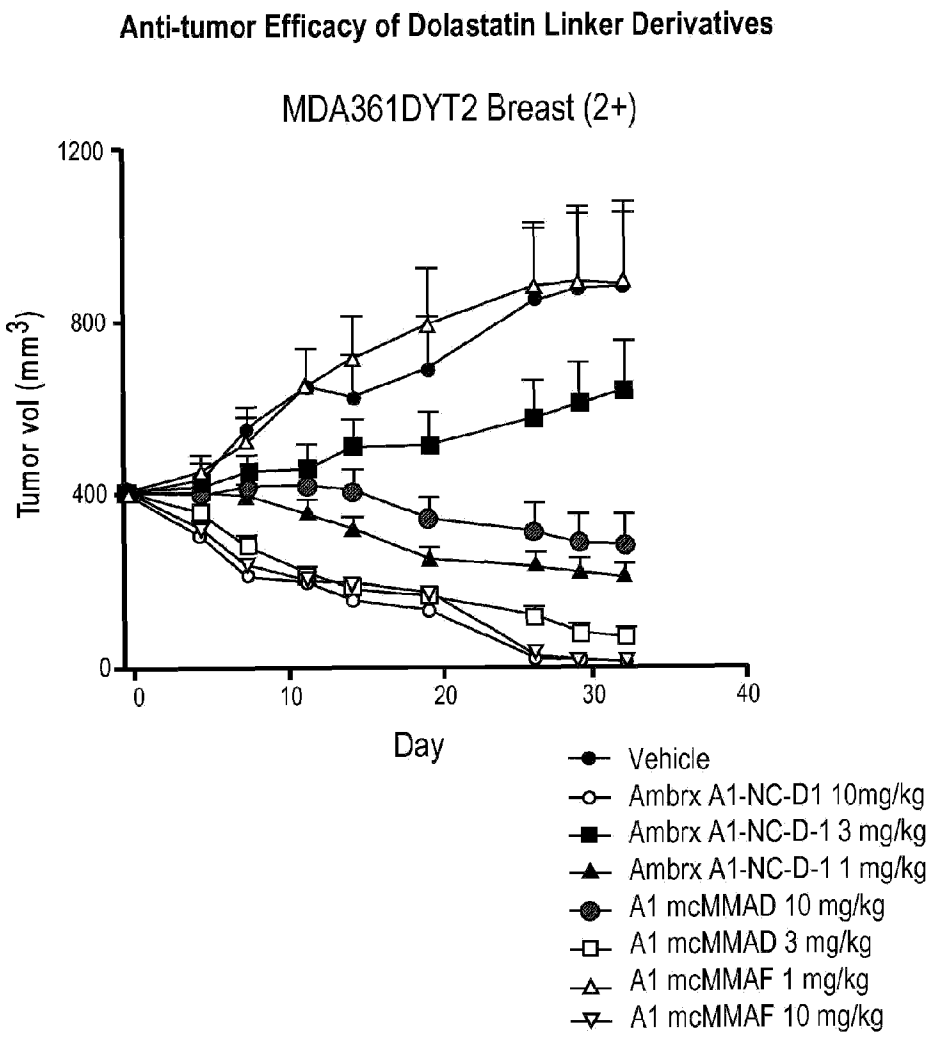

Anti-tumor Efficacy of Dolastatin Linker Derivatives
MDA361DYT2 Breast (2+)
Tumor vol (mm3)
1200
800
400
0
0
10
20
30
40
Day
Vehicle
Ambrx A1-NC-D1 10mg/kg
Ambrx A1-NC-D-1 3 mg/kg
Ambrx A1-NC-D-1 1 mg/kg
A1 mcMMAD 10 mg/kg
A1 mcMMAD 3 mg/kg
A1 mcMMAF 1 mg/kg
A1 mcMMAF 10 mg/kg

FIG. 17

Anti-tumor Efficacy of Dolastatin Linker Derivatives

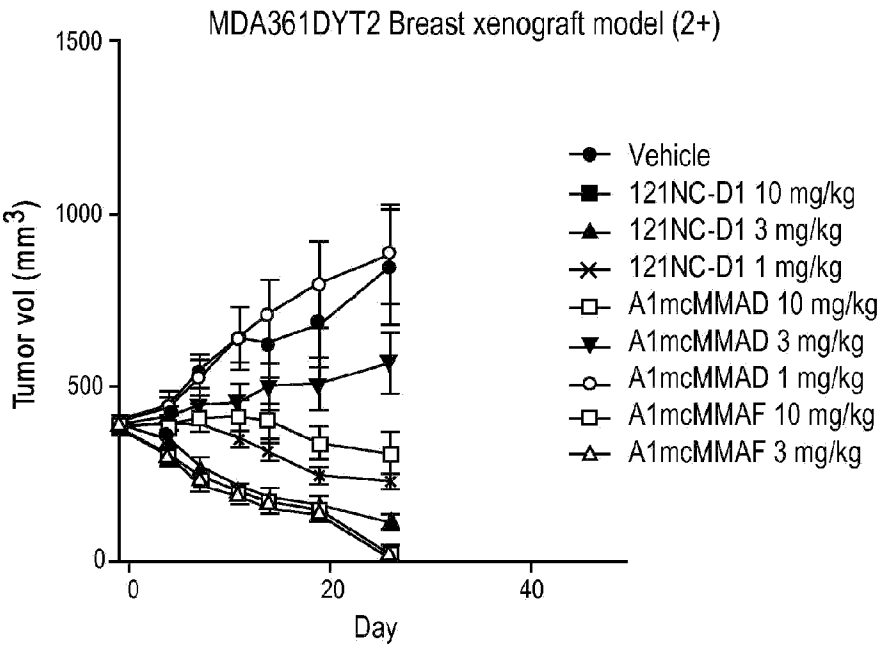


FIG. 18