ANTIPROLIFERATIVE COMPOUNDS, CONJUGATES THEREOF, METHODS THEREFOR, AND USES THEREOF

BACKGROUND OF THE INVENTION

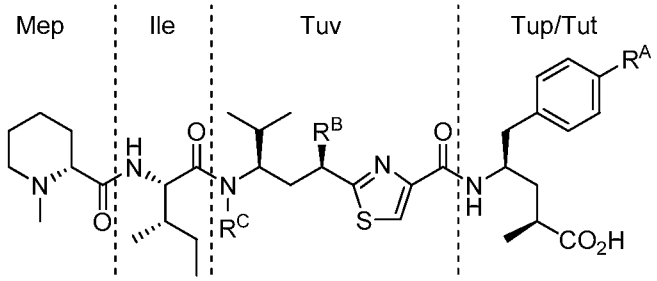
[0001] This invention relates to compounds structurally related to the tubulysins, conjugates thereof with a ligand, methods for making and using such compounds and conjugates, and compositions comprising such compounds and conjugates.

[0002] The tubulysins are cytotoxins originally isolated from cultures of the myxobacteria *Archangium gephyra* or *Angiococcus disciformis*, with each organism producing a different mixture of tubulysins (Sasse et al. 2000; Reichenbach et al. 1998). Their crystal structure and biosynthetic pathway have been elucidated (Steinmetz et al. 2004) and their biosynthesis genes have been sequenced (Hoefle et al. 2006b). Pretubulysin, a biosynthetic precursor of the tubulysins, also has been shown to possess significant activity in its own right (Ullrich et al. 2009). (Full citations for the documents cited herein by first author or inventor and year are listed at the end of this specification.)

[0003] The tubulysins belong to a group of naturally occurring antimitotic polypeptides and depsipeptides that includes the phomopsins, the dolastatins, and the cryptophycins (Hamel 2002). Antimitotic agents other than polypeptides or depsipeptides also exist, for example paclitaxel, the maytansines, and the epothilones. During mitosis, a cell's microtubules reorganize to form the mitotic spindle, a process requiring the rapid assembly and disassembly of the microtubule constituent proteins α- and β-tubulin. Antimitotic agents block this process and prevent a cell from undergoing mitosis, although at the molecular level the exact blockage mechanism may differ from one agent to another. The tubulysins prevent the assembly of the tubulins into microtubules, causing the affected cells to accumulate in the G_2/M phase and undergo apoptosis (Khalil et al. 2006). Conversely, paclitaxel effects the same end result by binding to microtubules and preventing their disassembly.

[0004] The tubulysins have a tetrapeptidyl scaffold constructed from one proteinogenic and three non-proteinogenic amino acid subunits: N-methylpipecolinic acid (Mep), isoleucine (Ile), tubuvaline (Tuv), and either tubuphenylalanine (Tup, R^A equals H in formula (I) below) or tubutyrosine (Tut, R^A equals OH). About a dozen naturally

occurring tubulysins (named A, B, etc.) are known, the sites of structural variation among them being at residues R^A, R^B and R^C as shown in Formula (I) and Table 1:



(I)

Table 1 – Naturally Occurring Tubulysins

Tubulysin	R^A	R^B	R^C
A	OH	OC(=O)Me	$CH_2OC(=O)i$-Bu
B	OH	OC(=O)Me	$CH_2OC(=O)n$-Pr
C	OH	OC(=O)Me	$CH_2OC(=O)$Et
D	H	OC(=O)Me	$CH_2OC(=O)i$-Bu
E	H	OC(=O)Me	$CH_2OC(=O)n$-Pr
F	H	OC(=O)Me	$CH_2OC(=O)$Et
G	OH	OC(=O)Me	$CH_2OC(=O)CH=CH_2$
H	H	OC(=O)Me	$CH_2OC(=O)$Me
I	OH	OC(=O)Me	$CH_2OC(=O)$Me
U	H	OC(=O)Me	H
V	H	OH	H
Z	OH	OH	H
Pretubulysin	H	H	Me

[0005] Kaur et al. 2006 studied the antiproliferative properties of tubulysin A and found that it was more potent than other antimitotic agents such as paclitaxel and vinblastine and was active in xenograft assays against a variety of cancer cell lines. Further, tubulysin A induced apoptosis in cancer cells but not normal cells and showed significant potential antiangiogenic properties in *in vitro* assays. The antimitotic properties of other tubulysins have also been evaluated and generally have been found to compare favorably against those of non-tubulysin antimitotic agents (see, e.g., Balasubramanian et al. 2009;

Steinmetz et al. 2004; Wipf et al. 2004). For these reasons, there is considerable interest in the tubulysins as anti-cancer agents (see, e.g., Domling et al. 2005c; Hamel 2002).

[0006] Numerous publications describe efforts directed at the synthesis of tubulysins, including: Balasubramanian et al. 2009; Domling et al. 2006; Hoefle et al. 2003; Neri et al. 2006; Peltier et al. 2006; Sani et al. 2007; Sasse et al. 2007; Shankar et al. 2009; Shibue et al. 2009; and Wipf et al. 2004. Other publications describe structure-activity relationship (SAR) studies, via the preparation and evaluation of tubulysin analogs or derivatives: Balasubramanian et al. 2008 and 2009; Domling 2006; Domling et al. 2005a; Ellman et al. 2009; Hoefle et al. 2001 & 2006a; Patterson et al. 2007 & 2008; Richter 2008; Vlahov et al. 2009; Wang et al. 2007; and Wipf et al. 2007 and 2010. The SAR studies mainly explored structural variations in the Mep ring, residues R^B and R^C of the Tuv subunit, and the aromatic ring or aliphatic carbon chain of the Tup/Tut subunit.

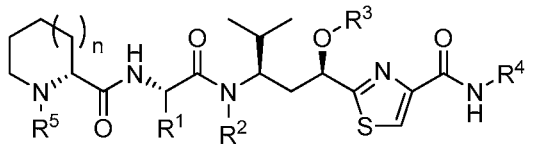
[0007] Domling et al. 2005 disclose conjugates of tubulysins with a partner molecule generically described as a polymer or a biomolecule, but with actual examples limited to polyethylene glycol (PEG) as the partner molecule. Other documents disclosing conjugates of tubulysins are Boyd et al. 2008 and 2010; Vlahov et al. 2008a, 2008b and 2010; Leamon et al. 2008 and 2009; Reddy et al. 2009; and Low et al. 2009. Leung et al. 2002 disclose polyanionic polypeptides that can be conjugated to drugs (including tubulysins) to improve their bioactivity and water solubility.

[0008] Davis et al. 2008 and Schluep et al. 2009 disclose cyclodextrin based formulations in which tubulysins are covalently attached to a cyclodextrin via a hydrazide-disulfide linker moiety bonded to the Tup/Tut carboxyl group.

BRIEF SUMMARY OF THE INVENTION

[0009] The present invention discloses novel antiproliferative compounds that are structurally related to the tubulysins, are cytotoxic or cytostatic against many cancer cells, and are believed to act by an antimitotic mechanism. These compounds can be conjugated to ligands such as antibodies for targeted delivery against cancer cells.

[0010] In one embodiment, this invention provides a compound having a structure represented by formula (II)



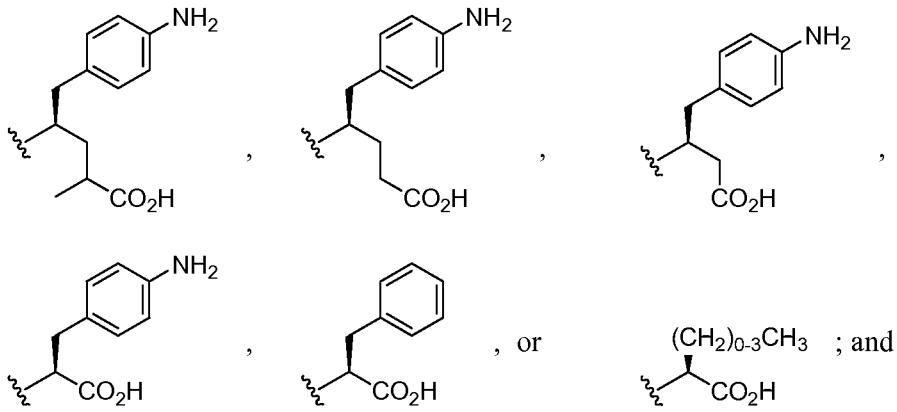
(II)

wherein

n is 0, 1, or 2;

R^1, R^2 and R^3 are independently H, unsubstituted or substituted C_1-C_{10} alkyl, unsubstituted or substituted C_2-C_{10} alkenyl, unsubstituted or substituted C_2-C_{10} alkynyl, unsubstituted or substituted aryl, unsubstituted or substituted heteroaryl, unsubstituted or substituted $(CH_2)_{1\text{-}2}O(C_1\text{-}C_{10}$ alkyl), unsubstituted or substituted $(CH_2)_{1\text{-}2}O(C_2\text{-}C_{10}$ alkenyl), unsubstituted or substituted $(CH_2)_{1\text{-}2}O(C_2\text{-}C_{10}$ alkynyl), $(CH_2)_{1\text{-}2}OC(=O)(C_1\text{-}C_{10}$ alkyl), unsubstituted or substituted $(CH_2)_{1\text{-}2}OC(=O)(C_2\text{-}C_{10}$ alkenyl), unsubstituted or substituted $(CH_2)_{1\text{-}2}OC(=O)(C_2\text{-}C_{10}$ alkynyl), unsubstituted or substituted $C(=O)(C_1\text{-}C_{10}$ alkyl), unsubstituted or substituted $C(=O)(C_2\text{-}C_{10}$ alkenyl), unsubstituted or substituted $C(=O)(C_2\text{-}C_{10}$ alkynyl), unsubstituted or substituted cycloaliphatic, unsubstituted or substituted heterocycloaliphatic, unsubstituted or substituted arylalkyl, or unsubstituted or substituted alkylaryl;

R^4 is

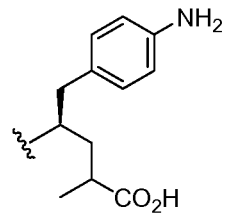


; and

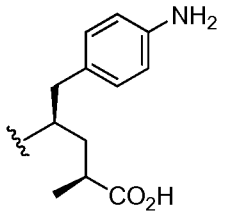
R^5 is H, C_1-C_5 alkyl, C_2-C_5 alkenyl, C_2-C_5 alkynyl, $CO(C_1\text{-}C_5$ alkyl), $CO(C_2\text{-}C_5$ alkenyl), or $CO(C_2\text{-}C_5$ alkynyl);

or a pharmaceutically acceptable ester thereof, a pharmaceutically acceptable amide thereof at the carboxyl group of R^4 with the α-amino group of an α-amino acid, or a pharmaceutically acceptable salt thereof.

[0011] A preferred R^4 is



with the stereochemistry at the methyl group alpha to the carboxyl being more preferably that corresponding to the natural tubulysins, that is:



[0012] This invention also provides novel intermediates useful for synthesizing compounds according to formula (II).

[0013] In another embodiment, this invention provides a compound of this invention conjugated via a linker moiety to a ligand (preferably an antibody, more preferably a monoclonal antibody, and most preferably a human monoclonal antibody) for its selective delivery to a target cell such as a cancer cell.

[0014] In another embodiment, there is provided a composition of matter comprising a compound of this invention and a linker moiety, suitable for conjugation to a ligand.

[0015] In another embodiment, this invention provides a method for inhibiting the proliferation of cancer cells in a subject suffering from cancer, comprising administering to the subject a therapeutically effective amount of a compound of this invention or a conjugate thereof with a ligand (particularly an antibody). In another embodiment, there is provided a method for inhibiting the proliferation of cancer cells, comprising contacting such cells with a compound of this invention or a conjugate thereof with a ligand (particularly an antibody), under conditions sufficient to inhibit the growth of such cancer cells. The cancer cells can be colorectal cancer, liver cancer, prostate cancer, breast cancer,

melanoma, glioblastoma, lung cancer, pancreatic cancer, ovarian cancer, multiple myeloma, renal cancer, leukemia, or lymphoma cells. Where the ligand is an antibody, it is preferred that the antibody binds to an antigen expressed by the cancer cells.

[0016] In another embodiment, there is provided a method of treating a cancer in a subject suffering from such cancer, comprising administering to the subject a therapeutically effective amount of a compound of this invention or a conjugate thereof with a ligand (particularly an antibody). In another embodiment, there is provided the use of a compound of this invention (or a conjugate thereof with a ligand (particularly an antibody) for the preparation of a medicament for the treatment of cancer. In these embodiments, the cancer can be colorectal cancer, liver cancer, prostate cancer, breast cancer, melanoma, glioblastoma, lung cancer, pancreatic cancer, ovarian cancer, multiple myeloma, renal cancer, leukemia, or lymphoma. Where the ligand is an antibody, it is preferred that the antibody binds to an antigen expressed by the cells of the cancer.

[0017] In another embodiment, there is provided the use of a compound of this invention or a conjugate thereof with a ligand (preferably an antibody) for the preparation of a medicament for treating a cancer in a subject suffering from such cancer.

BRIEF DESCRIPTION OF THE DRAWING(S)

[0018] **Figs. 1a** and **1b** depict, in combination, Scheme 1 for making compounds of this invention.

[0019] **Figs. 2** and **3** depict Schemes 2 and 3, respectively, also for making compounds of this invention.

[0020] **Fig. 4** depicts a Scheme 4 suitable for attaching a peptidyl linker and a maleimide reactive group to compounds of this invention.

[0021] **Figs. 5**, **6**, and **7** depict Schemes 5, 6, and 7, respectively, for making compounds of this invention.

[0022] **Figs. 8a**, **8b**, and **8c** show Schemes 8, 9, and 10, respectively, for making intermediates useful for preparing compounds of this invention.

[0023] **Figs. 9** and **10** show Schemes 11 and 12, respectively, illustrating how intermediates such as those shown in **Figs. 8a-8c** can be elaborated into compounds of this invention.

[0024] **Figs. 11a** and **11b** show the plots for ^{3}H thymidine proliferation assays for a first set of compounds of this invention, against two different types of cancer cells.

[0025] **Figs. 12a** and **12b** show the plots for ATP luminescence proliferation assays for a second set of compounds of this invention, against two different types of cancer cells. **Figs. 12c** and **12d** show the plots for ^{3}H thymidine proliferation assays for the same second set of compounds and against the same two types of cancer cells.

[0026] **Fig. 13** shows the activity against renal cancer cells of conjugates of compounds of this invention in ^{3}H thymidine proliferation assays.

[0027] **Fig. 14** shows the activity against renal cancer cells of conjugates of compounds of this invention in xenograft studies.

[0028] **Fig. 15** shows a Scheme 13 for making intermediates useful for making compounds of this invention.

[0029] **Fig. 16** shows a Scheme 14 for making compounds of this invention from intermediates made per Scheme 13.

[0030] **Figs. 17** and **18** show Schemes 15 and 16, respectively, for making conjugation-ready compounds of this invention.

[0031] **Fig. 19** shows Scheme 17 for making an intermediate useful for making compounds of this invention.

[0032] **Figs. 20a** and **20b** show in combination Scheme 18 for the preparation of compounds of this invention from the intermediate of Scheme 17.

[0033] **Fig. 21** shows Scheme 19 for making an intermediate used in Scheme 18.

[0034] **Fig. 22** shows Scheme 20 for the synthesis of an intermediate used in making compounds of this invention.

[0035] **Fig. 23** shows Scheme 21 for making compounds of this invention from the intermediate of Scheme 20.

[0036] **Fig. 24** shows the Scheme 22 for making yet another intermediate useful for making compounds of this invention.

[0037] **Fig. 25** shows Scheme 23 for making compounds of this invention from the intermediate of Scheme 22.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

[0038] "Antibody" means whole antibodies and any antigen binding fragment (*i.e.*, "antigen-binding portion") or single chains thereof. A whole antibody is a glycoprotein

comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain comprises a heavy chain variable region (V_H) and a heavy chain constant region comprising three domains, C_{H1}, C_{H2} and C_{H3}. Each light chain comprises a light chain variable region (V_L or V_k) and a light chain constant region comprising one single domain, C_L. The V_H and V_L regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDRs), interspersed with more conserved framework regions (FRs). Each V_H and V_L comprises three CDRs and four FRs, arranged from amino- to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. The variable regions contain a binding domain that interacts with an antigen. The constant regions may mediate the binding of the antibody to host tissues or factors, including various cells of the immune system (*e.g.*, effector cells) and the first component (Clq) of the classical complement system. An antibody is said to "specifically bind" to an antigen X if the antibody binds to antigen X with a K_D of 5 x 10^{-8} M or less, more preferably 1 x 10^{-8} M or less, more preferably 6 x 10^{-9} M or less, more preferably 3 x 10^{-9} M or less, even more preferably 2 x 10^{-9} M or less. The antibody can be chimeric, humanized, or, preferably, human. The heavy chain constant region can be engineered to affect glycosylation type or extent, to extend antibody half-life, to enhance or reduce interactions with effector cells or the complement system, or to modulate some other property. The engineering can be accomplished by replacement, addition, or deletion of one or more amino acids or by replacement of a domain with a domain from another immunoglobulin type, or a combination of the foregoing.

[0039] "Antibody fragment" and "antigen-binding portion" of an antibody (or simply "antibody portion") mean one or more fragments of an antibody that retain the ability to specifically bind to an antigen. It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody, such as (i) a Fab fragment, a monovalent fragment consisting of the V_L, V_H, C_L and C_{H1} domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fab' fragment, which is essentially an Fab with part of the hinge region (see, for example, Abbas et al., *Cellular and Molecular Immunology*, 6th Ed., Saunders Elsevier 2007); (iv) a Fd fragment consisting of the V_H and C_{H1} domains; (v) a Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody, (vi) a dAb fragment (Ward *et al.*, (1989) *Nature* 341:544-546), which consists of a V_H

domain; (vii) an isolated complementtarity determining region (CDR); and (viii) a nanobody, a heavy chain variable region containing a single variable domain and two constant domains. Furthermore, although the two domains of the Fv fragment, V_L and V_H, are encoded by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules (known as single chain Fv, or scFv); *see, e.g.,* Bird *et al.* (1988) *Science* 242:423-426; and Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883*)*. Such single chain antibodies are also encompassed within the term "antigen-binding portion" of an antibody.

[0040] An "isolated antibody" means an antibody that is substantially free of other antibodies having different antigenic specificities (*e.g.*, an isolated antibody that specifically binds antigen X is substantially free of antibodies that specifically bind antigens other than antigen X). An isolated antibody that specifically binds antigen X may, however, have cross-reactivity to other antigens, such as antigen X molecules from other species. In certain embodiments, an isolated antibody specifically binds to human antigen X and does not cross-react with other (non-human) antigen X antigens. Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals.

[0041] "Monoclonal antibody" or "monoclonal antibody composition" means a preparation of antibody molecules of single molecular composition, which displays a single binding specificity and affinity for a particular epitope.

[0042] "Human antibody" means an antibody having variable regions in which both the framework and CDR regions (and the constant region, if present) are derived from human germline immunoglobulin sequences. Human antibodies may include later modifications, including natural or synthetic modifications. Human antibodies may include amino acid residues not encoded by human germline immunoglobulin sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*). However, "human antibody" does not include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

[0043] "Human monoclonal antibody" means an antibody displaying a single binding specificity, which has variable regions in which both the framework and CDR regions are derived from human germline immunoglobulin sequences. In one embodiment, human

monoclonal antibodies are produced by a hybridoma that includes a B cell obtained from a transgenic nonhuman animal, *e.g.*, a transgenic mouse, having a genome comprising a human heavy chain transgene and a light chain transgene fused to an immortalized cell.

[0044] "Aliphatic" means a straight- or branched-chain, saturated or unsaturated, non-aromatic hydrocarbon moiety having the specified number of carbon atoms (*e.g.*, as in "C_3 aliphatic," "C_1-C_5 aliphatic," or "C_1 to C_5 aliphatic," the latter two phrases being synonymous for an aliphatic moiety having from 1 to 5 carbon atoms) or, where the number of carbon atoms is not explicitly specified, from 1 to 4 carbon atoms (2 to 4 carbons in the instance of unsaturated aliphatic moieties).

[0045] "Alkyl" means a saturated aliphatic moiety, with the same convention for designating the number of carbon atoms being applicable. By way of illustration, C_1-C_4 alkyl moieties include, but are not limited to, methyl, ethyl, propyl, isopropyl, isobutyl, *t*-butyl, 1-butyl, 2-butyl, and the like.

[0046] "Alkenyl" means an aliphatic moiety having at least one carbon-carbon double bond, with the same convention for designating the number of carbon atoms being applicable. By way of illustration, C_2-C_4 alkenyl moieties include, but are not limited to, ethenyl (vinyl), 2-propenyl (allyl or prop-2-enyl), cis-1-propenyl, *trans*-1-propenyl, *E*- (or *Z*-) 2-butenyl, 3-butenyl, 1,3-butadienyl (but-1,3-dienyl) and the like.

[0047] "Alkynyl" means an aliphatic moiety having at least one carbon-carbon triple bond, with the same convention for designating the number of carbon atoms being applicable. By way of illustration, C_2-C_4 alkynyl groups include ethynyl (acetylenyl), propargyl (prop-2-ynyl), 1-propynyl, but-2-ynyl, and the like.

[0048] "Cycloaliphatic" means a saturated or unsaturated, non-aromatic hydrocarbon moiety having from 1 to 3 rings, each ring having from 3 to 8 (preferably from 3 to 6) carbon atoms. "Cycloalkyl" means a cycloaliphatic moiety in which each ring is saturated. "Cycloalkenyl" means a cycloaliphatic moiety in which at least one ring has at least one carbon-carbon double bond. "Cycloalkynyl" means a cycloaliphatic moiety in which at least one ring has at least one carbon-carbon triple bond. By way of illustration, cycloaliphatic moieties include, but are not limited to, cyclopropyl, cyclobutyl, cyclopentyl, cyclopentenyl, cyclohexyl, cyclohexenyl, cycloheptyl, cyclooctyl, and adamantyl. Preferred cycloaliphatic moieties are cycloalkyl ones, especially cyclopropyl, cyclobutyl, cyclopentyl, and cyclohexyl.

[0049] "Heterocycloaliphatic" means a cycloaliphatic moiety wherein, in at least one ring thereof, up to three (preferably 1 to 2) carbons have been replaced with a heteroatom independently selected from N, O, or S, where the N and S optionally may be oxidized and the N optionally may be quaternized. Similarly, "heterocycloalkyl," "heterocycloalkenyl," and "heterocycloalkynyl" means a cycloalkyl, cycloalkenyl, or cycloalkynyl moiety, respectively, in which at least one ring thereof has been so modified. Exemplary heterocycloaliphatic moieties include aziridinyl, azetidinyl, 1,3-dioxanyl, oxetanyl, tetrahydrofuryl, pyrrolidinyl, piperidinyl, piperazinyl, tetrahydropyranyl, tetrahydrothiopyranyl, tetrahydrothiopyranyl sulfone, morpholinyl, thiomorpholinyl, thiomorpholinyl sulfoxide, thiomorpholinyl sulfone, 1,3-dioxolanyl, tetrahydro-1,1-dioxothienyl, 1,4-dioxanyl, thietanyl, and the like.

[0050] "Alkoxy," "aryloxy," "alkylthio," and "arylthio" mean –O(alkyl), -O(aryl), -S(alkyl), and -S(aryl), respectively. Examples are methoxy, phenoxy, methylthio, and phenylthio, respectively.

[0051] "Halogen" or "halo" means fluorine, chlorine, bromine or iodine.

[0052] "Aryl" means a hydrocarbon moiety having a mono-, bi-, or tricyclic ring system wherein each ring has from 3 to 7 carbon atoms and at least one ring is aromatic. The rings in the ring system may be fused to each other (as in naphthyl) or bonded to each other (as in biphenyl) and may be fused or bonded to non-aromatic rings (as in indanyl or cyclohexylphenyl). By way of further illustration, aryl moieties include, but are not limited to, phenyl, naphthyl, tetrahydronaphthyl, indanyl, biphenyl, phenanthryl, anthracenyl, and acenaphthyl.

[0053] "Heteroaryl" means a moiety having a mono-, bi-, or tricyclic ring system wherein each ring has from 3 to 7 carbon atoms and at least one ring is an aromatic ring containing from 1 to 4 heteroatoms independently selected from from N, O, or S, where the N and S optionally may be oxidized and the N optionally may be quaternized. Such at least one heteroatom containing aromatic ring may be fused to other types of rings (as in benzofuranyl or tetrahydroisoquinolyl) or directly bonded to other types of rings (as in phenylpyridyl or 2-cyclopentylpyridyl). By way of further illustration, heteroaryl moieties include pyrrolyl, furanyl, thiophenyl (thienyl), imidazolyl, pyrazolyl, oxazolyl, isoxazolyl, thiazolyl, isothiazolyl, triazolyl, tetrazolyl, pyridyl, N-oxopyridyl, pyridazinyl, pyrimidinyl, pyrazinyl, quinolinyl, isoquinolynyl, quinazolinyl, cinnolinyl, quinozalinyl,

naphthyridinyl, benzofuranyl, indolyl, benzothiophenyl, oxadiazolyl, thiadiazolyl, phenothiazolyl, benzimidazolyl, benzotriazolyl, dibenzofuranyl, carbazolyl, dibenzothiophenyl, acridinyl, and the like.

[0054] Where it is indicated that a moiety may be substituted, such as by use of "substituted or unsubstituted" or "optionally substituted" phrasing as in "substituted or unsubstituted C_1-C_5 alkyl" or "optionally substituted heteroaryl," such moiety may have one or more independently selected substituents, preferably one to five in number, more preferably one or two in number. Substituents and substitution patterns can be selected by one of ordinary skill in the art, having regard for the moiety to which the substituent is attached, to provide compounds that are chemically stable and that can be synthesized by techniques known in the art as well as the methods set forth herein.

[0055] "Arylalkyl," (heterocycloaliphatic)alkyl," "arylalkenyl," "arylalkynyl," "biarylalkyl," and the like mean an alkyl, alkenyl, or alkynyl moiety, as the case may be, substituted with an aryl, heterocycloaliphatic, biaryl, etc., moiety, as the case may be, with the open (unsatisfied) valence at the alkyl, alkenyl, or alkynyl moiety, for example as in benzyl, phenethyl, N-imidazoylethyl, N-morpholinoethyl, and the like. Conversely, "alkylaryl," "alkenylcycloalkyl," and the like mean an aryl, cycloalkyl, etc., moiety, as the case may be, substituted with an alkyl, alkenyl, etc., moiety, as the case may be, for example as in methylphenyl (tolyl) or allylcyclohexyl. "Hydroxyalkyl," "haloalkyl," "alkylaryl," "cyanoaryl," and the like mean an alkyl, aryl, etc., moiety, as the case may be, substituted with one or more of the identified substituent (hydroxyl, halo, etc., as the case may be).

[0056] By way of illustration, permissible substituents include, but are not limited to, alkyl (especially methyl or ethyl), alkenyl (especially allyl), alkynyl, aryl, heteroaryl, cycloaliphatic, heterocycloaliphatic, halo (especially fluoro), haloalkyl (especially trifluoromethyl), hydroxyl, hydroxyalkyl (especially hydroxyethyl), cyano, nitro, alkoxy, -O(hydroxyalkyl), -O(haloalkyl) (especially -OCF_3), -O(cycloalkyl), -O(heterocycloalkyl), -O(aryl), alkylthio, arylthio, =O, =NH, =N(alkyl), =NOH, =NO(alkyl), -C(=O)(alkyl), -C(=O)H, -CO_2H, -C(=O)NHOH, -C(=O)O(alkyl), -C(=O)O(hydroxyalkyl), -C(=O)NH_2, -C(=O)NH(alkyl), -C(=O)N(alkyl)$_2$, -OC(=O)(alkyl), -OC(=O)(hydroxyalkyl), -OC(=O)O(alkyl), -OC(=O)O(hydroxyalkyl), -OC(=O)NH_2, -OC(=O)NH(alkyl), -OC(=O)N(alkyl)$_2$, azido, -NH_2, -NH(alkyl),

-N(alkyl)$_2$, -NH(aryl), -NH(hydroxyalkyl), -NHC(=O)(alkyl), -NHC(=O)H, -NHC(=O)NH$_2$, -NHC(=O)NH(alkyl), -NHC(=O)N(alkyl)$_2$, -NHC(=NH)NH$_2$, -OSO$_2$(alkyl), -SH, -S(alkyl), -S(aryl), -S(cycloalkyl), -S(=O)alkyl, -SO$_2$(alkyl), -SO$_2$NH$_2$, -SO$_2$NH(alkyl), -SO$_2$N(alkyl)$_2$, and the like.

[0057] Where the moiety being substituted is an aliphatic moiety, preferred substituents are aryl, heteroaryl, cycloaliphatic, heterocycloaliphatic, halo, hydroxyl, cyano, nitro, alkoxy, -O(hydroxyalkyl), -O(haloalkyl), -O(cycloalkyl), -O(heterocycloalkyl), -O(aryl), alkylthio, arylthio, =O, =NH, =N(alkyl), =NOH, =NO(alkyl), -CO$_2$H, -C(=O)NHOH, -C(=O)O(alkyl), -C(=O)O(hydroxyalkyl), -C(=O)NH$_2$, -C(=O)NH(alkyl), -C(=O)N(alkyl)$_2$, -OC(=O)(alkyl), -OC(=O)(hydroxyalkyl), -OC(=O)O(alkyl), -OC(=O)O(hydroxyalkyl), -OC(=O)NH$_2$, -OC(=O)NH(alkyl), -OC(=O)N(alkyl)$_2$, azido, -NH$_2$, -NH(alkyl), -N(alkyl)$_2$, -NH(aryl), -NH(hydroxyalkyl), -NHC(=O)(alkyl), -NHC(=O)H, -NHC(=O)NH$_2$, -NHC(=O)NH(alkyl), -NHC(=O)N(alkyl)$_2$, -NHC(=NH)NH$_2$, -OSO$_2$(alkyl), -SH, -S(alkyl), -S(aryl), -S(=O)alkyl, -S(cycloalkyl), -SO$_2$(alkyl), -SO$_2$NH$_2$, -SO$_2$NH(alkyl), and -SO$_2$N(alkyl)$_2$. More preferred substituents are halo, hydroxyl, cyano, nitro, alkoxy, -O(aryl), =O, =NOH, =NO(alkyl), -OC(=O)(alkyl), -OC(=O)O(alkyl), -OC(=O)NH$_2$, -OC(=O)NH(alkyl), -OC(=O)N(alkyl)$_2$, azido, -NH$_2$, -NH(alkyl), -N(alkyl)$_2$, -NH(aryl), -NHC(=O)(alkyl), -NHC(=O)H, -NHC(=O)NH$_2$, -NHC(=O)NH(alkyl), -NHC(=O)N(alkyl)$_2$, and -NHC(=NH)NH$_2$.

[0058] Where the moiety being substituted is a cycloaliphatic, heterocycloaliphatic, aryl, or heteroaryl moiety, preferred substituents are alkyl, alkenyl, alkynyl, halo, haloalkyl, hydroxyl, hydroxyalkyl, cyano, nitro, alkoxy, -O(hydroxyalkyl), -O(haloalkyl), -O(aryl), -O(cycloalkyl), -O(heterocycloalkyl), alkylthio, arylthio, -C(=O)(alkyl), -C(=O)H, -CO$_2$H, -C(=O)NHOH, -C(=O)O(alkyl), -C(=O)O(hydroxyalkyl), -C(=O)NH$_2$, -C(=O)NH(alkyl), -C(=O)N(alkyl)$_2$, -OC(=O)(alkyl), -OC(=O)(hydroxyalkyl), -OC(=O)O(alkyl), -OC(=O)O(hydroxyalkyl), -OC(=O)NH$_2$, -OC(=O)NH(alkyl), -OC(=O)N(alkyl)$_2$, azido, -NH$_2$, -NH(alkyl), -N(alkyl)$_2$, -NH(aryl), -NH(hydroxyalkyl), -NHC(=O)(alkyl), -NHC(=O)H, -NHC(=O)NH$_2$, -NHC(=O)NH(alkyl), -NHC(=O)N(alkyl)$_2$, -NHC(=NH)NH$_2$, -OSO$_2$(alkyl), -SH, -S(alkyl), -S(aryl), -S(cycloalkyl), -S(=O)alkyl, -SO$_2$(alkyl), -SO$_2$NH$_2$, -SO$_2$NH(alkyl), and -SO$_2$N(alkyl)$_2$. More preferred substituents are alkyl, alkenyl, halo, haloalkyl, hydroxyl, hydroxyalkyl,

cyano, nitro, alkoxy, -O(hydroxyalkyl), -C(=O)(alkyl), -C(=O)H, -CO_2H, -C(=O)NHOH, -C(=O)O(alkyl), -C(=O)O(hydroxyalkyl), -C(=O)NH_2, -C(=O)NH(alkyl), -C(=O)N(alkyl)$_2$, -OC(=O)(alkyl), -OC(=O)(hydroxyalkyl), -OC(=O)O(alkyl), -OC(=O)O(hydroxyalkyl), -OC(=O)NH_2, -OC(=O)NH(alkyl), -OC(=O)N(alkyl)$_2$, -NH_2, -NH(alkyl), -N(alkyl)$_2$, -NH(aryl), -NHC(=O)(alkyl), -NHC(=O)H, -NHC(=O)NH_2, -NHC(=O)NH(alkyl), -NHC(=O)N(alkyl)$_2$, and -NHC(=NH)NH_2.

[0059] Where a range is stated, as in "C_1-C_5 alkyl" or "5 to 10%," such range includes the end points of the range, as in C_1 and C_5 in the first instance and 5% and 10% in the second instance.

[0060] Unless particular stereoisomers are specifically indicated (e.g., by a bolded or dashed bond at a relevant stereocenter in a structural formula, by depiction of a double bond as having E or Z configuration in a structural formula, or by use stereochemistry-designating nomenclature), all stereoisomers are included within the scope of the invention, as pure compounds as well as mixtures thereof. Unless otherwise indicated, individual enantiomers, diastereomers, geometrical isomers, and combinations and mixtures thereof are all encompassed by the present invention.

[0061] Those skilled in the art will appreciate that compounds may have tautomeric forms (*e.g.*, keto and enol forms), resonance forms, and zwitterionic forms that are equivalent to those depicted in the structural formulae used herein and that the structural formulae encompass such tautomeric, resonance, or zwitterionic forms.

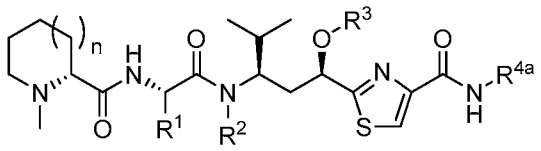
[0062] "Pharmaceutically acceptable ester" means an ester that hydrolyzes *in vivo* (for example in the human body) to produce the parent compound or a salt thereof or has *per se* activity similar to that of the parent compound. Suitable esters include C_1-C_5 alkyl, C_2-C_5 alkenyl or C_2-C_5 alkynyl esters, especially methyl, ethyl or n-propyl.

[0063] "Pharmaceutically acceptable salt" means a salt of a compound suitable for pharmaceutical formulation. Where a compound has one or more basic groups, the salt can be an acid addition salt, such as a sulfate, hydrobromide, tartrate, mesylate, maleate, citrate, phosphate, acetate, pamoate (embonate), hydroiodide, nitrate, hydrochloride, lactate, methylsulfate, fumarate, benzoate, succinate, mesylate, lactobionate, suberate, tosylate, and the like. Where a compound has one or more acidic groups, the salt can be a salt such as a calcium salt, potassium salt, magnesium salt, meglumine salt, ammonium salt, zinc salt, piperazine salt, tromethamine salt, lithium salt, choline salt, diethylamine

salt, 4-phenylcyclohexylamine salt, benzathine salt, sodium salt, tetramethylammonium salt, and the like. Polymorphic crystalline forms and solvates are also encompassed within the scope of this invention.

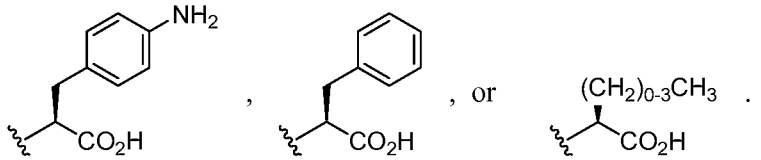
COMPOUNDS

[0064] A preferred embodiment of compounds of formula (II) is represented by formula (II-a)



(II-a)

wherein n, R^1, R^2, and R^3 are as defined hereinabove in respect of formula (II) and R^{4a} is

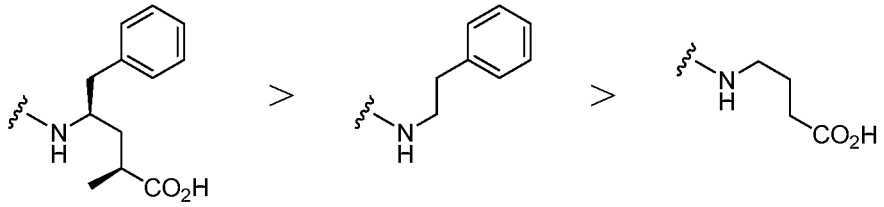


[0065] In the compounds of formula (II-a), the subunit corresponding to Tup/Tut in the naturally occurring tubulysins has been reduced in size and lipophilicity by at least two carbons, via deletion of the two aliphatic carbon atoms immediately following the carboxylic acid group – that is, the amino group is now α- to the carboxylic acid group, instead of γ- to it. In the instance in which R^4 is 4-aminophenylalanine, the amine group constitutes a polar moiety that further reduces lipophilicity. SAR studies show that lipophilicity is an important factor in the biological activity of the tubulysins and their analogs or derivatives. Steinmetz et al. 2004 and Neri et al. 2006 both disclose that the more lipophilic naturally occurring tubulysins – i.e., those having a Tup subunit (R^A equals H in formula I) instead of a Tut subunit (R^A equals OH in formula I) – possessed greater biological activity. Further, the differences in activities were retained regardless of the size and lipophilicity of the 11-acyloxy residue (group R^C in formula (I)) in the Tuv subunit (Steinmetz et al. 2004). These results indicate that a lipophilic Tup/Tut subunit is a particularly important SAR element.

[0066] The above observations are partially corroborated in two studies by Balasubramanian et al. In the first (Balasubramanian et al. 2008), analogs dimethylated at the carbon alpha to the carboxyl group in the Tup subunit were compared against otherwise

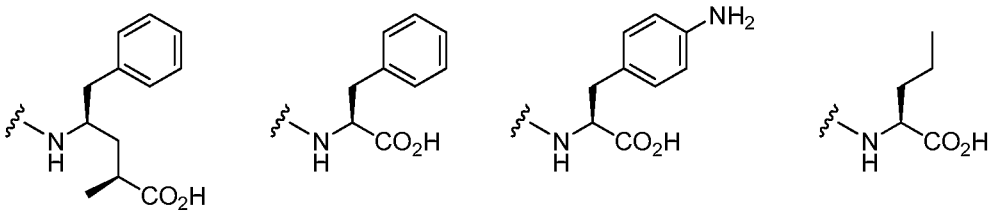
identical analogs desmethylated at the same position. The dimethylated analogs had greater antiproliferative activity – although the *in vitro* tubulin inhibition IC_{50}s were comparable – as might be expected based on their relative lipophilicities. However, this trend was not followed in the second study (Balasubramanian et al. 2009), in which three analogs (one α-carbon desmethylated, one monomethylated, and one dimethylated) were compared. There, the most active analog was the desmethylated one while the monomethylated one – i.e., with the natural Tup subunit – was by far the least active one. However, the latter analog had additional modifications elsewhere in the molecule rendering it essentially inactive, making it unclear what SAR inferences, if any, can be drawn.

[0067] Patterson et al. 2007 and Ellman et al. 2009 compared the cytotoxicities of tubulysin D and analogs in which either only the phenethyl or the γ-carboxy group was retained in the Tup subunit. The phenethyl-retained analog was 3.6 to 13.6 times less active than tubulysin D against three cancer cell lines but when the less lipophilic γ-carboxyl group was retained, there was an even greater loss of activity, from 25.7 to 62.5 times less active. That is, the order of activity was:



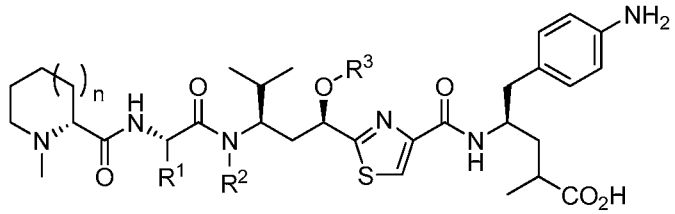
Tubulysin D

[0068] The above documents suggest, individually and in combination, that lipophilicity at the Tup locus is especially important for the biological activity of the tubulysins. Therefore, the prior art suggests that the replacement of the Tup subunit with a phenylalanine (Phe), 4-aminophenylalanine (4-NH_2Phe), norvaline or other R^4 subunit in accordance with formula (II-a) is undesirable, as each would lead to the loss of at least two aliphatic carbons and a consequent reduction in lipophilicity at the Tup/Tut locus.



Tup Phe 4-NH_2Phe Norvaline

[0069] Another preferred embodiment of compounds of formula (II) is represented by formula (II-b):

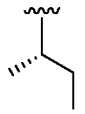


(II-b)

wherein n, R^1, R^2, and R^3 are as defined hereinabove in respect for formula (II).

Although chemical formulae can be found in the literature encompassing an $-NH_2$ group in the 4-position of the Tup aromatic ring (Domling 2005a and 2005b), there has been no disclosure on how a compound having such a feature might be made.

[0070] In formulae (II), (II-a), and (II-b), R^1 preferably is H, C_1-C_5 alkyl, C_2-C_5 alkenyl, or C_2-C_5 alkenyl, and is more preferably an isoleucyl residue, that is:

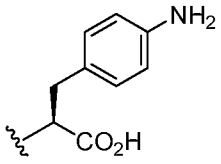


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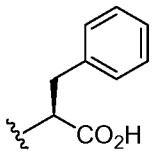
[0071] In formulae (II), (II-a), and (II-b), R^2 preferably is H, C_1-C_5 alkyl, C_2-C_5 alkenyl, $CH_2O(C_1$-C_5 alkyl), $CH_2O(C_2$-C_5 alkenyl), $CH_2O(C{=}O)(C_1$-C_5 alkyl), or $CH_2OC(=O)(C_2$-C_5 alkenyl); and more preferably is H, Me, Et, n-Pr, CH_2OMe, CH_2OEt, CH_2O(n-Pr), $CH_2OC(=O)$*i*-Bu, $CH_2OC(=O)$*n*-Pr, $CH_2OC(=O)CH{=}CH_2$, or $CH_2OC(=O)Me$, with Me, n-Pr, CH_2OMe, $CH_2OC(=O)$i-Bu, and CH_2O(n-Pr) being especially preferred.

[0072] In formulae (II), (II-a), and (II-b), R^3 preferably is H, C_1-C_5 alkyl, C_2-C_5 alkenyl, $C(=O)C_1$-C_5 alkyl, or $C(=O)C_2$-C_5 alkenyl; and more preferably is H, Me, Et, or C(=O)Me.

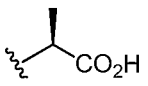
[0073] Preferably, in formulae (II) and (II-a), R^4 and R^{4a} are:



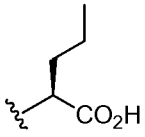
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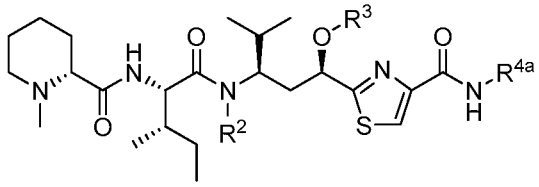
, or



.

[0074] In formulae (II), (II-a),and (II-b), n preferably is 1 and, in the instance of formula (II), R^5 preferably is methyl; that is, the ring in the Mep subunit preferably is an N-methyl piperidinyl one.

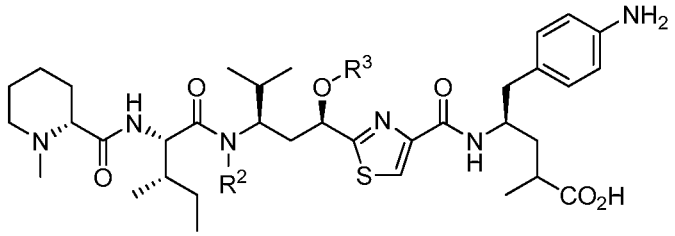
[0075] A preferred embodiment of compounds according to formula (II-a) is depicted by formula (II-a')



(II-a')

where R^{4a} is as defined above in respect of formula (II-a), R^2 is H, C_1-C_5 alkyl, C_2-C_5 alkenyl, $CH_2O(C_1$-C_5 alkyl), $CH_2O(C_2$-C_5 alkenyl), $CH_2O(C{=}O)(C_1$-C_5 alkyl), or $CH_2OC(=O)(C_2$-C_5 alkenyl); and R^3 is H, C_1-C_5 alkyl, C_2-C_5 alkenyl, $C(=O)C_1$-C_5 alkyl, or $C(=O)C_2$-C_5 alkenyl. Preferably, R^2 is H, Me, Et, n-Pr, CH_2OMe, CH_2OEt, $CH_2OC(=O)i$-Bu, $CH_2OC(=O)n$-Pr, $CH_2OC(=O)CH{=}CH_2$, or $CH_2OC(=O)Me$; more preferably Me, n-Pr, CH_2OMe, $CH_2OC(=O)$i-Bu, or CH_2O(n-Pr). Preferably, R^3 is H, Me, Et, or C(=O)Me.

[0076] A preferred embodiment of compounds according to formula (II-b) is depicted by formula (II-b')

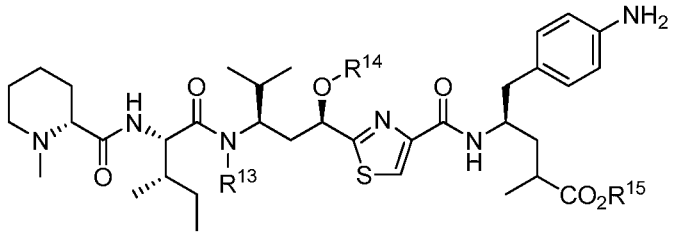


(II-b')

where R^2 is H, C_1-C_5 alkyl, C_2-C_5 alkenyl, $CH_2O(C_1$-C_5 alkyl), $CH_2O(C_2$-C_5 alkenyl), $CH_2O(C{=}O)(C_1$-C_5 alkyl), or $CH_2OC(=O)(C_2$-C_5 alkenyl); and R^3 is H, C_1-C_5 alkyl, C_2-C_5 alkenyl, $C(=O)C_1$-C_5 alkyl, or $C(=O)C_2$-C_5 alkenyl. Preferably, R^2 is H, Me, Et, n-Pr, CH_2OMe, CH_2OEt, $CH_2OC(=O)i$-Bu, $CH_2OC(=O)n$-Pr, $CH_2OC(=O)CH{=}CH_2$, or $CH_2OC(=O)Me$ and R^3 is H, Me, Et, or C(=O)Me.

[0077] Where a carboxyl group in R^4 is esterified, preferably the ester is a C_1-C_5 alkyl ester, such as a Me, Et, or Pr ester. Alternatively, the carboxyl group can be amidated with ammonia or an alkyl amine.

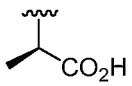
[0078] In another embodiment, this invention provides a compound having a structure represented by formula (II-c)



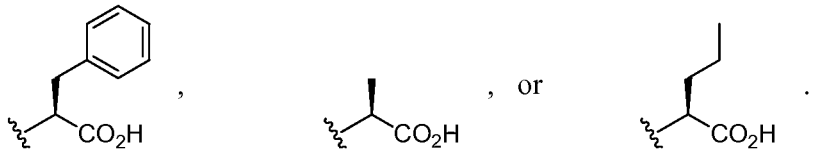
(II-c)

where R^{13} is Me, n-Pr, CH_2OMe, or $CH_2OC(=O)CH_2CH(Me)_2$; R^{14} is Me or C(=O)Me; and R^{15} is H or C_1-C_5 alkyl (preferably H, Me, or Et).

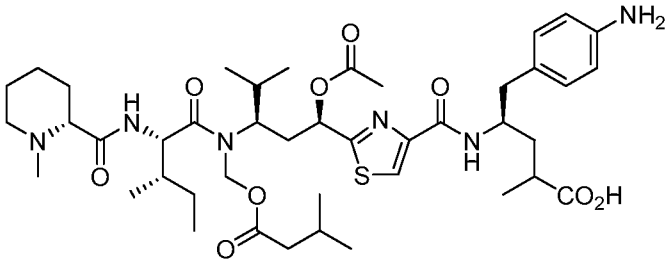
[0079] In formulae (II-b), (II-b'), and (II-c) the stereochemistry at the methyl alpha to the carboxyl preferably is that corresponding to the naturally-occurring tubulysins, i.e.:



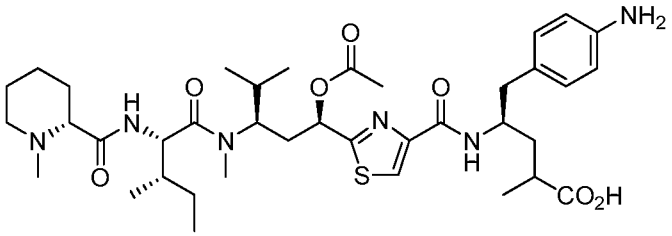
[0080] In a preferred embodiment, a compound of this invention is in the form of an amide of the carboxyl group in R^4 (or R^{4a}, as the case may be) with the α-amine group of an α-amino acid. The α-amino acid can be selected from the group consisting of a proteinogenic amino acid, 4-aminophenylalanine, norvaline, norleucine, and citrulline. Preferably, the α-amino acid is selected from the group consisting of alanine, norvaline, glycine, lysine, arginine, citrulline, norleucine, 4-aminophenylalanine, and phenylalanine. Also preferably, the absolute configuration of the α-amino acid is the proteinogenic one, i.e., L. In this preferred embodiment, R^4 (or R^{4a}) preferably is:



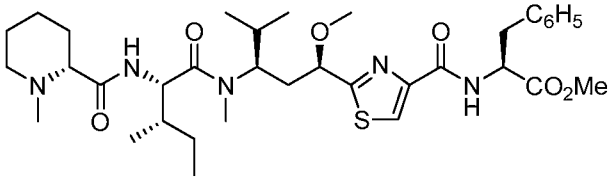
[0081] Specific examples of compounds of this invention according to formula (II) include compounds (III-a) through (III-y). Some of the compounds are depicted as a pharmaceutically acceptable ester or a pharmaceutically acceptable amide of the R^4 carboxyl group with the α-amine group of an α-amino acid or methyl ester thereof.



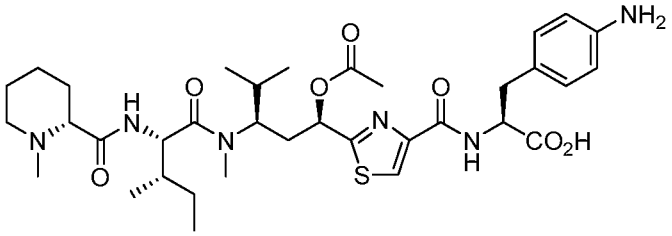
(III-a) ,



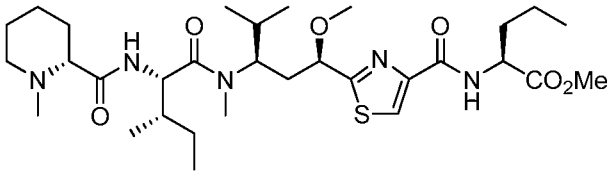
(III-b) ,



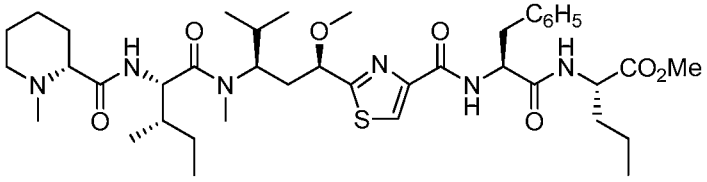
(III-c) ,



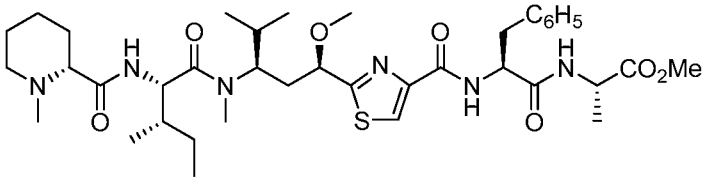
(III-d) ,



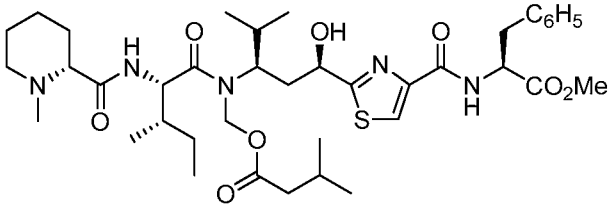
(III-e) ,



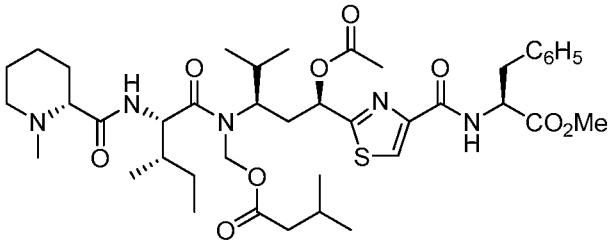
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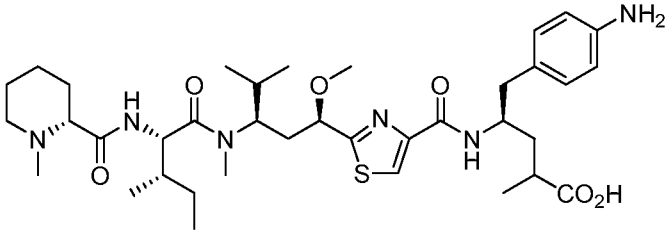
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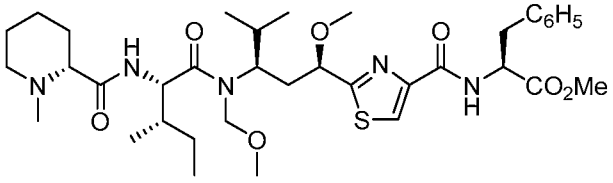
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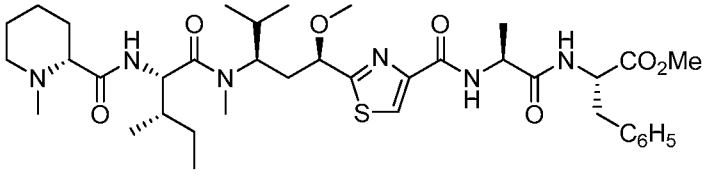
(III-i) ,



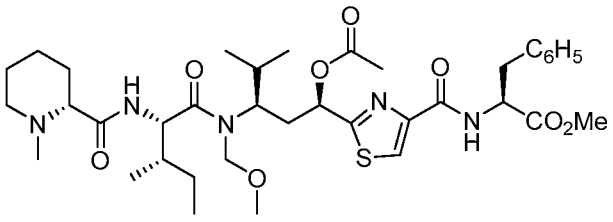
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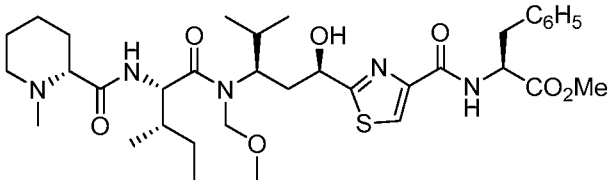
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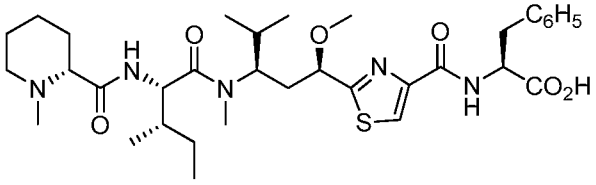
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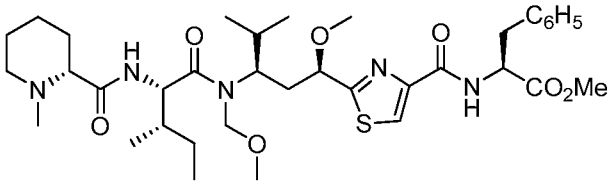
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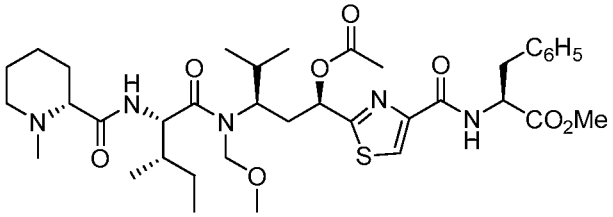
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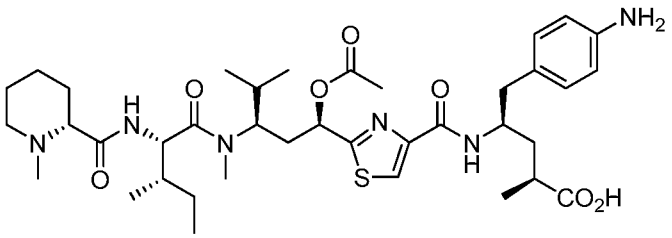
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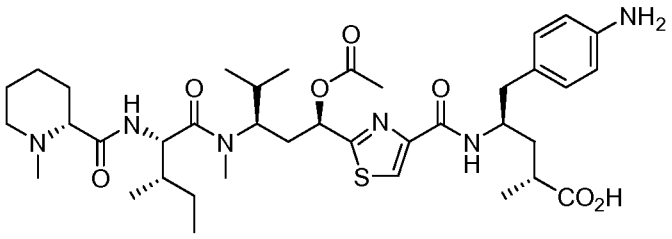
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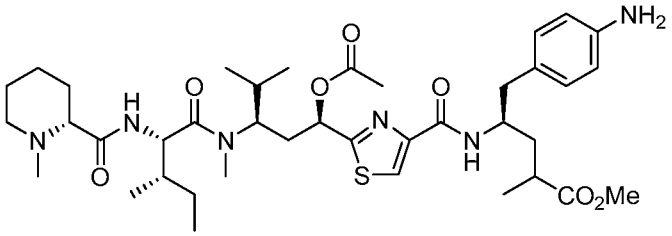
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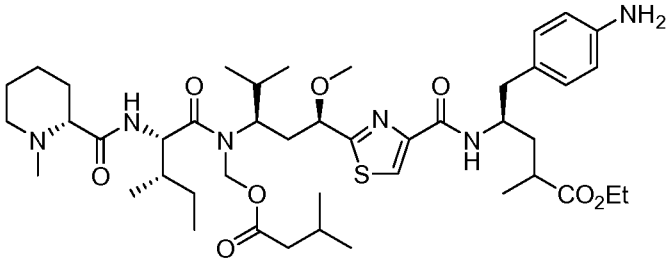
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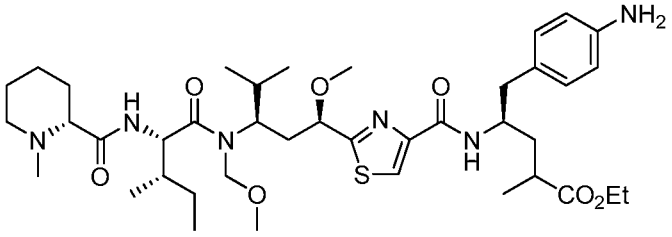
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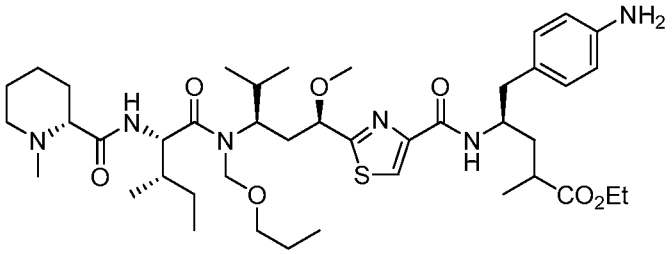
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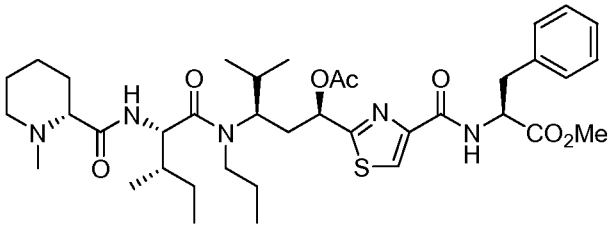
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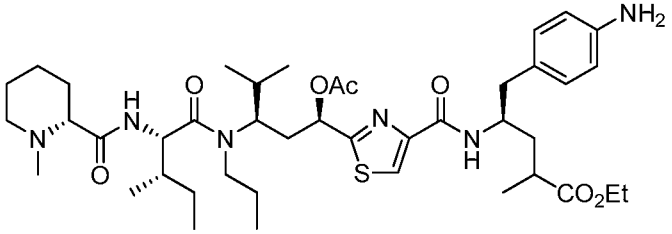
(III-v) ,



(III-w) ,

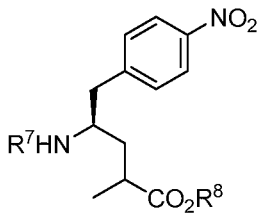


(III-x) , and



(III-y) .

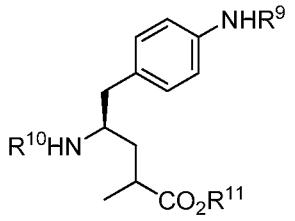
[0082] This invention also provides novel intermediates that can be utilized for the synthesis of compounds of this invention. Compounds according to formula (VIII-a) can be used for the making of compounds according to formula (II) or (II-b), as taught in the figures and examples herein.



(VIII-a)

[0083] In formula (VIII-a), R^7 is H or an amine protecting group and R^8 is H, C_1-C_{10} alkyl, C_2-C_{10} alkenyl, C_2-C_{10} alkynyl, aryl, cycloaliphatic, alkylcycloaliphatic, arylalkyl, or alkylaryl. Preferably, R^7 is H, Boc (*t*-butoxycarbonyl), Troc (2,2,2-trichloroethoxy carbonyl), Bpoc ((1-methyl-1-(4-biphenyl)ethoxycarbonyl)), Cbz (benzyloxy carbonyl), Aloc (allyloxycarbonyl), methyl amine, or Fmoc (9-fluorenylmethoxycarbonyl). Preferably, R^8 is H or C_1-C_5 alkyl (especially Me).

[0084] Another novel intermediate useful for synthesis of compounds of this inventtion has a structure according to formula (VIII-b) The use of compounds of formula (VIII-b) to make compounds of this invention is taught in the figures and examples herein.



(VIII-b)

[0085] In formula (VIII-b), R^9 and R^{10} are independently H or an amine protecting group and R^{11} is H, C_1-C_{10} alkyl, C_2-C_{10} alkenyl, C_2-C_{10} alkynyl, aryl, cycloaliphatic, alkylcycloaliphatic, arylalkyl, or alkylaryl. Preferably, R^9 and R^{10} are independently selected from H, Boc, Troc, Bpoc, Cbz, Aloc, methylamine, and Fmoc. Preferably, R^{11} is H or C_1-C_5 alkyl (especially Me). Preferably, where R^9 and R^{10} are each an amine protecting group, they are different amine protecting groups.

[0086] Additional suitable amine protecting groups for the compounds of formula (VIII-a) and (VIII-b) are disclosed in Greene and Wuts, *Protective Groups in Organic*

Synthesis, 3rd edition, pp. 464-653 (John Wiley & Sons, New York, 1999), the disclosure of which is incorporated herein by reference.

CONJUGATES

[0087] In another aspect, there is provided a conjugate comprising cytotoxic compound according to this invention and a ligand, represented by formula (IV)

$$[D(X^D)_aC(X^Z)_b]_mZ \qquad (IV)$$

where Z is ligand; D is a cytotoxic compound according to this invention; and $-(X^D)_aC(X^Z)_b-$ are collectively referred to as a "linker moiety" or "linker" because they link Z and D. Within the linker, C is a cleavable group designed to be cleaved at the site of intended biological action of compound D; X^D and X^Z are referred to as spacer moieties (or "spacers") because they space apart D and C and C and Z, respectively; subscripts a and b are independently 0 or 1 (that is, the presence of X^D and/or X^Z are optional); and subscript m is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 (preferably 1, 2, 3, or 4). D, X^D, C, X^Z and Z are more fully defined hereinbelow.

[0088] Ligand Z – for example an antibody – serves a targeting function. By binding to a target tissue or cell where its antigen or receptor is located, ligand Z directs the conjugate there. Preferably, the target tissue or cell is a cancer tissue or cell and the antigen or receptor is a tumor-associated antigen, that is, an antigen that is uniquely expressed by cancerous cells or is overexpressed by cancer cells, compared to non-cancerous cells. Cleavage of group C at the target tissue or cell releases compound D to exert its cytotoxic effect locally. In some instances, the conjugate is internalized into a target cell by endocytosis and cleavage takes place within the target cell. In this manner, precise delivery of compound D is achieved at the site of intended action, reducing the dosage needed. Also, compound D is normally biologically inactive (or significantly less active) in its conjugated state, thereby reducing undesired toxicity against non-target tissue or cells. As anticancer drugs are often highly toxic to cells in general, this is an important consideration.

[0089] As reflected by the subscript m, each molecule of ligand Z can conjugate with more than one compound D, depending on the number of sites D has available for conjugation and the experimental conditions employed. Those skilled in the art will appreciate that, while each individual molecule of ligand Z is conjugated to an integer

number of compounds D, a preparation of the conjugate may analyze for a non-integer ratio of compounds D to ligand Z, reflecting a statistical average.

Ligand Z and Conjugation Thereof

[0090] Preferably, ligand Z is an antibody. For convenience and brevity and not of limitation, the detailed subsequent discussion herein about the conjugation of ligand Z is written in the context of its being an antibody, but those skilled in the art will understand that other types of ligand Z can be conjugated, *mutatis mutandis*. For example, conjugates with folic acid as the ligand can target cells having the folate receptor on their surfaces (Vlahov et al. 2008a, 2008b and 2010; Leamon et al. 2009). For the same reason, the detailed discussion below is primarily written in terms of a 1:1 ratio of antibody Z to compound D.

[0091] Preferably, ligand Z is an antibody against a tumor associated antigen, allowing a conjugate comprising such a ligand Z to selectively target cancer cells. Examples of such antigens include: mesothelin, prostate specific membrane antigen (PSMA), CD19, CD22, CD30, CD70, B7H4 (also known as O8E), protein tyrosine kinase 7 (PTK7), RG1, CTLA-4, and CD44. The antibody can be animal (e.g., murine), chimeric, humanized, or, preferably, human. The antibody preferably is monoclonal, especially a monoclonal human antibody. The preparation of human monoclonal antibodies against some of the aforementioned antigens is disclosed in Korman et al., US 2009/0074660 A1 (B7H4); Rao-Naik et al., US 2009/0142349 A1 A2 (CD19); King et al., WO 2008/070569 A2 (CD22); Keler et al., US 7,387,776 B2 (2008) (CD30); Terrett et al., US 2009/0028872 A1 (CD70); Korman et al., US 6,984,720 B1 (2006) (CTLA-4); Korman et al., US 2009/0217401 A1 (PD-1); Huang et al., US 2008/0279868 A1 (PSMA); Lu et al., US 2010/0034826 A1 (PTK7); Harkins et al., US 7,335,748 B2(2008) (RG1); Terrett et al., WO 2009/045957 A1 (mesothelin); and Xu et al., US 2010/0092484 A1 (CD44); the disclosures of which are incorporated herein by reference.

[0092] Ligand Z can also be an antibody fragment or antibody mimetic, such as an affibody, a domain antibody (dAb), a nanobody, a unibody, a DARPin, an anticalin, a versabody, a duocalin, a lipocalin, or an avimer.

[0093] Any one of several different reactive groups on ligand Z can be a conjugation site, including ε-amino groups in lysine residues, pendant carbohydrate moieties, car-

boxylic acid groups, disulfide groups, and thiol groups. Each type of reactive group represents a trade-off, having some advantages and some disadvantages. For reviews on antibody reactive groups suitable for conjugation, see, e.g., Garnett, *Adv. Drug Delivery Rev.* 53 (2001), 171-216 and Dubowchik and Walker, *Pharmacology & Therapeutics* 83 (1999), 67-123, the disclosures of which are incorporated herein by reference.

[0094] In one embodiment, ligand Z is conjugated via a lysine ε-amino group. Most antibodies have multiple exposed lysine ε-amino groups, which can be conjugated via amide, urea, thiourea, or carbamate bonds using techniques known in the art, including modification with a heterobifunctional agent (as further described hereinbelow). However, it is difficult to control which and how many ε-amino groups react, leading to potential batch-to-batch variability in conjugate preparations. Also, conjugation may cause neutralization of a protonated ε-amino group important for maintaining the antibody's native conformation or may take place at a lysine near or at the antigen binding site, neither being a desirable occurrence.

[0095] In another embodiment, ligand Z can be conjugated via a carbohydrate side chain, as many antibodies are glycosylated. The carbohydrate side chain can be oxidized with periodate to generate aldehyde groups, which in turn can be reacted with amines to form an imine group, such as in a semicarbazone, oxime, or hydrazone. If desired, the imine group can be converted to a more stable amine group by reduction with sodium cyanoborohydride. For additional disclosures on conjugation via carbohydrate side chains, see, e.g., Rodwell et al., *Proc. Nat'l Acad. Sci. USA* 83, 2632-2636 (1986); the disclosure of which is incorporated herein by reference. As with lysine ε-amino groups, there are concerns regarding reproducibility of the location of the conjugation site(s) and stoichiometry.

[0096] In yet another embodiment, ligand Z can be conjugated via a carboxylic acid group. In one embodiment, a terminal carboxylic acid group is functionalized to generate a carbohydrazide, which is then reacted with an aldehyde-bearing conjugation moiety. See Fisch et al., *Bioconjugate Chemistry* **1992**, *3*, 147-153.

[0097] In yet another embodiment, antibody Z can be conjugated via a disulfide group bridging a cysteine residue on antibody Z and a sulfur on the other portion of the conjugate. Some antibodies lack free thiol (sulfhydryl) groups but have disulfide groups, for example in the hinge region. In such case, free thiol groups can be generated by

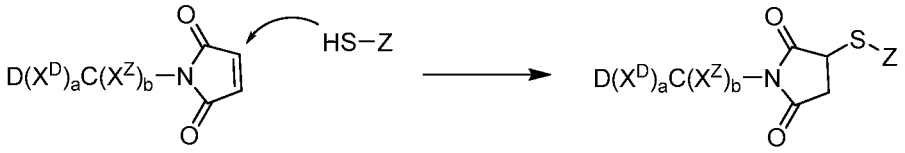
reduction of native disulfide groups. The thiol groups so generated can then be used for conjugation. See, e.g., Packard et al., *Biochemistry* **1986**, *25*, 3548-3552; King et al., *Cancer Res.* 54, 6176-6185 (1994); and Doronina et al., *Nature Biotechnol.* 21(7), 778-784 (2003); the disclosures of which are incorporated herein by reference. Again, there are concerns regarding conjugation site location and stoichiometry and the possible disruption of antibody native conformation.

[0098] A number of methods are known for introducing free thiol groups into antibodies without breaking native disulfide bonds, which methods can be practiced with a ligand Z of this invention. Depending on the method employed, it may be possible to introduce a predictable number of free sulfhydryls at predetermined locations. In one approach, mutated antibodies are prepared in which a cysteine is substituted for another amino acid. See, for example, Eigenbrot et al., US 2007/0092940 A1; Chilkoti et al., *Bioconjugate Chem.* **1994**, *5*, 504-507; Urnovitz et al., US 4,698,420 (1987); Stimmel et al., *J. Biol. Chem.*, 275 (39), 30445-30450 (2000); Bam et al., US 7,311,902 B2 (2007); Kuan et al., *J. Biol. Chem.*, 269 (10), 7610-7618 (1994); Poon et al., *J. Biol. Chem.*, 270 (15), 8571-8577 (1995). In another approach, an extra cysteine is added to the C-terminus. See, e.g. Cumber et al., *J. Immunol.*, 149, 120-126 (1992); King et al, *Cancer Res.*, 54, 6176-6185 (1994); Li et al., *Bioconjugate Chem.*, 13, 985-995 (2002); Yang et al., *Protein Engineering*, 16, 761-770 (2003); and Olafson et al., *Protein Engineering Design & Selection*, 17, 21-27 (2004). A preferred method for introducing free cysteines is that taught by Liu et al., WO 2009/026274 A1, in which a cysteine bearing amino acid sequence is added to the C-terminus of the heavy chain of an antibody. This method introduces a known number of cysteine residues (one per heavy chain) at a known location remote from the antigen binding site. The disclosures of the documents cited in this paragraph are all incorporated herein by reference.

[0099] In yet another embodiment, lysine ε-amino groups can be modified with heterobifunctional reagents such as 2-iminothiolane or N-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP), converting an ε-amino group into a thiol or disulfide group – creating a cysteine surrogate, as it were. However, this method suffers from the same conjugation location and stoichiometry limitations associated with ε-amino groups proper.

[00100] In yet another preferred embodiment, ligand Z is conjugated via the nucleophilic addition product of a thiol group to an acceptor moiety. A preferred acceptor moiety

is a maleimide group, whose reaction with an antibody thiol group is generically illustrated below. The thiol group can be a native one, or one introduced as described above.



Linker $-(X^D)_aC(X^Z)_b-$

[00101] As noted above, the linker portion of a conjugate of this invention comprises up to three elements: a cleavable group C and optional spacers X^Z and X^D.

[00102] Cleavable group C is a group cleavable under physiological conditions, preferably is selected such that it is relatively stable while the conjugate is in general circulation in the blood plasma, but is readily cleaved once the conjugate reaches its site of intended action, that is, near, at, or within the target cell. Preferably, the conjugate is internalized by endocytosis by a target cell upon binding of antibody Z to an antigen displayed on the surface of the target cell. Subsequently, cleavage of group C occurs in a vesicular body of the target cell (an early endosome, a late endosome, or, especially, a lysosome).

[00103] In one embodiment, group C is a pH sensitive group. The pH in blood plasma is slightly above neutral, while the pH inside a lysosome is acidic, circa 5. Thus, a group C whose cleavage is acid catalyzed will cleave at a rate several orders of magnitude faster inside a lysosome than in the blood plasma rate. Examples of suitable acid-sensitive groups include cis-aconityl amides and hydrazones, as described in Shen et al., US 4,631,190 (1986); Shen et al., US 5,144,011 (1992); Shen et al., *Biochem. Biophys. Res. Commun.* 102, 1048-1054 (1981) and Yang et al., *Proc. Natl Acad. Sci (USA)*, 85, 1189-1193 (1988); the disclosures of which are incorporated herein by reference.

[00104] In another embodiment, group C is a disulfide. Disulfides can be cleaved by a thiol-disulfide exchange mechanism, at a rate dependent on the ambient thiol concentration. As the intracellular concentration of glutathione and other thiols is higher than their serum concentrations, the cleavage rate of a disulfide will be higher intracellularly. Further, the rate of thiol-disulfide exchange can be modulated by adjustment of the steric and electronic characteristics of the disulfide (e.g., an alkyl-aryl disulfide versus an alkyl-alkyl disulfide; substitution on the aryl ring, etc.), enabling the design of disulfide linkages that have enhanced serum stability or a particular cleavage

rate. For additional disclosures relating to disulfide cleavable groups in conjugates, see, e.g., Thorpe et al., *Cancer Res.* 48, 6396-6403 (1988); Santi et al., US 2005/0287155 A1; Ng et al., US 6,989,452 B2 (2006); Ng et al., WO 2002/096910 A1; Boyd et al., US 7,691,962 B2; and Sufi et al., WO 2008/083312 A2; the disclosures of which are incorporated herein by reference.

[00105] A preferred group C comprises a peptide bond that is cleaved, preferentially by a protease, at the intended site of action, as opposed to by a protease in the serum. Typically, group C comprises from 1 to 20 amino acids, preferably from 1 to 6 amino acids, more preferably from 1 to 3 amino acids. The amino acid(s) can be natural and/or unnatural α-amino acids. Natural amino acids are those encoded by the genetic code, as well as amino acids derived therefrom, *e.g.*, hydroxyproline, γ-carboxyglutamate, citrulline, and O-phosphoserine. The term amino acid also includes amino acid analogs and mimetics. Analogs are compounds having the same general $H_2N(R)CHCO_2H$ structure of a natural amino acid, except that the R group is not one found among the natural amino acids. Examples of analogs include homoserine, norleucine, methionine-sulfoxide, and methionine methyl sulfonium. An amino acid mimetic is a compound that has a structure different from the general chemical structure of an α-amino acid but functions in a manner similar to one. The term "unnatural amino acid" is intended to represent the "D" stereochemical form, the natural amino acids being of the "L" form.

[00106] Preferably, group C contains an amino acid sequence that is a cleavage recognition sequence for a protease. Many cleavage recognition sequences are known in the art. *See, e.g.*, Matayoshi et al. *Science* 247: 954 (1990); Dunn et al. *Meth. Enzymol.* 241: 254 (1994); Seidah et al. *Meth. Enzymol.* 244: 175 (1994); Thornberry, *Meth. Enzymol.* 244: 615 (1994); Weber et al. *Meth. Enzymol.* 244: 595 (1994); Smith et al. *Meth. Enzymol.* 244: 412 (1994); and Bouvier et al. *Meth. Enzymol.* 248: 614 (1995); the disclosures of which are incorporated herein by reference.

[00107] For conjugates that are not intended to be internalized by a cell, a group C can be chosen such that it is cleaved by a protease present in the extracellular matrix in the vicinity of the target tissue, e.g., a protease released by nearby dying cells or a tumor-associated protease. Exemplary extracellular tumor-associated proteases are matrix metalloproteases (MMP), thimet oligopeptidase (TOP) and CD10.

[00108] For conjugates that are designed to be internalized by a cell, group C preferably comprises an amino acid sequence selected for cleavage by an endosomal or lysosomal protease, especially the latter. Non-limiting examples of such proteases include cathepsins B, C, D, H, L and S, especially cathepsin B. Cathepsin B preferentially cleaves peptides at a sequence -AA^2-AA^1- where AA^1 is a basic or strongly hydrogen bonding amino acid (such as lysine, arginine, or citrulline) and AA^2 is a hydrophobic amino acid (such as phenylalanine, valine, alanine, leucine, or isoleucine), for example Val-Cit (where Cit denotes citrulline) or Val-Lys. (Herein, amino acid sequences are written in the N-to-C direction, as in H_2N-AA^2-AA^1-CO_2H, unless the context clearly indicates otherwise.) For additional information regarding cathepsin-cleavable groups, see Dubowchik et al., *Biorg. Med. Chem. Lett.* **8**, 3341-3346 (1998); Dubowchik et al., *Bioorg. Med. Chem. Lett.*, **8** 3347-3352 (1998); and Dubowchik et al., *Bioconjugate Chem.* **13**, 855-869 (2002); the disclosures of which are incorporated by reference. Another enzyme that can be utilized for cleaving peptidyl linkers is legumain, a lysosomal cysteine protease that preferentially cleaves at Ala-Ala-Asn.

[00109] In one embodiment, Group C is a peptide comprising the two-amino acid sequence -AA^2-AA^1- wherein AA^1 is lysine, arginine, or citrulline and AA^2 is phenylalanine, valine, alanine, leucine or isoleucine. In another embodiment, C consists of a sequence of one to five amino acids, selected from the group consisting of Val-Cit, Ala-Val, Val-Ala-Val, Lys-Lys, Ala-Asn-Val, Val-Leu-Lys, Cit-Cit, Val-Lys, Ala-Ala-Asn, Lys, Cit, Ser, and Glu.

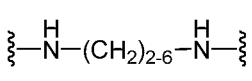
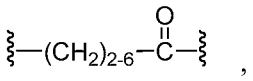
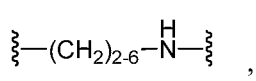
[00110] The preparation and design of cleavable groups C consisting of a single amino acid is disclosed in Chen et al., US 2010/0113476 A1, the disclosure of which is incorporated herein by reference.

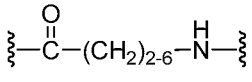
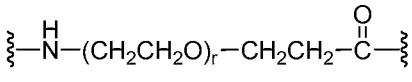
[00111] Group C can also be a photocleavable one, for example a nitrobenzyl ether that is cleaved upon exposure to light.

[00112] Group C can be bonded directly to antibody Z or compound D; that is, spacers X^Z and X^D, as the case may be, can be absent. For example, if group C is a disulfide, one of the two sulfurs can be a cysteine residue or its surrogate on antibody Z. Or, group C can be a hydrazone bonded to an aldehyde on a carbohydrate side chain of the antibody. Or, group C can be a peptide bond formed with a lysine ε-amino group of antibody Z. In

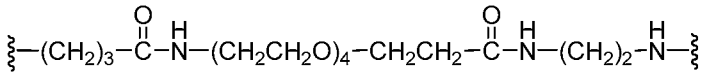
a preferred embodiment, compound D is directly bonded to group C via a peptidyl bond to a carboxyl or amine group in compound D.

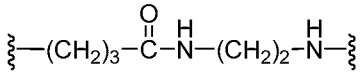
[00113] When present, spacer X^Z provides spatial separation between group C and antibody Z, lest the former sterically interfere with antigen binding by latter or the latter sterically interfere with cleavage of the former. Further, spacer X^Z can be used to confer increased solubility or decreased aggregation properties to conjugates. A spacer X^Z can comprise one or more modular segments, which can be assembled in any number of combinations. Examples of suitable segments for a spacer X^Z are:

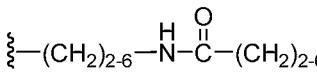

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where the subscript r is 1 to 24, preferably 2 to 4. These segments can be combined to make spacers X^Z such as:


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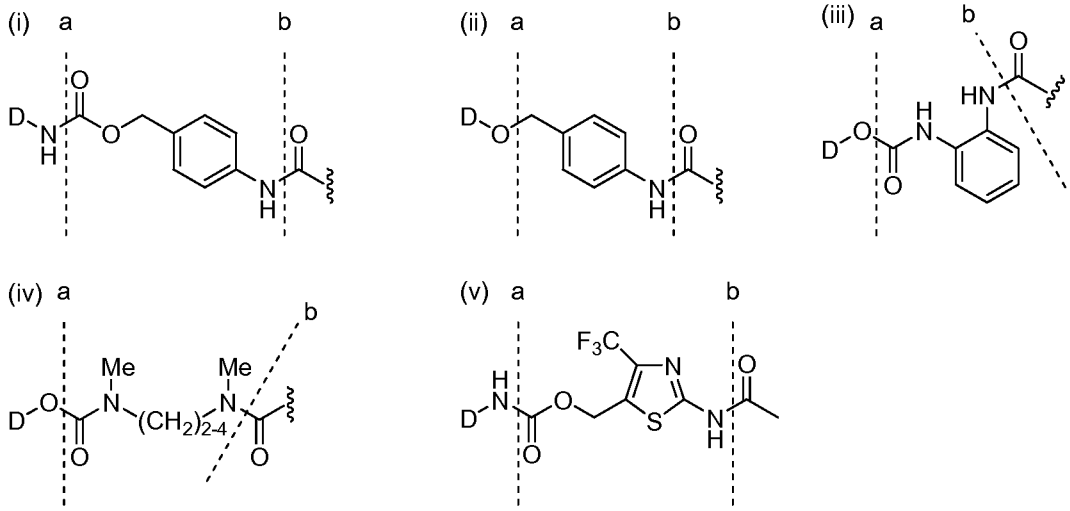
or

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[00114] Spacer X^D, if present, provides spatial separation between group C and compound D, lest the latter interfere sterically or electronically with cleavage of the former. Spacer X^D also can serve to introduce additional molecular mass and chemical functionality into a conjugate. Generally, the additional mass and functionality will affect the serum half-life and other properties of the conjugate. Thus, through judicious selection of spacer groups, the serum half-live of a conjugate can be modulated. Spacer X^D also can be assembled from modular segments, as described above in the context of spacer X^Z.

[00115] Either spacer X^Z or X^D, or both, can comprise a self-immolating moiety. A self-immolating moiety is a moiety that (1) is bonded to group C and either antibody Z or cytotoxin D and (2) has a structure such that cleavage from group C initiates a reaction sequence resulting in the self-immolating moiety disbonding itself from antibody Z or cytotoxin D, as the case may be. In other words, reaction at a site distal from antibody Z or cytotoxin D (cleavage from group C) causes the X^Z-Z or the X^D-D bond to rupture as

well. The presence of a self-immolating moiety is desirable in the case of spacer X^D because, if, after cleavage of the conjugate, spacer X^D or a portion thereof were to remain attached to cytotoxin D, the biological activity of the latter may be impaired. The use of a self-immolating moiety is especially desirable where cleavable group C is a polypeptide.

[00116] Exemplary self-immolating moieties (i)-(v) bonded to a hydroxyl or amino group on a partner molecule D are shown below:



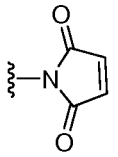
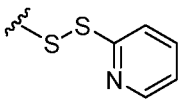
[00117] The self-immolating moiety is the structure between dotted lines a and b, with adjacent structural features shown to provide context. Self-immolating moieties (i) and (v) are bonded to a compound D-NH_2 (i.e., compound D is conjugated via an amino group), while self-immolating moieties (ii), (iii), and (iv) are bonded to a compound D-OH (i.e., compound D is conjugated via a hydroxyl or carboxyl group). Cleavage of the amide bond at dotted line b releases the amide nitrogen as an amine nitrogen, initiating a reaction sequence that results in the cleavage of the bond at dotted line a and the consequent release of D-OH or D-NH_2, as the case may be. For additional disclosures regarding self-immolating moieties, see Carl et al., *J. Med. Chem.*, 24 (3), 479-480 (1981); Carl et al., WO 81/01145 (1981); Dubowchik et al., *Pharmacology & Therapeutics*, **83**, 67-123 (1999); Firestone et al., US 6,214,345 B1 (2001); Toki et al., *J. Org. Chem.* **67**, 1866-1872 (2002); Doronina et al., *Nature Biotechnology* **21** (7), 778-784 (2003) (*erratum*, p. 941); Boyd et al., US 7,691,962 B2; Boyd et al., US 2008/0279868 A1; Sufi et al., WO 2008/083312 A2; Feng, US 7,375,078 B2; and Senter et al., US 2003/0096743 A1; the disclosures of which are incorporated by reference.

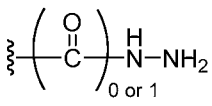
Compound D – Linker Compositions

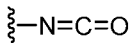
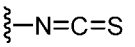
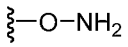
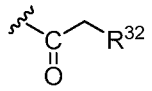
[00118] Conjugates of this invention preferably are prepared by first joining a compound D and linker $(X^D)_aC(X^Z)_b$ to form a drug-linker composition represented by formula (V-a):

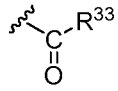
$$D\text{-}(X^D)_aC(X^Z)_b\text{-}R^{31} \qquad \text{(V-a)}$$

where R^{31} is a functional group suitable for reacting with a functional group on antibody Z to form the conjugate. Examples of suitable groups R^{31} include:


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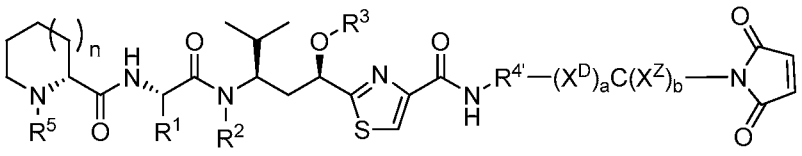
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where R^{32} is Cl, Br, F, mesylate, or tosylate and R^{33} is Cl, Br, I, F, OH, -O-N-succinimidyl, -O-(4-nitrophenyl), -O-pentafluorophenyl, or –O-tetrafluorophenyl. Chemistry generally usable for the preparation of suitable moieties $D\text{-}(X^D)_aC(X^Z)_b\text{-}R^{31}$ is disclosed in Ng et al., US 7,087,600 B2 (2006); Ng et al., US 6,989,452 B2 (2006); Ng et al., US 7,129,261 B2 (2006); Ng et al., WO 02/096910 A1; Boyd et al., US 7,691,962 B2; Chen et al., US 2006/0004081 A1; Gangwar et al., US 2006/0247295 A1; Boyd et al., US 2008/0279868 A1; Gangwar et al., US 2008/0281102 A1; Gangwar et al., US 2008/0293800 A1; Sufi et al., WO 2008/083312 A2; and Chen et al., US 2010/0113476 A1; the disclosures of which are incorporated herein by reference.

[00119] In a preferred embodiment, R^{31} is a maleimide group and the cytotoxic compound-linker molecule can be represented by formula (V-b):

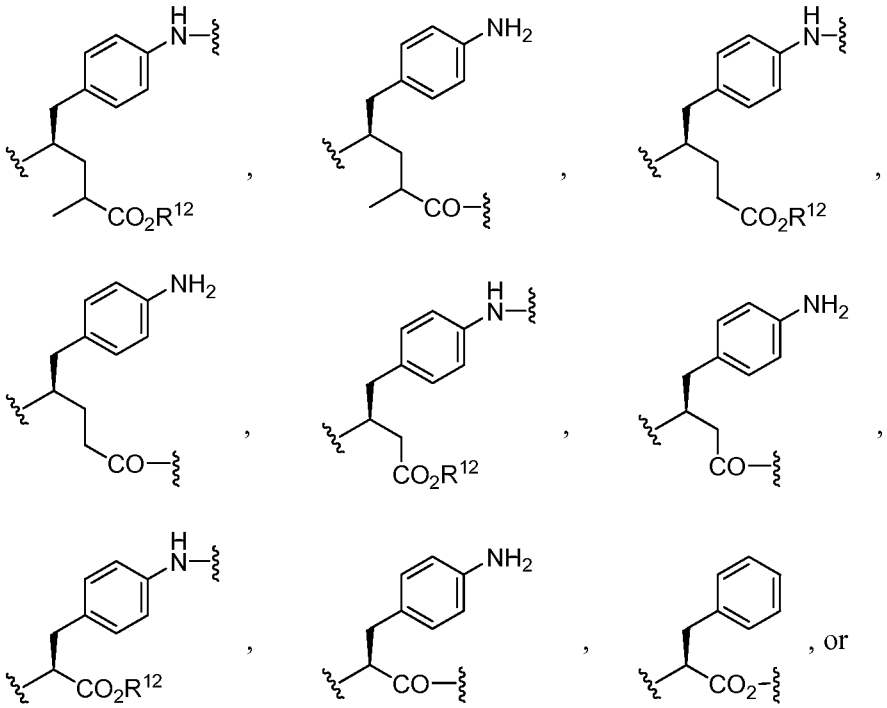

(V-b)

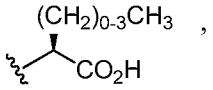
wherein

n is 0, 1, or 2;

R^1, R^2 and R^3 are independently H, unsubstituted or substituted C_1-C_{10} alkyl, unsubstituted or substituted C_2-C_{10} alkenyl, unsubstituted or substituted C_2-C_{10} alkynyl, unsubstituted or substituted aryl, unsubstituted or substituted heteroaryl, unsubstituted or substituted $(CH_2)_{1\text{-}2}O(C_1\text{-}C_{10}$ alkyl), unsubstituted or substituted $(CH_2)_{1\text{-}2}O(C_2\text{-}C_{10}$ alkenyl), unsubstituted or substituted $(CH_2)_{1\text{-}2}O(C_2\text{-}C_{10}$ alkynyl), $(CH_2)_{1\text{-}2}OC(=O)(C_1\text{-}C_{10}$ alkyl), unsubstituted or substituted $(CH_2)_{1\text{-}2}OC(=O)(C_2\text{-}C_{10}$ alkenyl), unsubstituted or substituted $(CH_2)_{1\text{-}2}OC(=O)(C_2\text{-}C_{10}$ alkynyl), unsubstituted or substituted $C(=O)(C_1\text{-}C_{10}$ alkyl), unsubstituted or substituted $C(=O)(C_2\text{-}C_{10}$ alkenyl), unsubstituted or substituted $C(=O)(C_2\text{-}C_{10}$ alkynyl), unsubstituted or substituted cycloaliphatic, unsubstituted or substituted heterocycloaliphatic, unsubstituted or substituted arylalkyl, or unsubstituted or substituted alkylaryl;

$R^{4'}$ is





wherein R^{12} is H, C_1-C_5 alkyl, C_2-C_5 alkenyl, or C_2-C_5 alkynyl; and

R^5 is H, C_1-C_5 alkyl, C_2-C_5 alkenyl, C_2-C_5 alkynyl, $CO(C_1$-C_5 alkyl), $CO(C_2$-C_5 alkenyl), or $CO(C_2$-C_5 alkynyl);

X^D and X^Z are spacer groups;

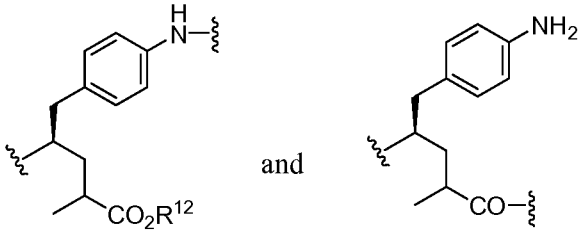
C is a cleavable group; and

a and b are independently 0 or 1;

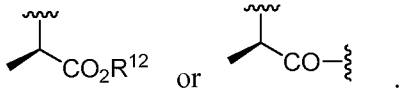
wherein group $R^{4'}$ is linked via a carboxyl or amine group therein to either group X^D in the event a is 1 or to group C in the event a is 0.

[00120] Preferably, the link between the carboxyl or amine group in $R^{4'}$ and group X^D or C, as the case may be, is via an amide bond.

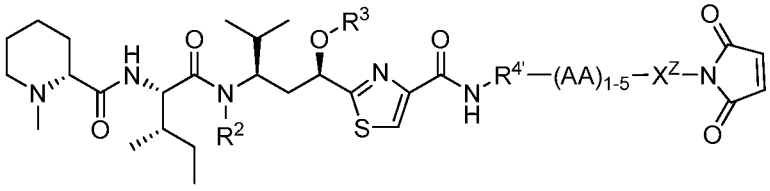
[00121] In the structures



the stereochemistry at the methyl group alpha to the carboxyl group preferably corresponds to that of the naturally occurring tubulysins, i.e.:



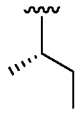
[00122] Preferably, in formula (V-b) n is 1, R^1 is an isoleucyl residue, a is 0, b is 1, and C comprises one to five amino acids (preferably one to two), and $R^{4'}$ is bonded to C by a peptidyl bond that is enzymatically cleavable, and R^5 is Me. This preferred embodiment is represented by formula (V-c):



(V-c)

where R^2, R^3, and $R^{4'}$ are as defined in respect of formula (V-b), each AA independently is a natural amino acid, and X^Z is $CH_2CH_2NHC(=O)(CH_2)_{2-5}$ or $C(=O)(CH_2)_{2-5}$. Preferred amino acids AA are lysine, citrulline, alanine, valine, glycine, and phenylalanine.

[00123] In formula (V-b), R^1 preferably is H, C_1-C_5 alkyl, C_2-C_5 alkenyl, or C_2-C_5 alkenyl, and is more preferably an isoleucyl residue, that is:

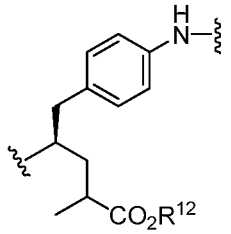


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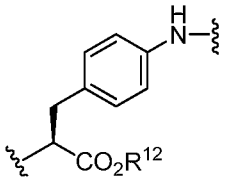
[00124] In formulae (V-b) and (V-c), R^2 preferably is H, C_1-C_5 alkyl, C_2-C_5 alkenyl, $CH_2O(C_1$-C_5 alkyl), $CH_2O(C_2$-C_5 alkenyl), $CH_2O(C=O)(C_1$-C_5 alkyl), or $CH_2OC(=O)(C_2$-C_5 alkenyl); and more preferably is H, Me, Et, CH_2OMe, CH_2OEt, $CH_2OC(=O)i$-Bu, $CH_2OC(=O)n$-Pr, $CH_2OC(=O)CH=CH_2$, or $CH_2OC(=O)Me$.

[00125] In formulae (V-b) and (V-c), R^3 preferably is H, C_1-C_5 alkyl, C_2-C_5 alkenyl, $C(=O)C_1$-C_5 alkyl, or $C(=O)C_2$-C_5 alkenyl; and more preferably is H, Me, Et, or C(=O)Me.

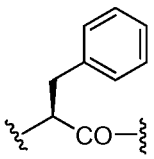
[00126] In formulae (V-b) and (V-c), $R^{4'}$ preferably is



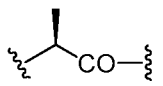
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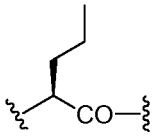
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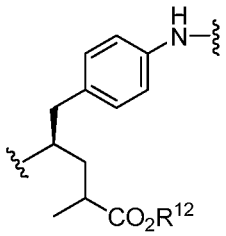


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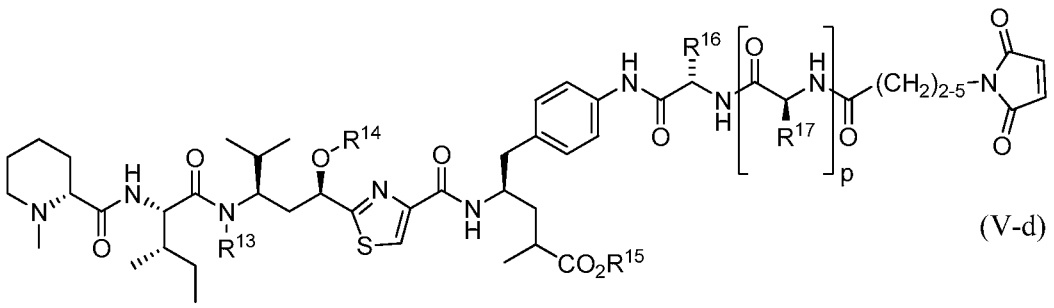
with $R^{4'}$ equaling



and R^{12} equaling H, Me, or Et being especially preferred.

[00127] In formula (V-b), n preferably is 1 and R^5 preferably is methyl; that is, the ring in the Mep subunit preferably is an N-methyl piperidinyl one.

[00128] In another embodiment, this invention provides a compound having a structure represented by formula (V-d)

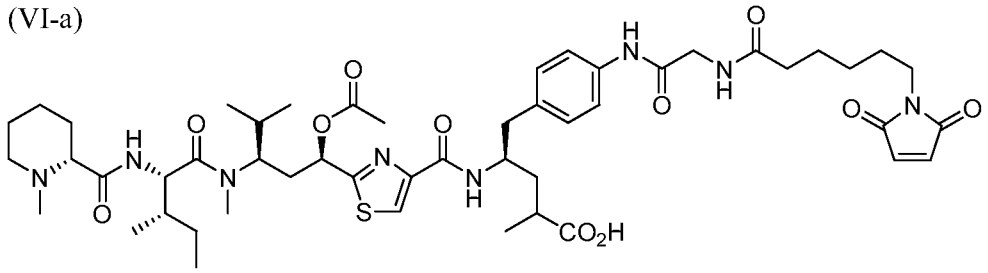


(V-d)

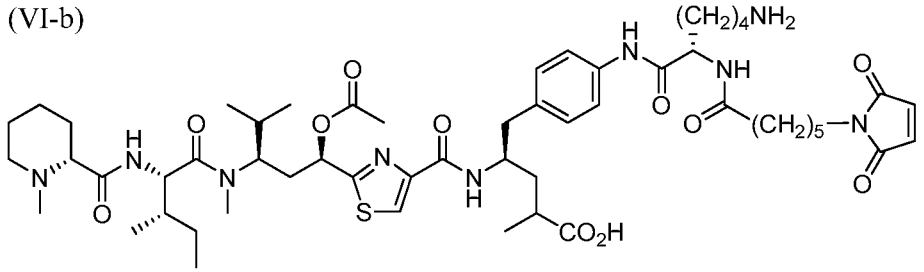
where R^{13} is Me, n-Pr, CH_2OMe, or $CH_2OC(=O)CH_2CH(Me)_2$; R^{14} is Me or C(=O)Me; and R^{15} is H or C_1-C_5 alkyl (preferably H, Me, or Et); R^{16} is a lysine ($(CH_2)_4NH_2$) or citrulline ($(CH_2)_3NHC(=O)NH_2$) side chain group; R^{17} is a valine ($C(Me)_2$) or alanine (Me) side chain group; and p is 0 or 1.

[00129] Examples of specific cytotoxic compound-linker constructs of this invention are shown below in formulae (VI-a) through (VI-t). Compound-linker (VI-n) is especially preferred. They have a maleimide group and are ready for conjugation to an antibody via a sulfhydryl group thereon, by a procedure such as that described below.

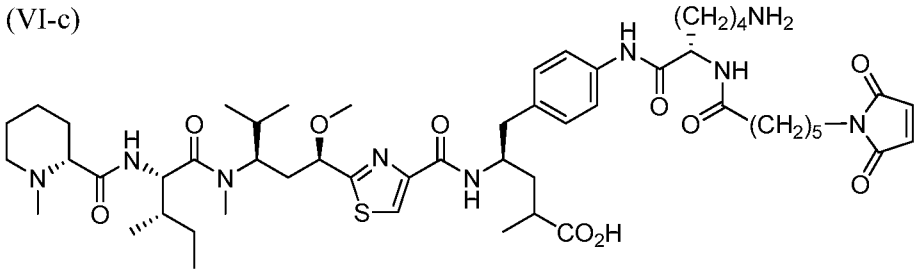
(VI-a)



(VI-b)

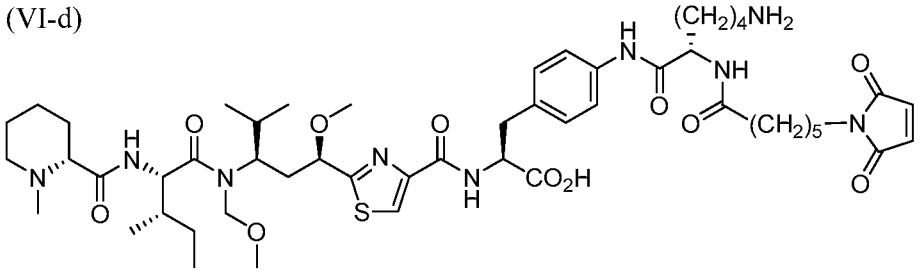


(VI-c)



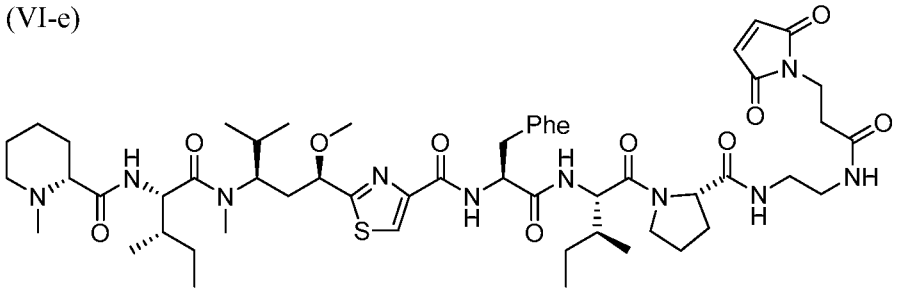
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(VI-d)



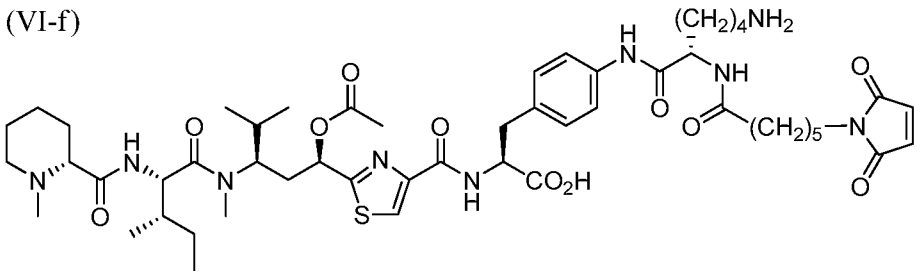
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(VI-e)



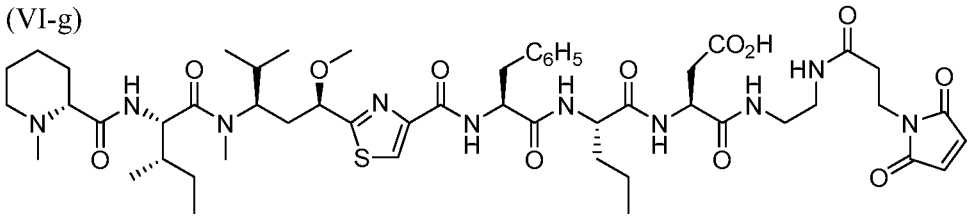
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(VI-f)



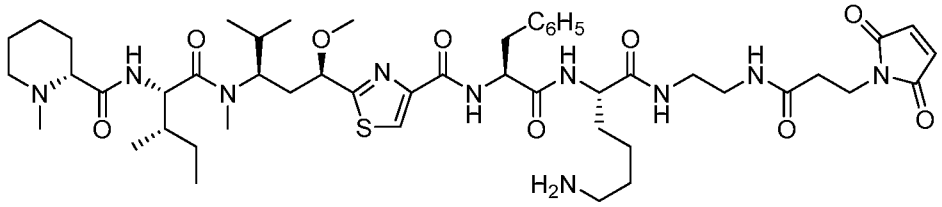
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(VI-g)



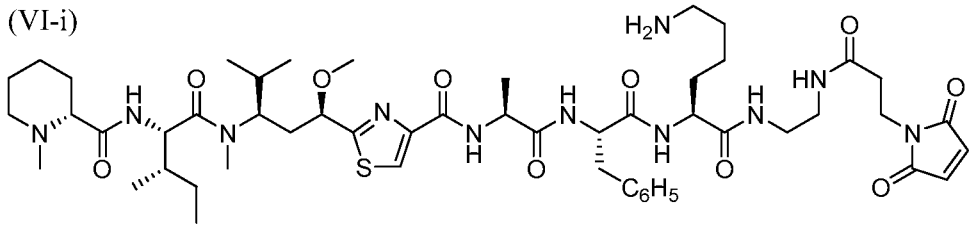
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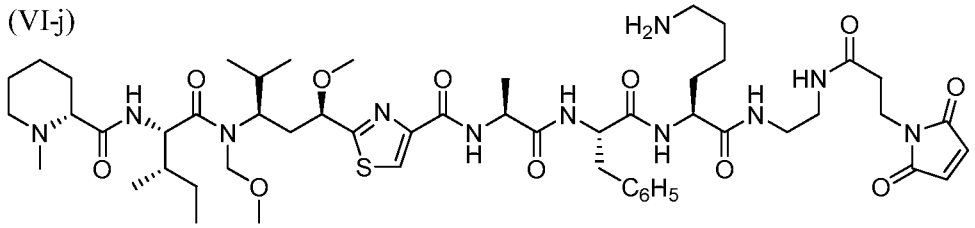
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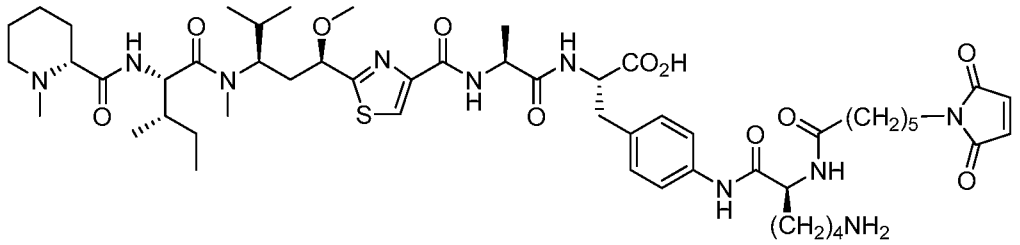
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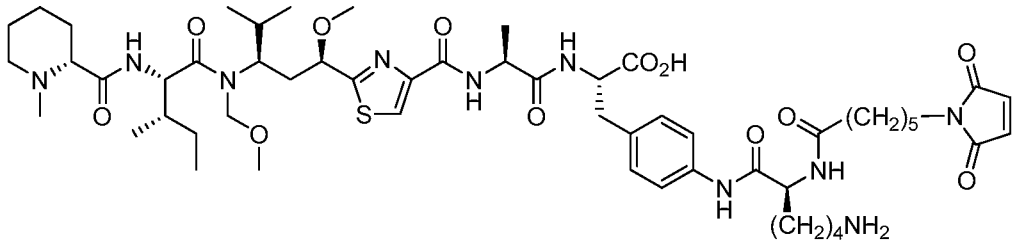
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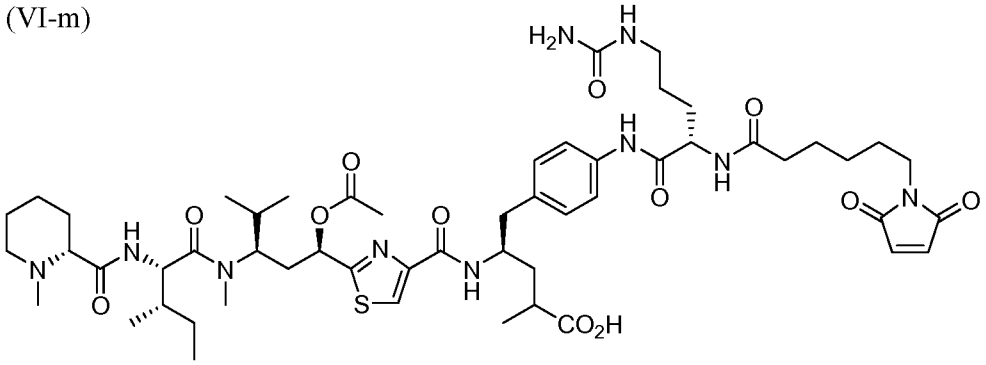
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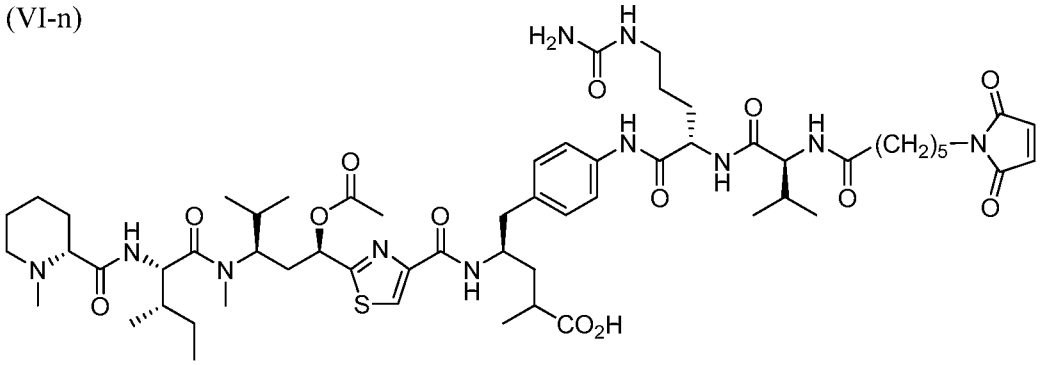
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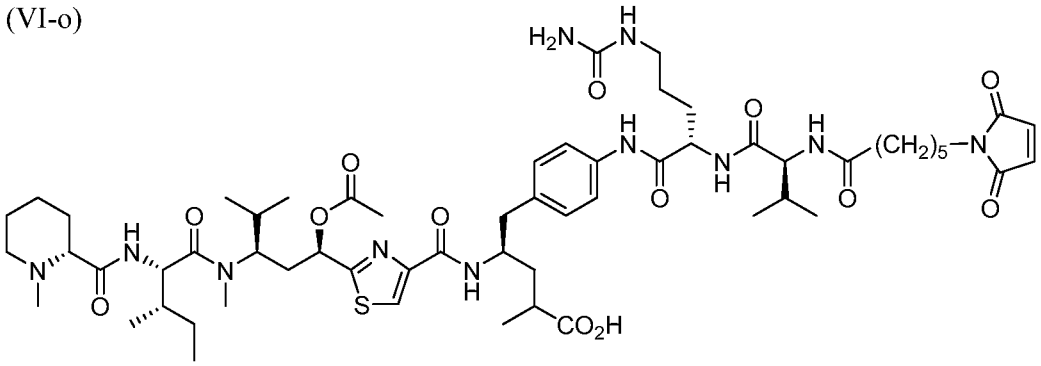
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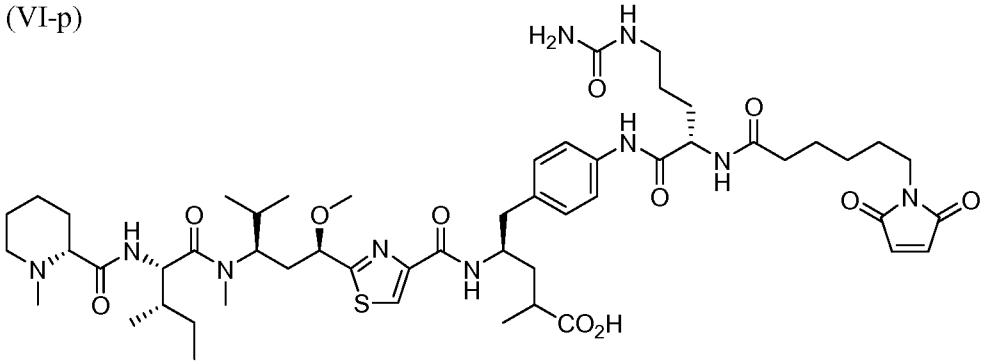
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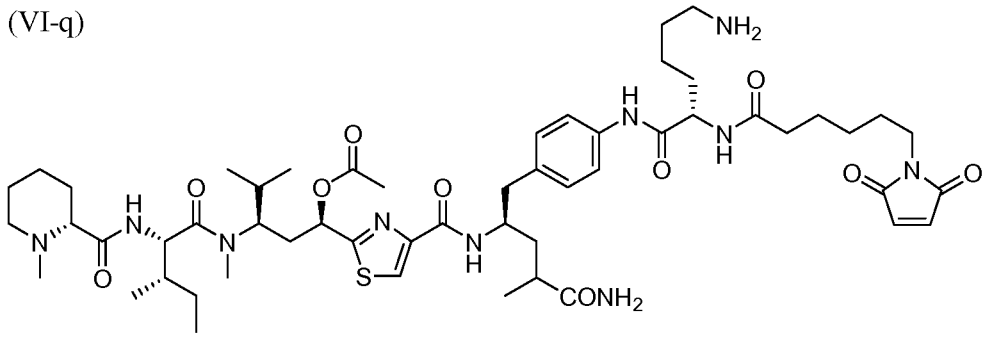
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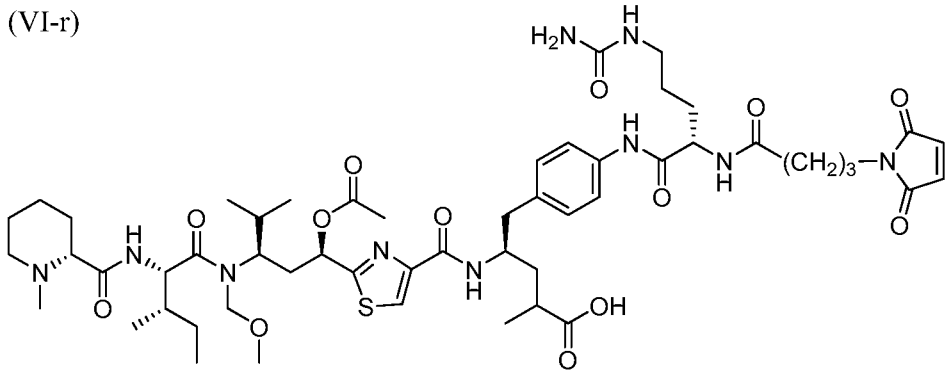
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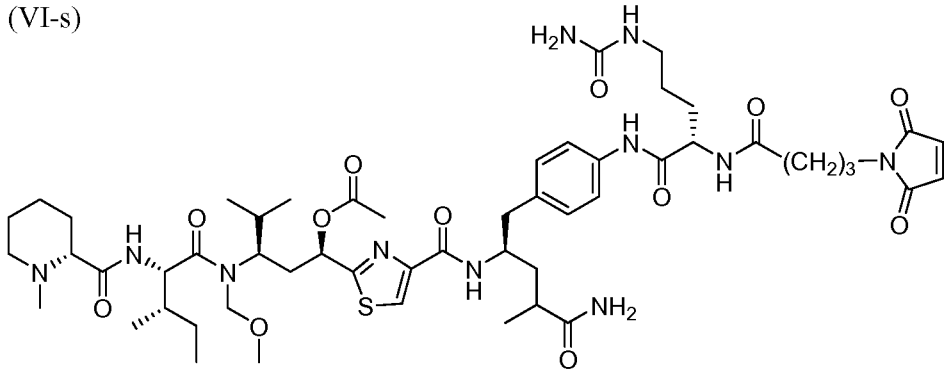
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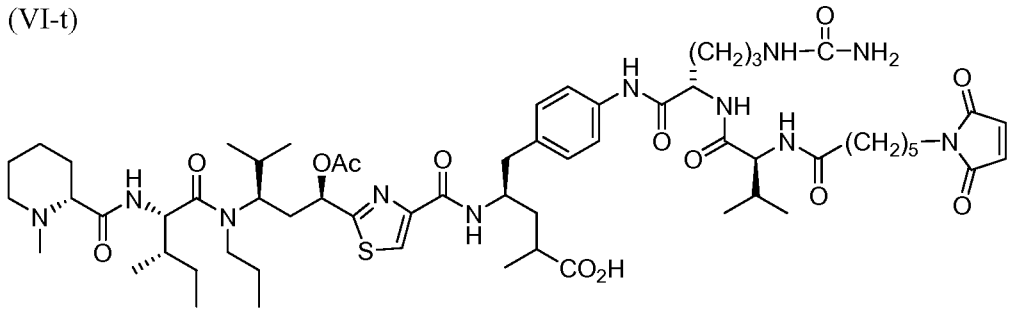
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, and

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Preparation of Conjugates

[00130] The following is an illustrative procedure, based on introduction of free thiol groups into an antibody by reaction of lysine ε-amino groups with 2-iminothiolane, followed by reaction with a maleimide-containing drug-linker moiety such as described above. Initially the antibody is buffer exchanged into 0.1 M phosphate buffer (pH 8.0) containing 50 mM NaCl and 2 mM diethylene triamine pentaacetic acid (DTPA) and concentrated to 5-10 mg/mL. Thiolation is achieved through addition of 2-iminothiolane to the antibody. The amount of 2-iminothiolane to be added can be determined by a preliminary experiment and varies from antibody to antibody. In the preliminary experiment, a titration of increasing amounts of 2-iminothiolane is added to the antibody, and following incubation with the antibody for 1 h at RT (room temperature, circa 25 °C), the antibody is desalted into 50 mM pH 6.0 HEPES buffer using a SEPHADEX™ G-25 column and the number of thiol groups introduced determined rapidly by reaction with dithiodipyridine (DTDP). Reaction of thiol groups with DTDP results in liberation of thiopyridine, which can be monitored spectroscopically at 324 nm. Samples at a protein concentration of 0.5-1.0 mg/mL are typically used. The absorbance at 280 nm can be used to accurately determine the concentration of protein in the samples, and then an aliquot of each sample (0.9 mL) is incubated with 0.1 mL DTDP (5 mM stock solution in ethanol) for 10 min at RT. Blank samples of buffer alone plus DTDP are also incubated alongside. After 10 min, absorbance at 324 nm is measured and the number of thiol groups is quantitated using an extinction coefficient for thiopyridine of 19,800 M^{-1}.

[00131] Typically a thiolation level of about three thiol groups per antibody is desirable. For example, with some antibodies this can be achieved by adding a 15-fold molar excess of 2-iminothiolane followed by incubation at RT for 1 h. The antibody is then incubated with 2-iminothiolane at the desired molar ratio and then desalted into conjugation buffer (50 mM pH 6.0 HEPES buffer containing 5 mM glycine and 2 mM DTPA). The thiolated material is maintained on ice while the number of thiols introduced is quantitated as described above.

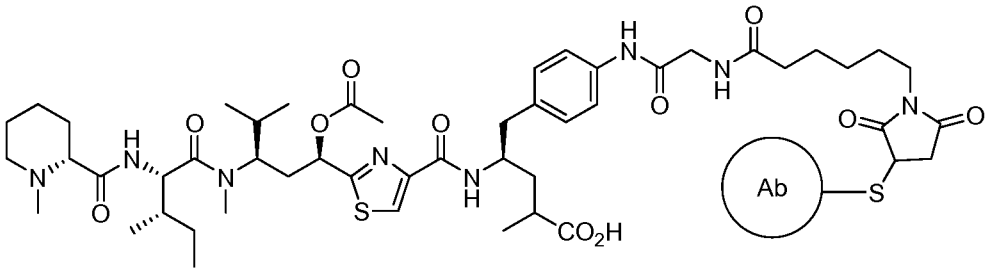
[00132] After verification of the number of thiols introduced, the drug-linker moiety is added at a 3-fold molar excess per thiol. The conjugation reaction is allowed to proceed in conjugation buffer also containing a final concentration of 5% dimethylsulfoxide (DMSO), or similar alternative solvent. Commonly, the drug-linker stock solution is

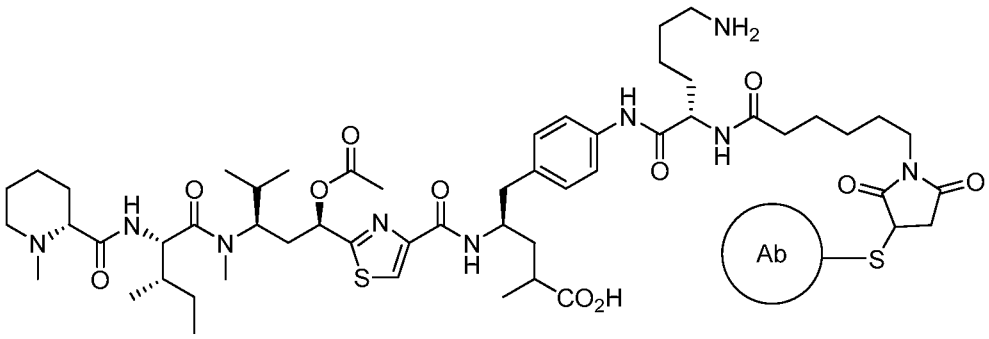
dissolved in 100% DMSO. The stock solution is added directly to the thiolated antibody, which has enough DMSO added to bring the final concentration to 10%, or pre-diluted in conjugation buffer containing a final concentration of 10% DMSO, followed by addition to an equal volume of thiolated antibody.

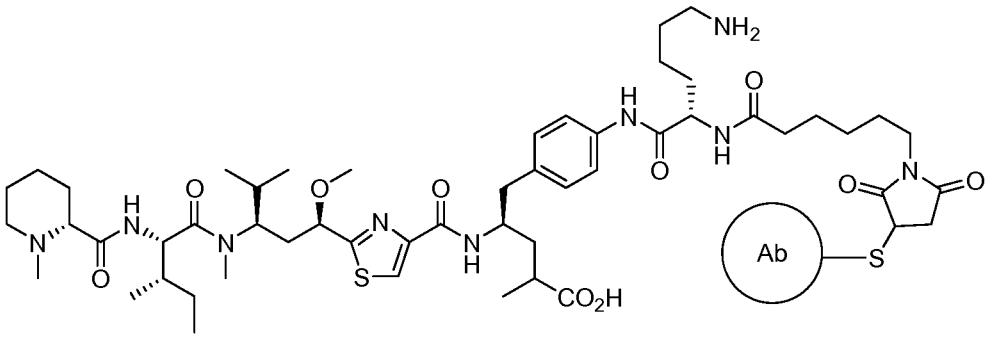
[00133] The conjugation reaction mixture is incubated at RT for 2 h with stirring. Following incubation, the conjugation reaction mixture is centrifuged and filtered through a 0.2 μm filter. Purification of the conjugate can be achieved through chromatography using a number of methods. In one method, the conjugate is purified using size-exclusion chromatography on a SEPHACRYL™ S200 column pre-equilibrated with 50 mM pH 7.2 HEPES buffer containing 5 mM glycine and 50 mM NaCl. Chromatography is carried out at a linear flow rate of 28 cm/h. Fractions containing conjugate are collected, pooled and concentrated. In an alternative method, purification can be achieved through ion-exchange chromatography. Conditions vary from antibody to antibody and should to be optimized in each case. For example, antibody-drug conjugate reaction mix is applied to an SP-SEPHAROSE™ column pre-equilibrated in 50 mM pH 5.5 HEPES containing 5mM glycine,. The antibody conjugate is eluted using a gradient of 0-1 M NaCl in equilibration buffer at pH 5.5. Relevant fractions containing the conjugate are pooled and dialyzed against formulation buffer (50 mM pH 7.2 HEPES buffer containing 5 mM glycine and 100 mM NaCl).

[00134] Those skilled in the art will understand that the above-described conditions and methodology are exemplary and non-limiting and that other approaches for conjugation are known in the art and usable in the present invention.

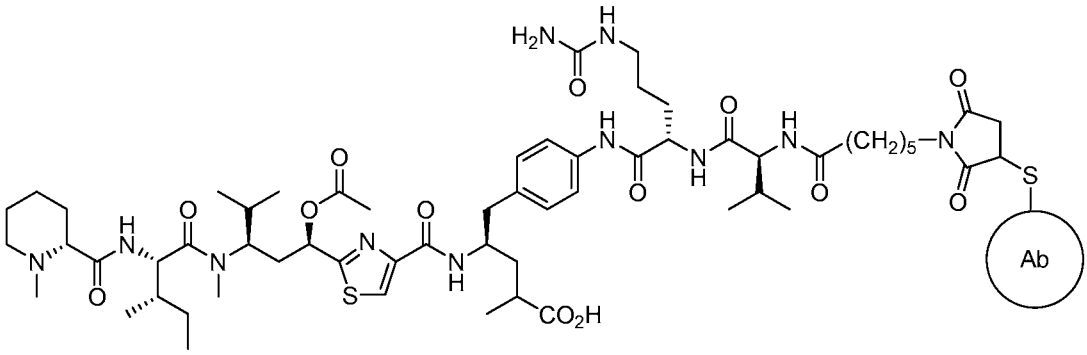
[00135] Using the foregoing techniques, compounds of this invention were conjugated using antibodies 2A10, an anti-PSMA antibody (Huang et al., US 2009/0297438); 2H5, an anti-CD70 antibody (Terrett et al., US 2009/0028872); 1F4, an anti-CD70 antibody (Coccia et al., WO 2008/074004); or 6A4, an anti-mesothelin antibody (Terrett et al., WO 2009/045957). The resulting conugates may be depicted by the following formulae, where Ab represents an antibody. Those skilled in the art will understand that in these formulae the cytotoxin-antibody compound ratio is shown as 1:1 for simplicity, but that in actuality the ratio is usually greater, such as between 2 to 3.

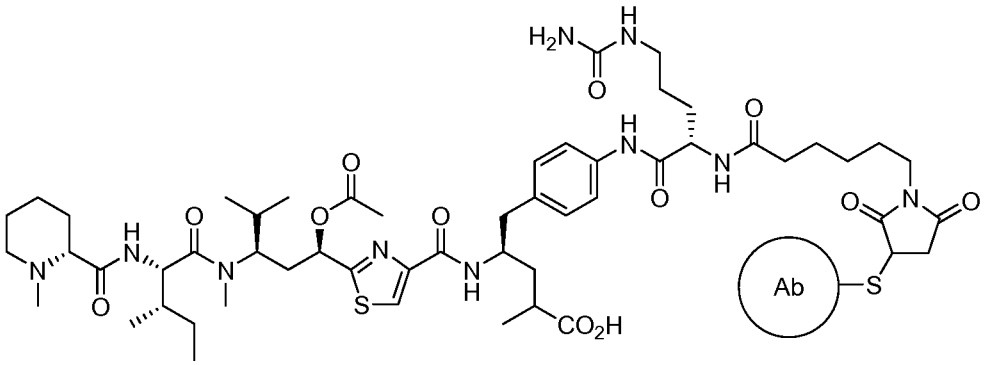


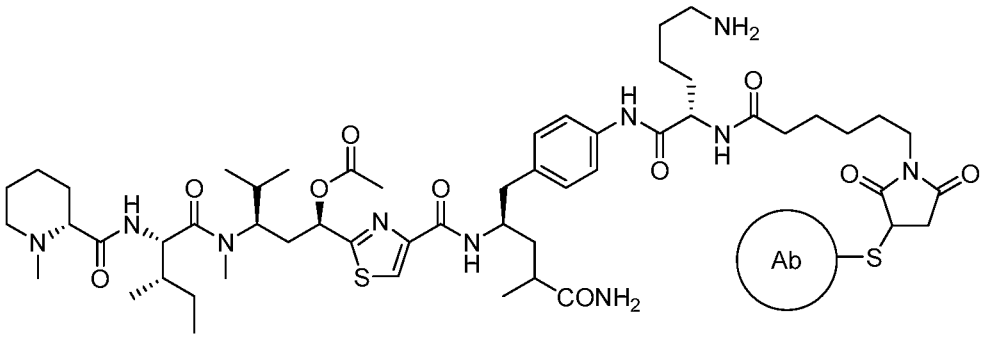


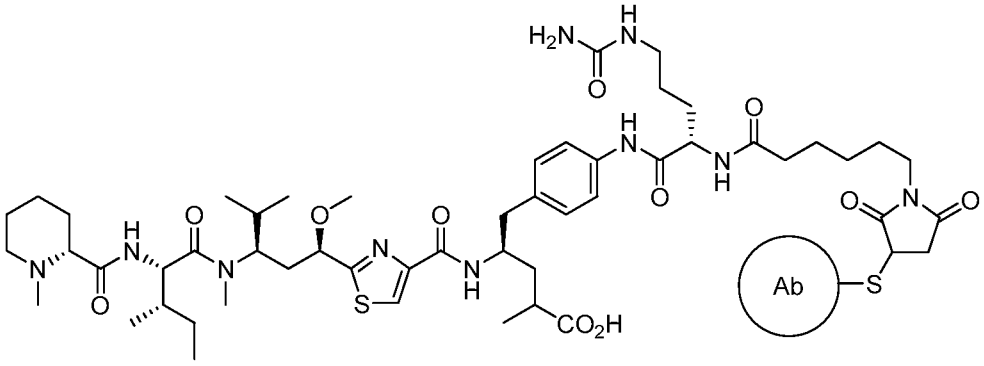


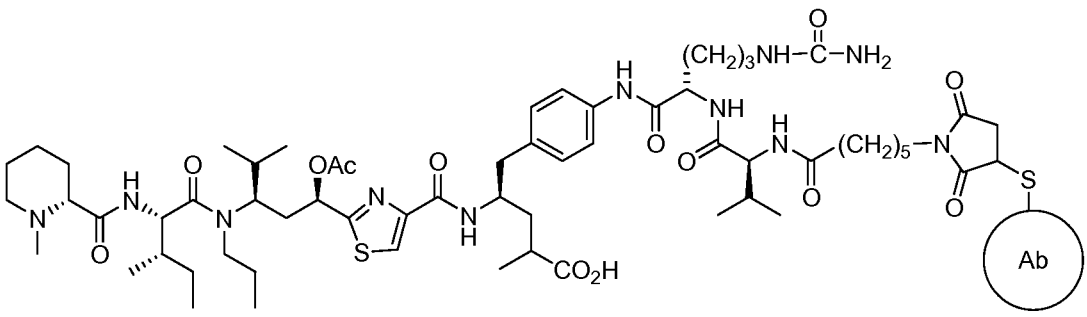
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PHARMACEUTICAL COMPOSITIONS

[00136] In another aspect, the present disclosure provides a pharmaceutical composition comprising a compound of the present disclosure formulated together with a pharmaceutically acceptable excipient. It may optionally contain one or more additional pharmaceutically active ingredients, such as an antibody or another drug. The pharmaceutical compositions can be administered in a combination therapy with another therapeutic agent, especially another anti-cancer agent.

[00137] The pharmaceutical composition may comprise one or more excipients. Excipients that may be used include carriers, surface active agents, thickening or emulsifying agents, solid binders, dispersion or suspension aids, solubilizers, colorants, flavoring agents, coatings, disintegrating agents, lubricants, sweeteners, preservatives, isotonic agents, and combinations thereof. The selection and use of suitable excipients is taught in Gennaro, ed., *Remington: The Science and Practice of Pharmacy*, 20th Ed. (Lippincott Williams & Wilkins 2003), the disclosure of which is incorporated herein by reference.

[00138] Preferably, a pharmaceutical composition is suitable for intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration (*e.g.,* by injection or infusion). Depending on the route of administration, the active compound may be coated in a material to protect it from the action of acids and other natural conditions that may inactivate it. The phrase "parenteral administration" means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion. Alternatively, the pharmaceutical composition can be administered via a non-parenteral route, such as a topical, epidermal or mucosal route of administration, for example, intranasally, orally, vaginally, rectally, sublingually or topically.

[00139] Pharmaceutical compositions can be in the form of sterile aqueous solutions or dispersions. They can also be formulated in a microemulsion, liposome, or other ordered structure suitable to achieve high drug concentration.

[00140] The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the subject being treated and the particular mode of administration and will generally be that amount of the

composition which produces a therapeutic effect. Generally, out of one hundred per cent, this amount will range from about 0.01 per cent to about ninety-nine percent of active ingredient, preferably from about 0.1 per cent to about 70 per cent, most preferably from about 1 per cent to about 30 per cent of active ingredient in combination with a pharmaceutically acceptable carrier.

[00141] Dosage regimens are adjusted to provide a therapeutic response. For example, a single bolus may be administered, several divided doses may be administered over time, or the dose may be proportionally reduced or increased as indicated by the exigencies of the situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. "Dosage unit form" refers to physically discrete units suited as unitary dosages for the subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic response, in association with the required pharmaceutical carrier.

[00142] The dosage ranges from about 0.0001 to 100 mg/kg, and more usually 0.01 to 5 mg/kg, of the host body weight. For example dosages can be 0.3 mg/kg body weight, 1 mg/kg body weight, 3 mg/kg body weight, 5 mg/kg body weight or 10 mg/kg body weight or within the range of 1-10 mg/kg. Exemplary treatment regimens are administration once per week, once every two weeks, once every three weeks, once every four weeks, once a month, once every 3 months, or once every three to 6 months. Preferred dosage regimens include 1 mg/kg body weight or 3 mg/kg body weight via intravenous administration, using one of the following dosing schedules: (i) every four weeks for six dosages, then every three months; (ii) every three weeks; (iii) 3 mg/kg body weight once followed by 1 mg/kg body weight every three weeks. In some methods, dosage is adjusted to achieve a plasma antibody concentration of about 1-1000 μg/mL and in some methods about 25-300 μg /mL.

[00143] A "therapeutically effective amount" of a compound of the invention preferably results in a decrease in severity of disease symptoms, an increase in frequency and duration of disease symptom-free periods, or a prevention of impairment or disability due to the disease affliction. For example, for the treatment of tumor-bearing subjects, a "therapeutically effective amount" preferably inhibits tumor growth by at least about 20%, more preferably by at least about 40%, even more preferably by at least about 60%, and still more preferably by at least about 80% relative to untreated subjects. A

therapeutically effective amount of a therapeutic compound can decrease tumor size, or otherwise ameliorate symptoms in a subject, which is typically a human but can be another mammal.

[00144] The pharmaceutical composition can be a controlled or sustained release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. See, *e.g., Sustained and Controlled Release Drug Delivery Systems*, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

[00145] Therapeutic compositions can be administered via medical devices such as (1) needleless hypodermic injection devices (e.g., US 5,399,163; 5,383,851; 5,312,335; 5,064,413; 4,941,880; 4,790,824; and 4,596,556); (2) micro-infusion pumps (US 4,487,603); (3) transdermal devices (US 4,486,194); (4) infusion apparati (US 4,447,233 and 4,447,224); and (5) osmotic devices (US 4,439,196 and 4,475,196); the disclosures of which are incorporated herein by reference.

[00146] In certain embodiments, the pharmaceutical composition can be formulated to ensure proper distribution *in vivo*. For example, to ensure that the therapeutic compounds of the invention cross the blood-brain barrier, they can be formulated in liposomes, which may additionally comprise targeting moieties to enhance selective transport to specific cells or organs. See, e.g. US 4,522,811; 5,374,548; 5,416,016; and 5,399,331; V.V. Ranade (1989) *J. Clin. Pharmacol.* 29:685; Umezawa *et al.*, (1988) *Biochem. Biophys. Res. Commun.* 153:1038; Bloeman *et al.* (1995) *FEBS Lett.* 357:140; M. Owais *et al.* (1995) *Antimicrob. Agents Chemother.* 39:180; Briscoe *et al.* (1995) *Am. J. Physiol.* 1233:134; Schreier *et al.* (1994) *J. Biol. Chem.* 269:9090; Keinanen and Laukkanen (1994) *FEBS Lett.* 346:123; and Killion and Fidler (1994) *Immunomethods* 4:273.

USES

[00147] Compounds of this invention or their conjugatescan be used for treating diseases such as, but not limited to, hyperproliferative diseases, including: cancers of the head and neck which include tumors of the head, neck, nasal cavity, paranasal sinuses, nasopharynx, oral cavity, oropharynx, larynx, hypopharynx, salivary glands, and paragangliomas; cancers of the liver and biliary tree, particularly hepatocellular

carcinoma; intestinal cancers, particularly colorectal cancer; ovarian cancer; small cell and non-small cell lung cancer (SCLC and NSCLC); breast cancer sarcomas, such as fibrosarcoma, malignant fibrous histiocytoma, embryonal rhabdomyosarcoma, leiomysosarcoma, neurofibrosarcoma, osteosarcoma, synovial sarcoma, liposarcoma, and alveolar soft part sarcoma; leukemias such as acute promyelocytic leukemia (APL), acute myelogenous leukemia (AML), acute lymphoblastic leukemia (ALL), and chronic myelogenous leukemia (CML); neoplasms of the central nervous systems, particularly brain cancer; multiple myeloma (MM), lymphomas such as Hodgkin's lymphoma, lymphoplasmacytoid lymphoma, follicular lymphoma, mucosa-associated lymphoid tissue lymphoma, mantle cell lymphoma, B-lineage large cell lymphoma, Burkitt's lymphoma, and T-cell anaplastic large cell lymphoma. Clinically, practice of the methods and use of compositions described herein will result in a reduction in the size or number of the cancerous growth and/ or a reduction in associated symptoms (where applicable). Pathologically, practice of the method and use of compositions described herein will produce a pathologically relevant response, such as: inhibition of cancer cell proliferation, reduction in the size of the cancer or tumor, prevention of further metastasis, and inhibition of tumor angiogenesis. The method of treating such diseases comprises administering a therapeutically effective amount of an inventive combination to a subject. The method may be repeated as necessary. Especially, the cancer can be colorectal cancer, liver cancer, prostate cancer, breast cancer, melanoma, glioblastoma, lung cancer, pancreatic cancer, ovarian cancer, multiple myeloma, renal cancer, leukemia (especially ALL, APL, or AML), or lymphoma.

[00148] Compounds of this invention or their conjugates can be administered in combination with other anti-cancer or cytotoxic agents, including antibodies, alkylating agents, angiogenesis inhibitors, antimetabolites, DNA cleavers, DNA crosslinkers, DNA intercalators, DNA minor groove binders, enediynes, heat shock protein 90 inhibitors, histone deacetylase inhibitors, immunomodulators, microtubule stabilizers, nucleoside (purine or pyrimidine) analogs, nuclear export inhibitors, proteasome inhibitors, topoisomerase (I or II) inhibitors, tyrosine kinase inhibitors, and serine/threonine kinase inhibitors. Specific anti-cancer or cytotoxic agents include β-lapachone, ansamitocin P3, auristatin, bicalutamide, bleomycin, bortezomib, busulfan, callistatin A, camptothecin, capecitabine, CC-1065, cisplatin, cryptophycins, daunorubicin, disorazole, docetaxel,

doxorubicin, duocarmycin, dynemycin A, epothilones, etoposide, floxuridine, floxuridine, fludarabine, fluoruracil, gefitinib, geldanamycin, 17-allylamino-17-demethoxy-geldanamycin (17-AAG), 17-(2-dimethylaminoethyl)amino17-demethoxygeldanamycin (17-DMAG), gemcitabine, hydroxyurea, imatinib, interferons, interleukins, irinotecan, maytansine, methotrexate, mitomycin C, oxaliplatin, paclitaxel, suberoylanilide hydroxamic acid (SAHA), thiotepa, topotecan, trichostatin A, vinblastine, vincristine, vindesine, lenalidomide (REVLIMID®), bevacizumab (AVASTIN®), trastuzumab (HERCEPTIN®), and cetuximab (ERBITUX®).

EXAMPLES

[00149] The practice of this invention can be further understood by reference to the following examples, which are provided by way of illustration and not of limitation.

Example 1 – Scheme 1

[00150] Scheme 1 (**Figs. 1a** and **1b**) depicts a method for making compounds of this invention.

[00151] *Thioamide 2.* 2,2-Diethoxyacetonitrile **1** (25 g, 193 mmol) was mixed with $(NH_4)_2S$ (40 mL, 265 mmol, 45% aq. solution in) in 300 mL of methanol (MeOH) at room temperature (RT). After keeping the reaction mixture overnight, it was concentrated under vacuum and the residue was taken up in ethyl acetate (EtOAc). The EtOAc solution was washed with saturated $NaHCO_3$ solution then brine and dried over anhydrous Na_2SO_4. The EtOAc was evaporated to give thioamide **2** (26 g, 159 mmol, 82%) as a white solid. ^{1}HNMR (400 MHz, $CDCl_3$) δ 5.01 (s, 1H), 3.67 (m, 4H), 1.22 (t, J = 7.2 Hz, 6H).

[00152] *Methyl 2-(diethoxymethyl)thiazole-4-carboxylate **3**.* 100 g of molecular sieves (3A) was added to a reaction mixture of thioamide **2** (25 g, 153 mmol) and methyl bromopyruvate (20 mL, 163 mmol) in 300 mL of MeOH. After the mixture was refluxed for 1.5 h, it was cooled and filtered through CELITE™. The filtrate was concentrated and passed through a column (dichloromethane (DCM):EtOAc, 8:1) to give thiazole carboxylate **3** (34.5 g, 140 mmol, 91%) as a solid, which was used for next step without further purification.

[00153] *Methyl 2-formylthiazole-4-carboxylate **4**.* Thiazole-4-carboxylate **3** (30 g, 122 mmol) was dissolved in 300 mL of acetone, to which was added aqueous HCl (21 mL,

2M). After keeping the reaction mixture at RT for overnight, the reaction mixture was heated up and kept at 60 °C for 2 h. The reaction mixture was then cooled and evaporated under vacuum to give a residue which was taken up in 200 mL of DCM. DCM solution was then washed with saturated $NaHCO_3$ solution and then brine and dried over anhydrous Na_2SO_4. DCM solution was filtered and concentrated under vacuum to give the concentrated solution which was triturated by ether to give methyl 2-formylthiazole-4-carboxylate **4** (14 g, 82 mmol, 54% for two steps) as a white solid. ^{1}HNMR (400 MHz, $CDCl_3$) δ 133-8-p110.16 (d, J = 1.3 Hz, 1H), 8.53 (d, J = 1.3 Hz, 1H), 4.01 (s, 3H).

[00154] *Sulfinimine 7.* (S)-2-Methylpropane-2-sulfinamide **5** (7.3 mL, 68 mmol) was dissolved in 100 mL tetrahydrofuran (THF), to which was added $Ti(OEt)_4$ (27 mL, 130 mmol) and 3-methyl-2-butanone **6** (8g, 41 mmol) at RT. The reaction mixture was refluxed overnight and then cooled and added to a brine solution. The resultant mixture was filtered and the cake was washed with EtOAc. The organic phase was concentrated to give a residue which was subjected silica gel column chromatography (DCM:EtOAc, 4:1) to give sulfinimine **7** (9.5 g, 37 mmol, 75%) as an oil. ^{1}HNMR (400 MHz, $CDCl_3$) δ 2.53 (m, 1H), 2.29 (s, 3H), 1.22 (s, 9H), 1.12 (d, J = 4.2 Hz, 3H), 1.10 (d, J = 4.2 Hz, 3H) MS (ES+) m/z, calculated: m+1, 190.12, found, 190.

[00155] *Compound 8.* Lithium diisopropyl amide ("LDA," 60 mL, 108 mmol, 1.8M) was added to 200mL of ether at -78 °C followed by the addition of sulfinimine **7** (18.9g, 100 mmol) in 200 mL ether and the resultant reaction mixture was stirred for 40 min. $ClTi(OiPr)_3$ (203 mmol, 48.4 mL) was added to the reaction mixture and the solution was stirred for 60 min. A solution of methyl 2-formylthiazole-4-carboxylate **4** (12.5 g, 72.6mmol) in 180 mL of THF was added slowly to the reaction mixture. After another 2 h at -78 °C, a mixture of acetic acid and THF (1/5 v/v, 4.9 mL) was added. The mixture was warmed to 5 °C over 1 h and then poured into brine solution. The desired product was then extracted from the brine solution with ether and EtOAc solution. The organic phase was then dried over anhydrous $MgSO_4$, filtered and evaporated. The residue was passed through 2 columns (DCM:EtOAc and hexane:EtOAc) to give compound **8** (19.6 g, 54 mmol, 75%) as an oil. MS (ES+) m/z, calculated: m+1, 361.12, found, 361.

[00156] *Compound 9.* A solution of compound **8** (19 g, 52.7 mmol) in 200 mL of THF was cooled to -78 °C, after which $Ti(OEt)_4$ (21.7 mL, 105 mmol) was added slowly. After 60 min, when the solution became clear, $NaBH_4$ (31 mmol, 1.17g) was added, after 2 h

(longer reaction time caused reduction of the ester) 10 mL MeOH was added. The mixture was then warmed to 0 °C, poured into 1 mL HOAc in a lot of ice. The mixture was filtered and the cake was washed with EtOAc. After separation, the organic phase was dried with Na_2SO_4 and evaporated. The final residue was passed through a column (DCM:EtOAc, 1:4) to give compound **9** (19 g, 52mmol, 99%) as an oil. ^{1}HNMR (400 MHz, $CDCl_3$) δ 8.1 (s, 1H), 5.54 (d, J = 6.7 Hz, 1H), 5.16 (m, 1H), 3.92 (s, 3H), 3.42 ((m, 1H), 3.32 (d, J = 8.4 Hz, 1H), 2.25 (m, 1H), 1.88 (m, 1H), 1.68 (m, 1H), 1.26 (s, 9H), 0.91 (d, J = 6.8 Hz, 3H), 0.87 (d, J = 6.7 Hz, 3H). ^{13}CNMR (100 MHz, $CDCl_3$) δ 177.9, 162.1, 146.6, 127.7, 67.9, 58.6, 56.4, 52.5, 40.8, 33.9, 23.1, 19.8, 17.4. MS (ES+) m/z, calculated: m+1, 363.13, found, 363.

[00157] *Dimethylated compound **10**.* Sodium hydride (9.69 mmol, 60%, 387 mg) was added to a solution of compound **9** in 6 mL N,N-dimethylformamide (DMF) at 5 °C followed by methyl iodide (607 uL, 9.7 mmol) after 60 minutes. After stirring the reaction mixture for 3 h, the mixture was poured into ice-cooled saturated NH_4Cl solution. Ethyl ether was added and the organic layer was washed with brine, dried over anhydrous $MgSO_4$ and concentrated to give a residue. The residue was passed through a column (hexane:EtOAc 1:4) to give dimethylated compound **10** (314 mg, 0.805 mmol, 33%) as a liquid. ^{1}HNMR (400 MHz, $CDCl_3$) δ 8.17 (s, 1H), 4.87 (dd, J = 11.0 Hz, J = 2.5 Hz, 1H), 3.94 (s, 3H), 3.50 (s, 3H), 3.40 (m, 1H), 2.58 (s, 3H), 1.99 (m, 1H), 1.83 (m, 2H), 1.25 (s, 9H), 0.98 (d, J = 6.8 Hz, 3H), 0.95 (d, J = 6.7 Hz, 3H) MS (ES+) m/z, calculated: m+1, 391.16, found, 391.

[00158] *Monomethyl amine **11**.* Aqueous HCl (4N, in dioxane, 0.5 mL) was added to a solution of dimethylated compound **10** (370 mg, 0.95 mmol) in 5 mL MeOH. After stirring the reaction mixture for 60 min, it was evaporated under vacuum to give monomethyl amine **11** (362 mg) as its HCl salt, which was used for next step without further purification. MS (ES+) m/z, calculated: m+1, 287.14, found, 287.

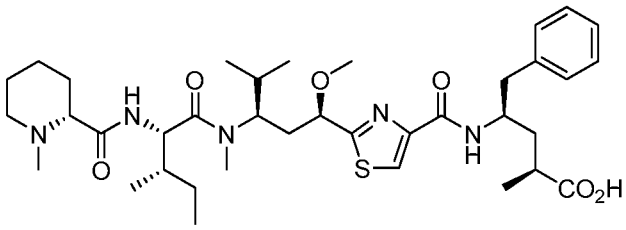
[00159] *Amide **12**.* Monomethyl amine **11** (362 mg, 1.12 mmol), Fmoc compound **22** (prepared per Wipf et al. 2007; 1.2 g, 3.38 mmol) and N,N-diisopropylethylamine (DIEA, 976 uL, 5.6 mmol) were mixed in 5 mL DMF at RT. After stirring for 24 h, the mixture was concentrated and the residue was dissolved in EtOAc. The organic phase was washed with $NaHCO_3$, brine, dried over anhydrous $MgSO_4$ and concentrated to give a residue.

The residue was passed through a column (hexane:EtOAt:MeOH 7:3:0.6) to give amide **12** (466 mg, 0.75 mmol, 67%) as an oil. (ES+) m/z, calculated: m+1, 622.2, found, 622.

[00160] *Compound 13.* Amide **12** (466 mg, 0.75 mmol) was dissolved in 8 mL DCM containing 5% piperidine at RT. After 1 h, the mixture was evaporated under vacuum and the residue was passed through a column to give an oil (150 mg) which was then mixed with(D)-*N*-methyl pipecolinic acid **23** ("D-Mep," prepared per Peltier et al., 2006; 50 mg, 0.35 mmol), *N,N,N',N'*-tetramethyl-*O*-(7-azabenzotriazol-1-yl)uronium hexafluoro-phosphate ("HATU," 126 mg, 0.33 mmol), DIEA (152 uL, 0.84 mmol) in 2 mL DCM. After stirring for 2.5 h, the solvent was evaporated to give a residue which was dissolved in EtOAc. The organic phase was then washed with saturated $NaHCO_3$, brine, dried over anhydrous $MgSO_4$ and concentrated to give a residue. The residue was passed through a column (DCM:MeOH 0-10%) to give compound **13** (99 mg, 0,188 mmol, 25%) as an oil. MS (ES+) m/z, calculated: m+1, 525.3, found, 525.

[00161] *Acid 14.* Compound **13** (99 mg, 0.18 mmol) was dissolved in a 3 mL mixture of MeOH and water (3:1 v:v), to which was added NaOH (370 uL, 0.37 mmol, 1M). After stirring for two h, the reaction mixture was neutralized and concentrated to give a residue. The residue was passed through a C-18 column (water (1% trifluoroacetic acid ("TFA")):acetonitrile (ACN) (1%TFA), 0-100%) to give acid **14** (78 mg, 0.125 mmol, 69%) as a TFA salt. MS (ES+) m/z, calculated: m+1, 511.29, found, 511.

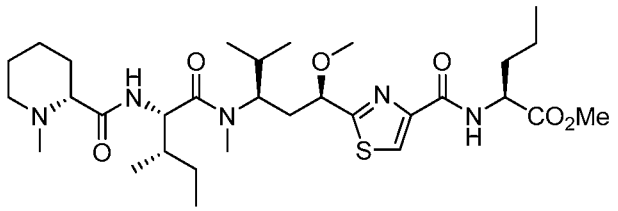
[00162] *Compound 15.* DIEA (24 uL, 137 μmol) was added to a solution of acid **14** (9 mg, 14.4 umol, TFA salt) and HATU (6 mg, 15 umol) in DMF (0.5 mL) at RT. After all of acid **14** was activated (monitored by HPLC), tubuphenylalanine (prepared per Peltier et al. 2006; 6.5 mg, 27 umol, HCl salt) was added. After stirring for 20 mins, the reaction mixture was passed through a C-18 column (water (1% TFA):ACN (1%TFA), 0-100%) to give compound **15** (2.5 mg, 3 umol, 21%) as a white TFA salt. MS (ES+) m/z, calculated: m+1, 700.4, found, 700.



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[00163] *Compound 16.* DIEA (20 uL, 0.11 mmol) was added to a solution of acid **14** (12 mg, 0.019 mmol, TFA salt), phenylalanine methyl ester (5.3 mg, 0.024 mmol, as HCl salt) and HATU (11.4 mg, 0.03 mmol) in 0.5 mL DMF. After 30 min, the reaction mixture was passed through a C-18 column (water (1% TFA):ACN (1%TFA) 0-100%) to give compound **16** (4.2 mg, 0.005 mmol, 26%) as a white TFA salt. MS (ES+) m/z, calculated: m+1, 672.89, found, 672.5. Compound **16** is also referred to as compound (III-c) hereinabove.

[00164] *Compound 17.* DIEA (7 uL, 0.04 mmol) was added to a solution of acid **14** (5 mg, 0.008 mmol), norvaline methyl ester ("NVaM," 2 mg, 0.012 mmol) and HATU (4.5 mg, 0.012 mmol) in DMF. After stirring the reaction mixture for 30 min the crude mixture was passed through a C-18 column (water (1% TFA):ACN (1%TFA) 0-100%) to give compound **17** (1.3 mg, 0.0017 mmol, 21%) as a white TFA salt. MS (ES+) m/z, calculated: m+1, 624.85, found, 624.5. Compound **17** is also referred to as compound (III-e) hereinabove.



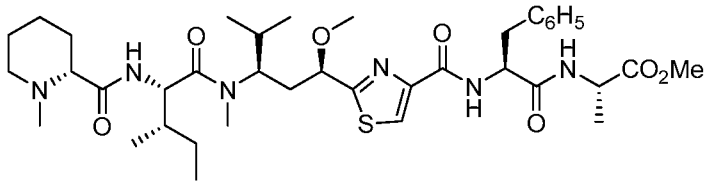
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[00165] *Acid 18.* Aqueous NaOH (75 uL, 0.75 mmol, 10M) was added to a solution of compound **16** (168 mg, 0.25 mmol) in a mixture of MeOH and THF. After stirring overnight, the mixture was neutralized and lyophilized to dryness. The solid was used for next step without further purification. MS (ES+) m/z, calculated: m+1, 658.36, found, 658.4. Acid **18** is also referred to as compound (III-o) hereinabove.

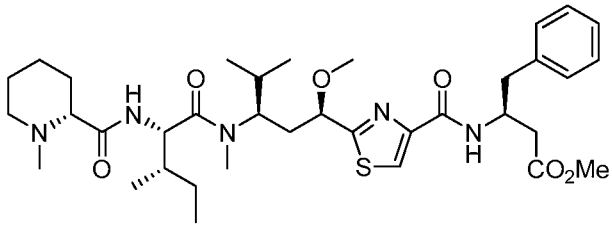
[00166] *Norvalyl amide 19.* DIEA (5, uL, 0.03 mmol) was added to a solution of acid **18** (5 mg, 0.006 mmol), HATU (3.5 mg, 0.009 mmol) and NVaM (1.5 mg, 0.009 mmol) in DMF. After stirring for 30 min, the reaction mixture was passed through a C-18 column (water (1% TFA):ACN (1%TFA) 0-100%) to give norvalyl amide **19** (2.2, 0.0025 mmol, 40%) as a white TFA salt. MS (ES+) m/z, calculated: m+1, 772.02, found, 771.5. Norvalyl amide **19** is also referred to as compound (III-f) hereinabove.

[00167] *Compounds 24, 25, and 26.* These three compounds were synthesized from acid **14** or **18** using procedures analogous to those described above. The products were all

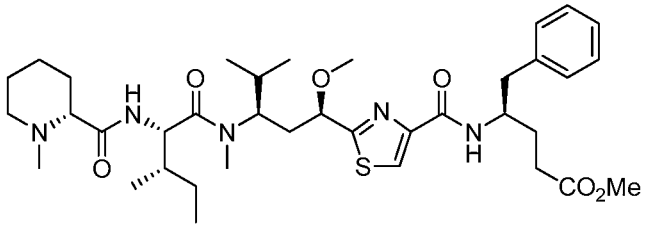
purified by a C-18 column (water (1% TFA):ACN (1%TFA) 0-100%). The yields varied from 25-50%. Compound **24**: MS (ES+) m/z, calculated: m+1, 743.4, found 743. Compound **25**: MS (ES+) m/z, calculated: m+1, 686.39, found 686.5. Compound **26**: MS (ES+) m/z, calculated: m+1, 700.40, found 700.5.



24



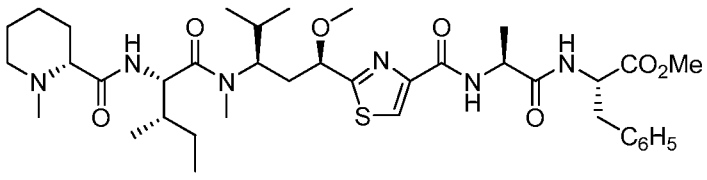
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[00168] Compound **24** is also referred to as compound (III-g) hereinabove.

[00169] *Compound 26a.* Compound **14** was coupled with Ala-Phe OMe following the same procedure as described for compound **16**. The product was purified by a C-18 column (water (1% TFA):ACN (1%TFA), 0-100%). MS (ES+) m/z, calculated: m+1, 743, found 743.4. Compound **26a** is also referred to as compound (III-l) hereinabove.



26a

Example 2 – Scheme 2

[00170] This example describes the synthesis of compounds shown in Scheme 2 (**Fig. 2**).

[00171] *Compound 28.* Phenylalanine methyl ester (10 mg, 46.5 μmol) and HATU (14.7 mg, 38.6 umol) were added to a solution of compound **27** (prepared per Peltier et al., 2006; 10 mg, 15.5 μmol, formate salt) in 0.5 mL DMF followed by DIEA at RT. After stirring for 30 minutes, DMSO (2 mL) was added and the reaction mixture was directly subjected to a C-18 column (water (5 mM ammonium formate, pH 7.2):ACN 0-100%), to give compound **28** (3.2 mg, 25%) as a white solid (formate salt). MS (ES+) m/z, calculated: m+1, 758.41, found 758.4. Compound **28** is also referred to as compound (III-h) hereinabove.

[00172] *Compound 29.* Acetic anhydride (30 μL, 290 μmol) was added to a solution of compound **28** (3.2 mg, 3 μmol, formate salt) in 0.5 mL pyridine at 0 °C. After stirring for 24 h, solvent was was evaporated from the reaction mixture and the residue was passed through a regular silica column (DCM:MeOH 0-10%) to give compound **29** (2.0 mg, 83%) as an oil. MS (ES+) m/z, calculated: m+1, 800.42, found 800.4. Compound **29** is also referred to as compound (III-i) hereinabove.

[00173] *O-Acetyl, N,O-acetal **29a**.* Compound **29** (2 mg, 2.4 μmol) was dissolved in 0.5 mL MeOH, to which was added a drop of 4N HCl in dioxane. After stirring the reaction mixture overnight at RT, the mixture was concentrated and the residue was dissolved in DMSO, which was then passed through a C-18 column (water (20 mM ammonium formate, pH 6.1): ACN (0-100%) to give O-acetyl, N,O-acetal **29a** (1.38 mg, 75%) as a white solid (formate salt) after lyophilization. MS (ES+) m/z, calculated: m+1, 730, found 730.4. O-Acetyl, N,O-acetal **29a** is also referred to as compound (III-m) hereinabove.

[00174] *N,O-Acetal **29b**.* Compound **28** (5 mg, 6 umol) was dissolved in MeOH, to which was added 1 drop of 4N HCl in dioxane. After stirring the reaction mixture for 24 hrs, the solution was concentrated and used for next step reaction without further purification. MS (ES+) m/z, calculated: m+1, 687, found 688.4. N,O-Acetal **29b** is also referred to as compound (III-n) hereinabove.

[00175] *O-Methyl, N,O-acetal **29c**.* N,O-Acetal **29b** (about 5 mg, 7.2 umol) was dissolved in DMF, to which was added dimethylsulfate (3 μL, 37 μmol) and NaH (2 mg, 50 μmol, 60% in mineral oil) at 0 °C. After 1 h, the mixture was taken up in DMSO and passed through a C-18 column (water (20 mM ammonium formate, pH 6.1): ACN (0-100%) to give O-methyl, N,O-acetal **29c** as a semi-solid (0.31 mg, 5%; mixture contain-

ing an unidentified compound of same MW). MS (ES+) m/z, calculated: m+1, 702, found 702.4. O-methyl, N,O-acetal **29c** is also referred to as compound (III-k) hereinabove.

Example 3 – Scheme 3

[00176] Scheme 3 (**Fig. 3**) shows a procedure for making compounds according to formula (II-b).

[00177] *Alcohol **30**.* TFA (171 mL) was added to a solution of (*S*)-*tert*-butyl-1-hydroxy-3-(4-nitrophenyl)propan-2-ylcarbamate **20** (Erlanson et al., US 7,214,487 B2; 13.9 g, 46.9 mmol) in DCM (272 mL) at 0 °C. The reaction mixture was warmed to RT and reaction was allowed to proceed for 25 minutes. The solution was concentrated to afford 9.2 g of crude (*S*)-2-amino-3-(4-nitrophenyl)propan-1-ol as a white solid. To a solution of this crude product and sodium carbonate (12.4 g, 117.3 mmol) in THF (87 mL) and water (87 mL) was added *N*-carbethoxyphthalimide ("CEPT," 12.3 g, 56.3 mmol). After the reaction mixture was stirred at RT for 4 h, EtOAc (150 mL) was added. The aqueous phase was extracted with EtOAc. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated under vacuum to give a crude residue which was purified by flash chromatography eluting from silica gel with a gradient of 0-100% EtOAc in hexanes to give 12.3 g of alcohol **30.** MS: (+) m/z 327.0 (M+1).

[00178] *Triflate **31**.* To a solution of alcohol **30** (1 g, 3.06 mmol) in anhydrous DCM (18 mL) was added pyridine (0.274 mL, 3.36 mmol) at -78 °C. After the reaction mixture was stirred at -78 °C for 5 min, trifluoromethanesulfonic anhydride (0.568 mL, 3.36 mmol) was added. The reaction mixture was stirred at -78 °C for 45 min, and then at RT for 45 min. After the precipitate was filtered off, the filtrate was purified by flash chromatography eluting from silica gel with DCM to yield 0.84 g of triflate **31**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.10 (2H, d, J = 8.8 Hz), 7.81 (2H, m), p7.74 (2H, m), 7.36 (2H, J = 8.8 Hz), 5.13 (1H, t, J = 10.0 Hz), 4.99 (1H, m), 4.80 (1H, dd, J = 4.8, 5.6 Hz), 3.52 (1H, dd, J = 3.2, 11.2 Hz), and 3.27 (1H, dd, J = 5.6, 8.8 Hz).

[00179] *Diester **32**.* Diethyl methylmalonate (0.71 mL, 4.12 mmol) was added dropwise to a suspension of sodium hydride (0.161 g, 60% dispersion in mineral oil, 4.03 mmol) in anhydrous THF (4.7 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 10 min, and then at RT for 10 min. The resulting solution was added slowly to a solution of triflate **31** (0.84 g, 1.83 mmol), in anhydrous THF (9.4 mL) at 0 °C. After the reaction

mixture was stirred at 0 °C overnight, saturated NH_4Cl aqueous solution (20 mL) was added. The aqueous solution was extracted with EtOAc, and the combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and concentrated under vacuum to give a residue. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-50% EtOAc in hexanes to afford 0.57 g of diester **32**. MS: (+) m/z 483.3 (M+1).

[00180] *Monoester 33.* A solution of diester **32** in 6 N HCl (10 mL) and acetic acid (10 mL) was heated at 145 °C for 2 days. The organic solution was concentrated to afford 0.41 g of the hydrochloride salt of the crude (*R*)-4-amino-2-methyl-5-(4-nitrophenyl)pentanoic acid as a white solid.

[00181] 2,2-Dimethoxypropane ("DMP," 4 mL, 32.6 mmol) was added to a solution of the hydrochloride salt of the crude product and concentrated HCl (1 mL) in anhydrous MeOH (20 mL). The reaction mixture was heated at 60 °C overnight. The organic solution was concentrated to afford 0.43 g of the hydrochloride salt of the crude (*R*)-methyl 4-amino-2-methyl-5-(4-nitrophenyl)pentanoate as a white solid.

[00182] Triethylamine (0.44 mL, 3.1 mmol) was added to a solution of the hydrochloride salt of the crude (*R*)-methyl 4-amino-2-methyl-5-(4-nitrophenyl)pentanoate and di-*tert*-butyl dicarbonate (0.369 g, 1.69 mmol) in ACN (10 mL) at RT. After the reaction mixture was stirred at RT for 4 h, the solvent was evaporated. Water (20 mL) was added, and the aqueous solution was extracted with EtOAc. The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-30% EtOAc in hexanes to afford 0.31 g of monoester **33** as a colorless oil. MS: (+) m/z 267.3 (M-99).

[00183] *Carboxylic acid 34.* A solution of monoester **33** (0.31 g, 0.846 mmol) in 6 N HCl was heated at 130 °C for 1.5 h. The organic solution was concentrated to afford 0.244 g of carboxylic acid **34** as a white solid. MS: (+) m/z 253.1 (M+1).

[00184] *Nitro acid 35.* Compound **34a** (80.4 mg, 0.149 mmol, prepared per Patterson et al. 2008) was added to a 0.2 M solution of pentafluorophenol (41.1 mg, 0.224 mmol) and *N*, *N*'-diisopropylcarbodiimide ("DIC," 0.0255 mL, 0.164 mmol) in DCM (0.76 mL) at 0 °C. The reaction mixture was warmed to RT and stirred at RT overnight. The solvent was evaporated. EtOAc (18 mL) was added and the crude product was filtered, with rinsing of the reaction vessel with EtOAc. The filtrate was concentrated under reduced

pressure and the crude material was used without further purification. DMF (0.6 mL) was added to the crude product, followed by carboxylic acid **34** (0.129 g, 0.448 mmol) and DIEA (0.13 mL, 0.745 mmol). The reaction mixture was stirred at RT overnight and the solvent was evaporated off. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 10-20% MeOH in DCM containing 1% NH_4OH to afford 0.11 g of nitro acid **35** as a white solid. MS: (+) m/z 773.4 (M+1).

[00185] *Amino acid **36**.* A solution of nitro acid **35** (0.11 g, 0.142 mmol) and palladium on carbon (10%, 15 mg) in MeOH (5 mL) was stirred under a hydrogen atmosphere for 4 h. The catalyst was filtered off, and the filtrate was concentrated to afford 91 mg of amino acid **36** as a white solid. MS: (+) m/z 743.5 (M+1). Amino acid **36** is also referred to as compound (III-b) hereinabove.

[00186] *Methyl ester **36a**.* HCl (1 drop, 37%) was added to a solution of amino acid **36** (1.9 mg, 2.5 mmol) and 2, 2-dimethoxypropane ("DMP," 0.05 mL, 0.41 mol) in MeOH (0.5 mL). The reaction mixture was stirred at RT for 2 h and then concentrated. The crude product was purified by preparative HPLC to afford 1.7 mg of methyl ester **36a** as a white solid. MS: (+) m/z 757.5 (M+1). Ester **36a** is also depicted in this specification by formula (III-t).

Example 4 – Scheme 4

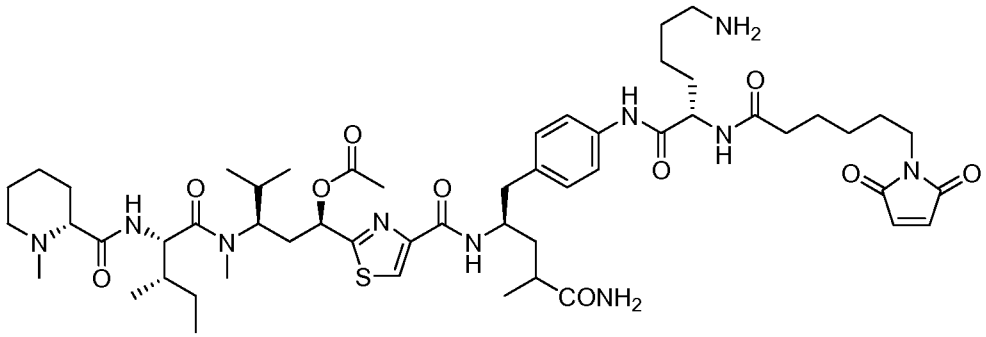
[00187] Scheme 4 (**Fig. 4**) shows a method for attaching peptidyl linkers and reactive functional groups to compounds of this invention, ready for conjugation.

[00188] *Compound **37**.* A solution of DIEA, Fmoc-Lys(Boc)-OH (17.3 mg, 0.037 mmol), and HATU (12.8 mg, 0.0336 mmol) in DMF (0.3 mL) was stirred at RT for 5 min. The pH of the solution was maintained between 8 and 9. Then a solution of amino acid **36** (25 mg, 0.0336 mmol) in DMF (2 mL) and DIEA was added to the reaction mixture, maintaining the pH between 8 and 9. After stirring at RT for 15 min, saturated NH_4Cl solution (5 mL) was added to quench the reaction. The aqueous solution was extracted with EtOAc, and the combined organic layers were dried, filtered, and concentrated. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-20% MeOH in DCM to afford 36.1 mg of compound **37**. MS: (+) m/z 1193.6 (M+1).

[00189] *Compound 38.* Piperidine was added to a solution of compound **37** (36.1 mg, 0.0302 mmol) in DMF (2 mL), maintaining pH between 9 and 10. After stirring at RT for 20 min, the organic solution was concentrated to afford 29.3 mg of the crude free α-amino compound.

[00190] DIEA was added to a solution of 6-maleimidohexanoic acid (7.0 mg, 0.0332 mmol) and HATU (11.5 mg, 0.0302 mmol) in DMF (0.3 mL) maintaining the pH between 8 and 9. The reaction mixture was stirred at RT for 5 min. Then DIEA and the crude free amino compound in DMF (2 mL) were added, maintaining the pH between 8 and 9. After the reaction mixture was stirred at RT for 15 min, the crude product was purified by preparative HPLC to afford 9.1 mg of compound **38** as a white solid. MS: (+) m/z 1164.6 (M+1).

[00191] *Compound 39.* TFA (1.5 mL) was added to a solution of compound **38** (9.1 mg, 0.0078 mmol) in DCM (1.5 mL). After the reaction mixture was stirred at RT for 15 min, the crude product was purified by preparative HPLC to afford 5.0 mg of the TFA salt of the desired compound **39** as a white solid. MS: (+) m/z 1064.8 (M+1). The free base structure of compound **39** is also shown hereinabove, as compound (VI-b). Some amide of compound **39** was also isolated as a by-product in its preparation. MS: (+) m/z 1063.6 (M+1). The amide is also depicted in this specification by formula (VI-q).



Amide of compound **39**

Example 5 – Scheme 5

[00192] Scheme 5 (**Fig. 5**) shows an alternative procedure for making compounds according to formula (II-b).

[00193] *Amino ester 42.* 4.0 N HCl in 1,4-dioxane (6.7 mL) was added to a solution of compound **41** (prepared per Patterson et al. 2008; 1 g, 2.66 mmol) in ethanol (17 mL).

The reaction mixture was stirred at RT for 2 h, and then concentrated to afford 0.82 g of amino ester **42** as a white solid. MS: (+) m/z 273.3 (M+1).

[00194] *Azido ester **43**.* Oxalyl chloride (1.71 mL, 19.95 mmol) and DMF (0.33 mL, 4.26 mmol) were added to a solution of azido isoleucine (Lundquist et al., *Org. Lett.* **2001**, *3*, 781; 0.669 g, 4.26 mmol) in hexanes (176 mL). The reaction mixture was stirred at RT for 1 h, filtered, and concentrated to afford the acid chloride. The acid chloride and DIEA (2.32 mL, 13.3 mmol) were added to a solution of amino ester **42** (0.82 g, 2.66 mmol) in DCM (26.7 mL) at 0 °C. The reaction mixture was allowed to warm to RT and stirred at RT overnight. Brine was added to quench the reaction, and the aqueous solution was extracted with EtOAc. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated under vacuum. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-50% EtOAc in hexanes to afford 0.86 g of azido ester **43** as a white solid. MS: (+) m/z 412.3 (M+1).

[00195] *Triethylsilyl compound **44**.* 2,6-Lutidine (1.22 mL, 10.45 mmol) and triethylsilyl trifluoromethanesulfonate (1.14 mL, 5.02 mmol) were added to a solution of azido ester **43** (0.86 g, 2.09 mmol) in DCM (11 mL) at 0 °C. The reaction mixture was allowed to warm to RT over 1 h, and then stirred at RT for an additional hour. Brine was added to quench the reaction, and the aqueous solution was extracted with EtOAc. The combined organic layers were dried, filtered, and concentrated. The crude product was purified by silica gel flash chromatography eluting with a gradient of 0-30% EtOAc in hexanes to afford 1.1 g of triethylsilyl compound **44**. MS: (+) m/z 526.4 (M+1).

[00196] *N-Methyl compound **45**.* A solution of triethysilyl compound **44** (1.04 g, 1.98 mmol) in THF (6.5 mL) was cooled at -45 °C, and potassium hexamethyldisilazide (0.5 M in toluene, 4.75 mL, 2.37 mmol) was added. The resulting mixture was stirred for 20 min at -45 °C. Methyl iodide (0.37 mL, 5.94 mmol) was added, and the reaction mixture was allowed to warm to RT over 4 h at which time the reaction was quenched with ethanol (10 mL). The crude product was diluted with EtOAc and washed with brine, and the aqueous layer was extracted with EtOAc. The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under vacuum. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-30% EtOAc in hexanes to afford 0.91 g of N-methyl compound **45**. MS: (+) m/z 540.4 (M+1).

[00197] *Compound 46.* A solution of N-methyl compound **45** (1.0 g, 1.85 mmol) in deoxygenated AcOH/H_2O/THF (65 mL, 3:1:1, v/v/v) was stirred at RT for 36 h. Toluene (250 mL) was added and the solution was concentrated. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-100% EtOAc in hexanes to afford 0.46 g of compound **46** as oil. MS: (+) m/z 426.3 (M+1).

[00198] *Methyl ether 47.* Potassium hexamethyldisilazide ("KHMDS," 0.5 M in toluene, 2.54 mL, 1.27 mmol) was added to a solution of compound **46** (0.45 g, 1.06 mmol) in THF (5 mL) at -78 °C. The reaction mixture was stirred for 20 min at -78 °C. Methyl iodide (0.2 mL, 3.18 mmol) was added, and the reaction mixture was allowed to warm to -20 °C over 2 h at which time the reaction was quenched with saturated NH_4Cl solution. The aqueous solution was extracted with EtOAc. The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under vacuum. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-50% EtOAc in hexanes to afford 0.41 g of compound **47** as colorless oil. MS: (+) m/z 440.3 (M+1).

[00199] *Compound 48.* To a solution of D-Mep (0.45 g, 3.14 mmol) in EtOAc (10 mL) were added pentafluorophenol (0.64 g, 3.47 mmol) and N,N'-dicyclohexylcarbodiimide ("DCC," 0.72 g, 3.47 mmol). After the reaction mixture was stirred at RT overnight, the precipitate was filtered, and washed with EtOAc. To the resulting filtrate waa added compound **47** (0.46 g, 1.05 mmol) and palladium on carbon (10 wt%, 0.36 g). The reaction mixture was stirred under a hydrogen atmosphere overnight. The catalyst was filtered off, and then the filtrate was concentrated under vacuum. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-5% MeOH in EtOAc to afford 0.43 g of compound **48** as colorless oil. MS: (+) m/z 539.4 (M+1).

[00200] *Carboxylic acid 49.* To a solution of compound **48** (0.43 g, 0.80 mmol) in deoxygenated 1,4-dioxane (8 mL) was added deoxygenated lithium hydroxide aqueous solution (0.6 M, 4 mL) at RT. The reaction mixture was stirred at RT for 2 h, and then concentrated under vacuum. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 10-30% MeOH in DCM containing 1% NH_4OH to afford 0.3 g of carboxylic acid **49** as a white solid. MS: (+) m/z 511.4 (M+1).

[00201] *Nitro acid 50.*_Carboxylic acid **49** (80 mg, 0.157 mmol) was added to a 0.2 M solution of pentafluorophenol (43.3 mg, 0.235 mmol) and DIC (0.0269 mL, 0.173 mmol)

in DCM (0.8 mL) at 0°C. The reaction mixture was warmed to RT, and stirred at such temperature overnight. The solvent was evaporated. Ethyl acetate (18 mL) was added, and the crude product was filtered, with EtOAc rinse of the reaction vessel. The filtrate was concentrated under reduced pressure, and the crude material was used without further purification. DMF (0.6 mL) was added to the crude product, followed by carboxylic acid **34** (0.136 g, 0.47 mmol), and DIEA (0.137 mL, 0.785 mmol). The reaction mixture was stirred at RT overnight, and the solvent was then evaporated under vacuum. The crude product was purified by flash chromatography, eluting from silica gel with a gradient of 10-20% MeOH in DCM containing 1% NH_4OH to afford 0.1 g of nitro acid **50** as a white solid. MS: (+) m/z 745.4 (M+1).

[00202] *Amino acid **51**.* A mixture of nitro acid **50** (0.1 g, 0.134 mmol) and palladium on carbon (10%, 14 mg) in MeOH (5 mL) was stirred under a hydrogen atmosphere for 4 h. The catalyst was filtered off, and the filtrate was concentrated under vacuum to afford 87.3 mg of amino acid **51** as a white solid. MS: (+) m/z 715.5 (M+1). Amino acid **51** is also referred to as compound (III-j) hereinabove.

Example 6 – Scheme 6

[00203] Scheme 6 (**Fig. 6**) shows yet another procedure for making compounds according to formula (II-b).

[00204] *Hydroxy nitro compound **52**.* Compound **27** (Scheme 2) (16.4 mg, 0.0275 mmol), was added to a 0.2 M solution of pentafluorophenol (7.6 mg, 0.0413 mmol) and DIC (0.0094 mL, 0.0606 mmol) in DCM (0.2 mL) at 0 °C. The reaction mixture was warmed to RT, and stirred at RT overnight. The solvent was evaporated. EtOAc (3 mL) was added and the crude product was filtered, with rinsing of the reaction vessel with EtOAc. The filtrate was concentrated under reduced pressure, and the crude material was used without further purification. DMF (0.1 mL) was added to the crude product, followed by carboxylic acid **34** (Scheme 3) (20.8 mg, 0.083 mmol), and *N,N*-diisopropylethylamine (0.024 mL, 0.138 mmol). The reaction mixture was stirred at RT overnight, and the solvent was then evaporated. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-10% MeOH in DCM to afford 14.9 mg of hydroxy nitro compound **52** as a white solid. MS: (+) m/z 831.5 (M+1).

[00205] *Acetyl nitro compound 53.* A 0.1 M solution of hydroxy nitro compound **52** (14.9 mg, 0.018 mmol) in pyridine (0.23 mL) was cooled at 0 °C, and acetic anhydride (0.054 mL, 0.57 mmol) was added. The reaction mixture was allowed to warm to RT over 2 h, and stirred at RT for 24 h. The reaction mixture was cooled to 0 °C, and 1:1 mixture of 1,4-dioxane and water was added. The reaction mixture was allowed to warm to RT, followed by stirring at this temperature for 12 h. The solvent was evaporated, and the residue was purified by preparative HPLC to afford 2.2 mg of acetyl nitro compound **53** as a white solid. MS: (+) m/z 873.2 (M+1).

[00206] *Acetyl amino compound 54.* A mixture of acetyl nitro compound **53** (2.2 mg, 0.0025 mmol) and palladium on carbon (10%, 1 mg) in methanol (0.2 mL) was stirred under a hydrogen atmosphere for 4 h. The catalyst was filtered off, and the filtrate was concentrated. The crude product was purified by preparative HPLC to afford 0.1 mg of acetyl nitro compound **54** as a white solid. MS: (+) m/z 843.2 (M+1). Acetyl amino compound **54** is also referred to as compound (III-a) hereinabove.

Example 7 – Scheme 7

[00207] Scheme 7 (**Fig. 7**) shows yet another procedure for making compounds of this invention.

[00208] *Compound 55.* Compound **34a** (Scheme 3) (70 mg, 0.13 mmol) was added to a 0.2 M solution of pentafluorophenol (35.9 mg, 0.195 mmol) and DIC (0.0223 mL, 0.143 mmol) in DCM (0.66 mL) at 0 °C. The reaction mixture was warmed to RT and stirred at RT overnight. The solvent was evaporated. EtOAc(16 mL) was added and the crude product was filtered, with rinsing of the reaction vessel with EtOAc. The filtrate was concentrated under reduced pressure, and the crude material was used without further purification. DMF (0.5 mL) was added to the crude product, followed by p-nitro-phenylalanine (82.0 mg. 0.39 mmol) and DIEA (0.114 mL, 0.65 mmol). The reaction mixture was stirred at RTovernight, and the solvent was then evaporated. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 10-20% MeOH in DCM containing 1% NH_4OH to afford 65.2 mg of compound **55** as a white solid. MS: (+) m/z 731.0 (M+1).

[00209] *Compound 56.* A mixture of compound **55** (65.2 mg, 0.089 mmol) and palladium on carbon (10%, 9.4 mg) in MeOH (3 mL) was stirred under a hydrogen

atmosphere for 4 h. The catalyst was filtered off, and the filtrate was concentrated to afford 33.8 mg of compund **56** as a white solid. MS: (+) m/z 701.2 (M+1). Compound **56** is also referred to as compound (III-d) hereinabove.

Example 8 – Scheme 8

[00210] Scheme 8 (**Fig. 8a**) shows a method for making a compound according to formula (VIII-b), useful as an intermediate for making compounds of this invention.

[00211] *Boc ester **58**.* To a solution of amino ester **57** (Chem-Impex, 5 g, 19.18 mmol) and di-*tert*-butyl dicarbonate ("$(Boc)_2O$," Aldrich, 4.6 g, 21.10 mmol) in DMF (Acros, anhydrous, 50 mL), triethylamine ("TEA," Aldrich, 8.36 mL, 60 mmol) was added. The reaction mixture was stirred for 0.5 h. HPLC analysis showed reaction was completed. The reaction mixture was diluted with EtOAc (500 mL) and the organic layer was washed with water (200 mL) and then brine (200 mL), dried over anhydrous $MgSO_4$ and concentrated. The crude product was purified on 120 g CombiFlash column with 0-5% MeOH in DCM to yield white solid Boc ester **58** (5.6 g, 81%). ^{1}HNMR (DMSO) δ 8.18 (d, 2 H), 7.47 (d, 2 H), 7.38 (d, 1H), 4.23 (m, 1 H), 3.60 (s, 3 H), 3.15 (m, 1 H), 2.95 (m, 1 H), 1.23 (s, 9 H).

[00212] *Alkene **59**.* To a solution of Boc ester **58** (230 mg, 0.68 mmol) in DCM (Acros, anhydrous, 2 mL) cooled to -78 °C in a dry ice–acetone, DIBAL (Aldrich, 1 M in DCM, 1 mL) was added slowly. The reaction mixture was stirred and warmed up to -20 °C over 3 h. (1-Ethoxycarbonylethyliden)-triphenylphosphoran (Aldrich, 492 mg, 1.36 mmol) was added. The reaction mixture was stirred at -20 °C for 1 h. The reaction mixture was diluted with EtOAc (100 mL) and the resultant organics was washed with water (50 mL) and then brine (50 mL), dried over anhydrous $MgSO_4$ and concentrated. The crude product was purified on 10 g COMBIFLASH™ column with 0-50% EtOAc in hexane to yield white solid alkene **59** (151 mg, 59%). ^{1}HNMR (DMSO) δ 8.18 (d, 2 H), 7.47 (d, 2 H), 7.22 (d, 1H), 6.51 (d, 1 H), 4.48 (m, 1 H), 4.11 (q, 2 H), 2.80-2.94 (m, 2 H), 1.62 (s, 3 H), 1.23 (s, 9 H), 1.16 (t, 3 H).

[00213] *Aryl amine **60**.* A solution of alkene **59** (148 mg, 0.39 mmol) in EtOH (Acros, anhydrous, 3 mL) and Pd on charcoal (Aldrich, 10%, 50 mg) was stirred under H_2 overnight. The reaction mixture was diluted with DCM (10 mL) and filtered through CELITE™. The filtrate was concentrated and the crude product was purified on 4 g

COMBIFLASH™ column with 0-20% MeOH in DCM to yield aryl amine **60** as a white solid (102 mg, 75%). ^{1}HNMR (DMSO) δ 7.18 (d, 2 H), 7.11 (s, 2 H), 6.71 (d, 1 H), 3.98 (q, 2 H), 3.51 (m, 1 H), 2.57 (m, 2 H), 2.41 (m, 1 H), 1.63 (m, 1 H), 1.37 (m, 1 H), 1.29 (s, 9 H), 1.09 (t, 3 H), 0.99 (d, 3 H), MS (ES+) m/z, calculated: m+1, 351.2, found 351.2.

Example 9 – Scheme 9

[00214] Scheme 9 (**Fig. 8b**) shows another method for making a compound according to formula (VIII-b), useful as an intermediate for making compounds of this invention.

[00215] *Amino acid 61.* A mixture of carboxylic acid **24** (Scheme 3, **Fig. 3**) (4.4 mg, 0.0025 mmol) and palladium on carbon (10%, 1 mg) in MeOH (0.5 mL) was stirred under a hydrogen atmosphere overnight. The catalyst was filtered off, and the filtrate was concentrated to afford 3.5 mg of amino acid **61** as a white solid. MS: (+) m/z 223.3 (M+1).

Example 10 – Schemes 10, 11, and 12

[00216] Scheme 10 (**Fig. 8c**) shows another method for making a compound according to formula (VIII-b), useful as an intermediate for making compounds of this invention.

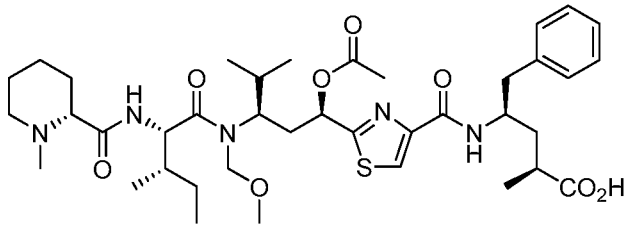
[00217] *Compound 62.* A mixture of monoester **33** (Scheme 3, **Fig. 3**) (0.34 g, 0.93 mmol) and palladium on carbon (10%, 50 mg) in methanol (20 mL) was stirred under a hydrogen atmosphere overnight. The catalyst was filtered off, and the filtrate was concentrated to afford 0.29 g of compound **62** as a white solid. MS: (+) m/z 237.3 (without Boc).

[00218] Scheme 11 (**Fig. 9**) illustrates how compounds according to formula (VIII-b) can be used to make compounds of this invention. Boc ester **62** is converted to Bpoc ester **62a** by first protecting the aromatic amine group with an Fmoc group, treatment with TFA to remove the Boc group from the aliphatic amine group, treatment with carbonic acid α,α-dimethyl-p-phenylbenzyl phenyl ester (8Cl) to install a Bpoc group there, and removal of the Fmoc group with piperidine. Bpoc ester **62a** is coupled with carboxylic acid **63** with HATU to yield an intermediate ester that is then hydrolyzed with LiOH to produce compound **64**. Hydrogenation to remove the Cbz protecting group from compound **64**, followed by HATU-mediated coupling with 6-maleimidohexanoic acid and removal of the Bpoc group with acetic acid yields amino acid **65**. Another HATU-mediated coupling with compound **34a** (Scheme 3, **Fig. 3**) yields compound **66**. Removal of the Boc protecting group with TFA affords compound **67**, ready for conjugation.

[00219] Yet another mode of utilizing compounds of formula (VIII-b) is shown in Scheme 12 (**Fig. 10**). Starting from compound **34a**, HATU-mediated coupling with compound **68** affords Boc ester **69**. Removal of the Boc group with TFA and hydrolysis of the ester with LiOH affords amino acid **36**, which can be elaborated as shown in Scheme 4 (**Fig. 4**) to prepare a composition suitable for conjugation.

Example 11 – Compound 70

[00220] *Compound 70.* Tubulysin D (made per Peltier et al. 2006; 2 mg, 2.4 umol) was dissolved in MeOH at 0 °C. To this solution was added a drop of HCl (0.1 M). After stirring the reaction mixture for overnight at RT the solution was evaporated under vacuum to give a residue that was passed through a short column (DCM:MeOH 0-10%) to give compound **70** (1.3 mg, 1.6 umol, 67%) as an oil. MS (ES+) m/z, calculated: m+1, 772.42, found, 772.



70

[00221] Those skilled in the art will appreciate that the general methodology of the Schemes can be adapted to make compounds of this invention other than those specifically described above. For example, compound **14** (same as compound **49**) can be used to make numerous other compounds of this invention by coupling it with other replacements for the Tup subunit. As another example, by changing the reagents used with compound **9** (Scheme 1), compounds **44** and **46** (Scheme 5), variations on groups R^2 and R^3 in formula (II) beyond those specifically exemplified can be synthesized.

Example 12 – Preparation of a Conjugate

[00222] This example describes the preparation of a conjugate of cytotoxin-linker construct (VI-b) and anti-CD70 monoclonal antibody 2H5 (Terrett et al., US 2009/0028872 A1; Coccia et al., WO 2008/074004 A2). It is representative of the procedure used in the preparation of other conjugates.

[00223] Anti-CD70 antibody 2H5 at ~5 mg/mL in 20 mM sodium phosphate, 50 mM NaCl, 100 μM DTPA, pH 7.5, was thiolated with a 13-fold molar excess of 2-

iminothiolane. The thiolation reaction was allowed to proceed for 1 h at RT with continuous mixing.

[00224] Following thiolation, the antibody was buffer exchanged into conjugation buffer (50 mM HEPES, 5 mM glycine, 2 mM DTPA, pH 6.0) via a PD10 column (Sephadex G-25). The concentration of the thiolated antibody was determined at 280 nm. The thiol concentration was measured using a dithiodipyridine assay.

[00225] A 5 mM stock of construct (VI-b) in DMSO was added at a 3-fold molar excess per thiol of antibody and mixed for 90 min at RT. Following conjugation, 100 mM N-ethylmaleimide in DMSO was added at a 10-fold molar excess of thiol per antibody to quench any unreacted thiol groups. This quenching reaction was done for one h at RT with continuous mixing.

[00226] The anti-CD70 antibody drug conjugate was 0.2 μm filtered prior to cation-exchange chromatographic purification. The SP SEPHAROSE™ High Performance Cation Exchange column (CEX) was regenerated with 5 column volumes (CVs) of 50 mM HEPES, 5 mM glycine, 1M NaCl, pH 6.0. Following regeneration, the column was equilibrated with 3 CVs of equilibration buffer (50 mM HEPES, 5 mM glycine, pH 6.0). The conjugate was loaded and the column and was washed once with the equilibration buffer. The conjugate was eluted with 50 mM HEPES, 5 mM glycine, 200 mM NaCl, pH 6.0. The eluate was collected in fractions. The column was then regenerated with 50 mM HEPES, 5 mM glycine, 1M NaCl, pH 6.0 to remove protein aggregates and any unreacted (VI-b).

[00227] Fractions containing monomeric antibody conjugate were pooled. Antibody conjugate concentration and substitution ratios were determined by measuring absorbance at 280 and 252 nm.

[00228] The purified eluate pool was buffer exchanged into 30 mg/mL sucrose, 10 mg/mL glycine, pH 6.0, by dialysis. Dextran 40 was added to the sample at 10 mg/mL post-dialysis. The concentration and substitution ratio (SR) were determined by measuring absorbance at 280 and 252 nm. The SR was 2.2 moles of cytotoxin per mole of antibody.

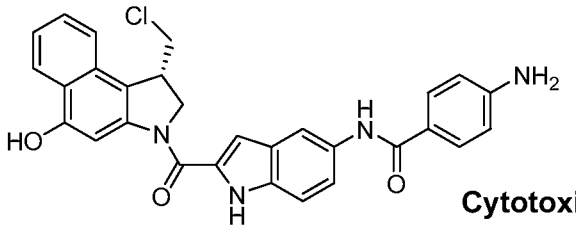
Example 13 – Proliferation Assays

[00229] This example generally describes the procedures used to assay the antiproliferative activity of compounds of this invention or their conjugates. Human tumor cell lines were obtained from the American Type Culture Collection (ATCC), P.O. Box 1549, Manassas, VA 20108, USA, and cultured according to instruction from the ATCC. Cells were seeded at 1.0×10^3 or 1.0×10^4 cells/well in 96-well plates for 3 h for ATP assays or ^{3}H thymidine assays, respectively. 1:3 serial dilutions of free (unconjugated) compounds or their conjugates were added to the wells. Plates were allowed to incubate for 24 to 72 h. The ^{3}H thymidine plates were pulsed with 1.0 μCi of ^{3}H-thymidine per well for the last 24 hours of the total incubation period, harvested, and read on a Top Count Scintillation Counter (Packard Instruments, Meriden, CT). ATP levels in the ATP plates were measured using the CELLTITER-GLO® Luminescent Cell Viability kit following the manufacturer's manual and read on a GLOMAX® 20/20 luminometer (both from Promega, Madison,WI, USA). The EC_{50} values – the concentration at which an agent inhibits or reduces cell proliferation by 50% – were determined using PRISM™ software, version 4.0 (GraphPad Software, La Jolla, CA, USA).

Example 14 – Cytotoxin In Vitro Activity

[00230] Using the ^{3}H thymidine or the ATP luminescence assay, or both, the activities of compounds of this invention were assayed against the following cancer cell lines: HCT-15 (colorectal cancer, multi-drug resistant (MDR)); Hep3B (liver cancer); LNCaP (prostate cancer, androgen receptor positive (AR^+)); MDA-MB-231 (breast cancer, estrogen receptor, progesterone receptor, and Her2 negative (triple negative)); A2058 (melanoma); U-87 MG (glioblastoma); NCI-H460 (NSCLC); A549 (NSCLC); HPAC (pancreatic cancer, primary); PC3 (prostate cancer, AR^-); BT474 (breast cancer, Her2 highly positive (Her2hi)); SKOV3 (ovarian cancer, Her2hi); 786-O (renal cancer); UO-31 (renal cancer, MDR); NCI-H740 (SCLC); DMS53 (SCLC); SK-BR3 (breast cancer, Her2hi); ZR-75 (breast cancer, estrogen receptor positive); OVCAR3 (ovarian cancer); HL-60 (APL); OVCAR8/Adr (ovarian cancer, MDR); CEM-C1 (ALL); Nomo-1 (AML); RPMI-8226 (MM)); Raji (lymphoma); SW-480 (colorectal cancer, metastatic); SW-620 (colorectal cancer); and H226 (lung cancer). (Not all compounds were assayed against all cell lines.)

[00231] The following compounds were used as reference or comparison cytotoxins: doxorubicin (Dox), Cytotoxin CBI (a DNA minor groove alkylating agent of the cyclopropa[c]benz[e]indol-4-one class), tubulysin D (Tub D, Table 1), and the methyl ester of MMAF ("MMAF,"an auristatin-related compound; see Sutherland et al., *J. Biol. Chem.* **2006**, 281 (15), 10540-10547).



Cytotoxin CBI

[00232] **Figs. 11a** and **11b** show illustrative plots for ^{3}H thymidine proliferation assays for compounds of this invention, against HL-60 and 786-O cells, respectively, with Cytotoxin CBI and tubulysin D as comparative compounds, with an incubation period of 48 h.

[00233] **Figs. 12a** and **12b** show illustrative plots for ATP luminescence proliferation assays for a second set of compounds of this invention, against HL-60 and 786-O cells, respectively, with an incubation period of 72 h. **Figs. 12c** and **12d** show the plots for ^{3}H thymidine proliferation assays for the same set of compounds, again against HL-60 and 786-O cells, respectively, with a 72 h incubation period. In each instance, doxorubicin was used as a comparative compound.

[00234] Table 2 provides the data for proliferation assays using the ^{3}H thymidine method, with an incubation period of 72 h.

Table 2 (Part 1) – ^{3}H Thymidine Proliferation Assays							
Com-pound	Cell Line and EC_{50} (nM)						
	HCT-15	Hep3B	LNCaP	MDA-MB-231	A2058	U-87 MG	NCI-H460
Dox	149	44	130	133	—	106	101
CBI	0.040	0.020	0.051	0.019	—	0.14	0.042
Tub D	0.062	0.022	0.36	0.091	—	0.014	0.032
MMAF	1.3	0.11	1.7	0.29	—	0.12	0.37
15	67	1.6	27	1.3	1.6	0.42	2.0
16	284	>100	>100	>100	>100	>100	>100
17	392	>100	>100	84	>100	12	>100
19	1067	52	>100	—	0.13	>100	>100
24	704	32	76	33	0.12	18	>100
25	280	305	930	236	409	271	306
26	31	18	>3000	15	25	5.2	18
26a	>100	36	80	>100	>100	71	>100
28	223	24	64	>100	0.96	>100	>100
29	0.18	0.041	0.54	0.13	0.40	0.064	0.21
29c	2.4	3.0	5.2	4.8	5.6	0.51	15
36	64	39	342	>100	60	15	52
51	101	4.2	41	8.7	0.40	0.75	7.2
54	0.44	0.30	16	1.6	0.64	0.11	0.31
56	2477	>100	>100	>100	>100	>100	>100

Table 2 (Part 2) – ^{3}H Thymidine Proliferation Assays							
Com-pound	Cell Line and EC_{50} (nM)						
	A549	HPAC	PC3	BT474	SKOV3	786-O	UO-31
Dox	128	126	276	424	163	168	267
CBI	0.072	0.063	0.049	1.2	0.047	0.035	0.035
Tub D	0.014	0.015	0.038	0.51	0.039	0.15	0.10
MMAF	0.21	0.29	0.38	1.7	0.24	1.4	7.3
(III-q)	—	—	—	—	—	7.61	—
15	2.1	3.0	1.7	7.1	2.6	—	15
16	>100	>100	>100	>100	44	19	>100
17	>100	>100	82	>100	91	32	>100
19	>100	>100	>100	>100	>100	>100	>100
24	38	>100	63	>100	68	>100	>100
25	270	206	319	804	180	1862	435
26	18	11	16	11	4.6	26	50
26a	>100	>100	>100	7	47	>100	>100
28	>100	34	>100	>100	9.9	61	>100
29	0.44	0.069	0.21	0.57	0.029	1.2	0.13
29c	6.5	32	11	12	1.9	3.9	1.0
36	44	83	66	>100	32	>100	>100
51	7.8	6.4	5.6	30	4.4	10	35
54	0.24	0.83	0.82	8.8	0.34	6.9	1.3
56	>100	>100	>100	>100	>100	29	>100

Table 2 (Part 3) – ^{3}H Thymidine Proliferation Assays							
Com-pound	Cell Line and EC_{50} (nM)						
	H740	DMS53	SK-BR3	ZR-75	OVCAR3	HL-60	OVCAR8
Dox	255	979	—	794	258	56	2044
CBI	0.098	1.5	—	1.5	0.071	0.027	0.16
Tub D	2.2	0.21	—	1.7	0.051	0.032	0.17
MMAF	13	10	—	42	0.16	0.25	25
(III-q)	—	—	—	—	—	15.05	—
15	2.3	37	17	—	>100	0.60	423
16	>100	>100	>100	>100	>100	8.9	1869
17	32	>100	>100	>100	>100	17	2970
19	3.8	>100	>100	34	53	37	69
24	51	>100	>100	3.6	90	6.9	>100
25	347	515	1714	>3000	379	308	946
26	3.0	99	32	>300	3.2	0.45	201
26a	3.2	>100	158	56	21	10	100
28	5.8	>100	>100	4.3	3.1	7.7	27
29	0.41	0.28	1.1	3.8	0.02	0.0099	0.70
29c	5.3	76	72	44	3.6	1.8	1.4
36	>100	>100	>100	>100	>100	107	>100
51	37	24	>100	1.3	2.5	4.2	20
54	10	2.0	1.1	2.2	3.9	0.53	2.1
56	>100	>100	>100	>100	>100	>100	>3000

Table 2 (Part 4) – ^{3}H Thymidine Proliferation Assays							
Com-pound	Cell Line and EC_{50} (nM)						
	CEM-C1	Nomo-1	RPMI-8226	Raji			
Dox	144	105	68	61			
CBI	0.11	0.016	0.12	0.013			
Tub D	0.018	0.042	0.037	0.013			
MMAF	0.17	0.27	0.24	0.12			
15	0.93	2.3	3.3	0.47			
16	11	1.8	22	28			
17	43	6.8	47	53			
19	22	1.3	23	32			
24	13	1.1	17	11			
25	225	116	396	231			
26	22	0.36	7.7	7.0			
26a	10	2.0	22	20			
28	10	0.20	8.7	13			
29	0.019	0.0075	0.034	0.024			
29c	45	0.43	16	8.5			
36	44	124	48	15			
51	2.0	3.5	2.2	1.5			
54	0.38	0.77	0.36	0.20			
56	>100	>100	>100	>100			

[00235] Table 3 provides the data for the ATP luminescence proliferation assays, with an incubation period of 48, 72, or 96 h, as noted.

Table 3 (Part 1) – ATP Luminescence Proliferation Assays (Incubation Period as Noted))

Com-pound	Cell Line and EC_{50} (nM)						
	HCT-15 (48 h)	Hep3B (48 h)	MDA-MB-231 (48 h)	A2058 (48 h)	NCI-H460 (48 h)	A549 (48 h)	PC3 (48 h)
Dox	—	443	—	—	—	149	1082
CBI	—	2.6	—	—	—	3.0	12
Tub D	—	0.0012	—	—	—	0.020	0.039
MMAF	—	0.053	—	—	—	0.33	0.43
15	40	6.3	5.6	3.5	6.7	—	>100
16	157	26	28	30	69	—	>100
17	443	>100	65	30	69	—	>100
19	—	18	—	—	—	—	~33
24	—	10	—	—	—	—	13
25	—	246	—	—	—	111	155
26	113	100	120	93	—	—	>3000
26a	—	—	—	—	—	—	—
28	—	17	—	—	—	—	5.8
29	—	1.6	—	—	—	3.9	3.4
29c	200	75	52	32	—	—	66
36	—	86	—	—	—	1.9	78
51	—	3.8	—	—	—	—	16
54	—	1.5	—	—	—	3.0	3.1
56	>3000	>100	>100	>100	>100	—	>100

Table 3 (Part 2) – ATP Luminescence Proliferation Assays (Incubation Period as Noted))

Com-pound	Cell Line and EC_{50} (nM)						
	BT474 (48 h)	SKOV3 (48 h)	786-O (48 h)	UO-31 (48 h)	DMS53 (48 h)	SK-BR3 (48 h)	ZR-75 (48 h)
Dox	—	674	360	908	—	—	—
CBI	—	3.0	1.6	4.2	—	—	—
Tub D	—	0.029	0.022	0.099	—	—	—
MMAF	—	0.19	0.71	2.6	—	—	—
15	3.5	4.2	—	—	32	3.9	13
16	19	18	—	—	50	6.6	11
17	23	58	—	>100	~80	24	24
19	8.8	—	—	>100	—	3.9	20
24	4.6	—	—	13	—	8.9	8.4
25	166	—	—	759	366	>3000	1003
26	19	—	—	240	—	253	208
26a	—	—	—	—	—	—	—
28	12	—	—	5.8	—	7.8	16
29	1.5	—	—	2.7	5.3	3.6	15
29c	492	—	—	26	—	278	136
36	~120	—	—	8.8	~300	>300	>300
51	11	—	—	>100	—	13	19
54	4.8	—	—	—	7.9	6.2	9.6
56	>100	>100	—	—	>100	>100	>100
84a	—	—	6.12	—	—	—	—
84b	—	—	4.10	—	—	—	—
109	—	—	1.03	—	—	—	—

Table 3 (Part 3) – ATP Luminescence Proliferation Assays (Incubation Period as Noted))

Com-pound	Cell Line and EC_{50} (nM)						
	OVCAR3 (48 h)	HL-60 (48 h)	CEM-C1 (48 h)	Nomo-1 (48 h)	RPMI-8226 (48 h)	HCT-15 (72 h)	LNCaP (72 h)
Dox	632	—	—	—	—	600	592
CBI	4.1	—	—	—	—	3.4	3.3
Tub D	0.085	—	—	—	—	0.014	0.093
MMAF	0.17	—	—	—	—	0.075	0.98
15	>100	—	—	—	—	—	—
16	>100	—	—	—	—	—	—
17	>100	—	—	—	—	—	—
19	25	—	—	—	—	245	—
24	29	—	—	—	—	188	—
25	>3000	224	—	83	—	366	—
26	—	—	61	3.9	45	—	412
26a	—	—	—	—	—	—	—
28	14	—	—	—	—	132	—
29	>100	0.59	—	0.24	—	4.5	—
29c	—	—	20	2.9	70	—	255
36	>300	114	—	34	—	96	>300
36a		0.048	—	—	—	—	—
51	5.5	—	—	—	—	93	—
54	>100	2.5	—	3.1	—	2.6	11
56	>100	—	—	—	—	—	—
84a	8.19	10-28	—	—	—	138	—
84b	4.91	16-27	—	—	—	22.8	—
109		0.019	—	—	—	—	—
112		0.05-.09	—	—	—	—	—

Table 3 (Part 4) – ATP Luminescence Proliferation Assays (Incubation Period as Noted))

Com-pound	Cell Line and EC_{50} (nM)						
	MDA-MB-231 (72 h)	A2058 (72 h)	U-87 MG (72 h)	NCI-H460 (72 h)	A549 (72 h)	HPAC (72 h)	BT474 (72 h)
Dox	518	101	324	92	—	215	388
CBI	3.1	0.20	3.3	0.64	—	6.0	5.5
Tub D	0.047	0.0064	0.0054	0.0076	—	—	0.021
MMAF	0.33	0.039	0.052	0.13	—	0.067	0.024
15	—	—	8.6	—	—	—	—
16	—	—	>100	—	—	—	—
17	—	—	22	—	—	—	—
19	32	26	>100	43	—	0.33	—
24	13	14	18	23	—	1.1	—
25	202	212	357	177	—	97	—
26	—	—	94	90	—	279	—
26a	—	—	—	—	—	—	—
28	16	20	18	35	—	5.9	—
29	5.1	2.9	4.9	3.0	—	4.9	—
29c	—	—	136	6.0	—	4.6	—
36	9.6	43	37	16	—	2.9	—
51	—	5.5	7.6	14	—	4.5	—
54	5.6	1.4	1.6	1.6	—	0.96	—
56	—	—	>100	—	—	—	—

Table 3 (Part 5) – ATP Luminescence Proliferation Assays (Incubation Period as Noted))

Com-pound	Cell Line (EC_{50}, nM)						
	SKOV3 (72 h)	786-O (72 h)	H740 (72 h)	DMS53 (72 h)	SK-BR3 (72 h)	ZR-75 (72 h)	OVCAR3 (72 h)
Dox	—	—	—	755	441	236	—
CBI	—	—	2.2	3.8	3.1	2.2	—
Tub D	—	—	0.022	0.069	0.025	0.034	—
MMAF	—	—	—	4.5	0.16	0.25	—
(III-q)	—	103.6	—	—	—	—	—
15	—	9.4	—	—	—	—	—
16	—	36	—	—	—	—	—
17	—	73	—	—	—	—	—
19	15	40	—	87	—	—	—
24	13	36	—	>100	—	—	—
25	109	306	—	—	—	—	—
26	38	126	—	193	—	—	49
26a	—	—	—	—	—	—	—
28	11	29	—	58	—	—	—
29	1.3	2.8	—	—	—	—	—
29c	17	34	—	17	—	—	99
36	45	183	—	—	—	—	—
36a	—	0.434	—	—	—	—	—
51	10	19	—	46	—	—	—
54	1.3	2.6	—	—	—	—	—
56	—	>100	—	—	—	—	—
109	—	—	—	—	—	—	0.202
112	—	0.67	—	—	—	—	0.049
133	—	4.17	—	—	—	—	—
134	—	9.89	—	—	—	—	—
135	—	12.94	—	—	—	—	—

Table 3 (Part 6) – ATP Luminescence Proliferation Assays (Incubation Period as Noted))

Com-pound	Cell Line and EC_{50} (nM)						
	HL-60 (72 h)	CEM-C1 (72 h)	Nomo-1 (72 h)	RPMI-8226 (72 h)	Raji (72 h)	SW480 (96 h)	SW-620 (96 h)
Dox	147	161	338	345	301	215	145
CBI	0.76	0.55	0.37	4.1	2.1	0.92	0.33
Tub D	0.0096	0.0030	0.013	0.0051	0.0048	0.020	0.0058
MMAF	0.12	0.11	0.14	0.068	0.084	0.17	0.057
(III-q)	14.27	—	—	—	—	—	—
15	2.7	2.6	2.7	2.6	2.5	5.6	3.7
16	2.8	2.5	3.1	15	3.0	48	29
17	7.4	26	3.9	15	27	92	65
19	7.7	11	5.2	9.6	9.2	52	14
24	6.4	8.7	4.0	8.4	7.9	42	10
25	—	176	—	235	209	261	193
26	27	—	—	—	46	67	48
26a	—	—	—	—	—	—	—
28	2.9	11	0.82	10	15	36	21
29	—	0.85	—	1.0	2.1	2.7	1.5
29c	8.6	—	—	—	36	41	8.4
36	—	40	—	62	24	81	45
51	—	4.8	7.4	5.2	5.0	13	6.4
54	—	1.2	—	1.6	1.2	1.6	1.1
56	>100	>100	>100	5.7	24	>100	28

[00236] Additionally the following EC_{50} values were measured for the following compounds against the H226 cell line, using the ATP assay and 72 h incubation period: compound **36a** (0.307 nM); compound **109** (1.609 nM); and compound **112** (0.67-1.16 nM). The following EC_{50} values were measured for the following compounds against the OVACAR8/Adr cell line, using the ATP assay and 48 h incubation period: compound

36a (17.05 nM); compound **84a** (>300 nM); compound **84b** (47.2 nM); compound **109** (24.9 nM); and compound **112** (12 nM).

Example 15 – Conjugate In Vitro Activity

[00237] **Fig. 13** shows the activity of conjugates of this invention in ^{3}H thymidine proliferation assays, measured against 786-O renal cancer cells, which are CD70 positive. The incubation period was 72 h. The EC_{50} values extracted from the curves of **Fig. 13** are given in Table 4, along data from other experiments. Cell line LNCap is a prostate cancer cell line that expresses prostate specific membrane antigen (PSMA); H226 is a lung cancer cell line that expresses mesothelin. The antibodies used for conjugation were 2A10, an anti-PSMA antibody (Huang et al., US 2009/0297438); 2H5, an anti-CD70 antibody (Terrett et al., US 2009/0028872); 1F4, an anti-CD70 antibody (Coccia et al., WO 2008/074004); and 6A4, an anti-mesothelin antibody (Terrett et al., WO 2009/045957). As controls, Compound J of Sufi et al., WO 2008/083312 ("Cpd. J," a DNA minor groove binding/alkylating agent) was used as a conjugation partner and diphtheria toxin ("DTX") was used as an unconjugated non-specific control.

Table 4 – In Vitro Activity of Conjugates			
Conjugate or Test Compound		Cell Line	EC_{50} (nM)
Designation	Description		
2A10-Cpd. J	Conjugate of 2A10 and Cpd. J	786-O	116.7
DTX	Unconjugated non-specific control	786-O	104.4
2H5-Cpd. J	Conjugate of 2H5 and Cpd. J	786-O	0.08592
1F4-Cpd. J	Conjugate of 1F4 and Cpd. J	786-O	0.09951
2H5-(VI-a)	Conjugate of 2H5 and Compound (VI-a)	786-O	0.1151 to 0.0749
2A10-(VI-a)	Conjugate of 2A10 and Compound (VI-a)	786-O	>> 100
2H5-(VI-b)	Conjugate of 2H5 and Compound (VI-b)	786-O	0.06554
2A10-(VI-b)	Conjugate of 2A10 and Compound (VI-b)	786-O	>> 100
2H5-(VI-n)	Conjugate of 2H5 and Compound (VI-n)	786-O	0.4384
2H5-(VI-m)	Conjugate of 2H5 and Compound (VI-m)	786-O	0.5899
2H5-(VI-q)	Conjugate of 2H5 and Compound (VI-q)	786-O	inactive
2H5-(VI-p)	Conjugate of 2H5 and Compound (VI-p)	786-O	inactive
2H5-(VI-t)	Conjugate of 2H5 and Compound (VI-t)	786-O	0.310
6A4-(VI-t)	Conjugate of 6A4 and Compound (VI-t)	H226	0.360
2A10-(VI-t)	Conjugate of 2A10 and Compound (VI-t)	LNCap	0.570

[00238] The data show that a CD70-specific antibody is needed for a conjugate to be able to effectively deliver a cytotoxin to CD70-positive 786-O cells: conjugates of antibody 2A10, which is specific for a different antigen (PSMA), have low or no activity. Conversely, all the conjugates of an anti-CD70 antibody were active. The conjugates of Compounds (VI-a) and (VI-b) were comparable in activity to the Compound J conjugates, a well known conjugation partner and one of whose conjugates is undergoing clinical trials. It is noteworthy that the conjugates of Compounds (VI-a) and (VI-b) exhibit very little non-specific toxicity: compare the activities of 2A10-(VI-a) and 2A10-(VI-b) against that of 2A10-CBI.

Example 16 – Conjugate In Vivo Activity

[00239] CD70-positive human renal cancer 786-O cells (Cat. CRL-1932 originally acquired from ATCC) were cultured *in vitro* per ATCC instructions. The cells were harvested, and 2.5 million cells per 200 μL of DPBS/MATRIGEL™ (1:1) were implanted subcutaneously in the flank region of CB17.SCID mice. Tumors were measured weekly in 3 dimensions with a Fowler Electronic Digital Caliper (Model 62379-531; Fred V. Fowler Co., Newton, MA, USA) and data were electronically recorded using StudyDirector software from Studylog Inc. (South San Francisco, CA, USA). Animals were checked daily for postural, grooming, and respiratory changes, as well as lethargy. Animals were also weighed weekly and euthanized if weight loss was ≥ 20%. When tumors reached an average size of 194 mm^3, groups of 6 mice each were treated with a single intraperitoneal (IP) dose of a test conjugate (e.g., 2H5-(VI-b)) and an isotype control (2A10-(VI-b)) at 0.3 μmol/kg body weight. Tumor volumes (LWH/2) and weights of mice were recorded throughout the course of each study, which were allowed to proceed for approximately 2 months post initial dosing. An Excel spreadsheet macro was used to calculate the mean, SD, and median values of tumor sizes. Data were graphed using Prism software version 4.0.

[00240] The xenograft study results are shown in **Fig. 14**, where legend labels have the same meaning as in the previous Example and in **Fig. 13**. The data demonstrate the in vivo activity of conjugates of compounds of this invention against $CD70^+$ 786-O cells. Both conjugates of compounds (VI-a) and (VI-b) with the anti-CD70 antibody 2H5 caused a reduction in mean tumor size to less than half over the course of the study, while, when the vehicle control or a conjugate with the anti-PSMA antibody 2A10 was administered, tumor mean volume more than doubled.

Example 17 – Scheme 13

[00241] Scheme 13 (**Fig. 15**) shows a method for making enantiomerically pure 4-nitrotubuphenylalanines (4-NO_2Tup) **82a** and **82b**, which are useful for making compounds of this invention.

[00242] *Compound **80***. Di-*tert*-butyl dicarbonate (90.5 mg, 0.42 mmol) was added to a mixture of compound **34** of Scheme 3 (0.1 g, 0.35 mmol) in 0.7 M aq. NaOH (1 mL). The reaction mixture was stirred at RT for 3 h, and then acidified to pH 3 with 0.5 N HCl.

After the aqueous solution was extracted with EtOAc three times, the combined organic layers were dried, filtered, and concentrated. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-20% methanol in DCM to afford 0.117 g of compound **80** as a white solid. MS: (+) m/z 253.1 (M+1 without Boc).

[00243] *(-)-Menthol esters **81a** and **81b**.* DCC (87.8 mg, 0.43 mmol) was added to a solution of compound **80**, (-)-menthol (66.6 mg, 0.43 mmol), and 4-(dimethylamino)-pyridine ("DMAP," 10.4 mg, 0.085 mmol) in DCM (1.5 mL) at RT. After the reaction mixture was stirred at RT for 3 h, the precipitate was filtered off. The filtrate was then concentrated. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-20% EtOAc in hexanes to afford 55.7 mg of ester **81a** as a white solid, and 55.7 mg of ester **81b** as a white solid. MS for ester **81a**: (+) m/z 391.2 (M+1 without Boc); MS for ester **81b**: (+) m/z 391.2 (M+1 without Boc).

[00244] *4-NO_2Tup **82a** and **82b**.* A solution of ester **81a** in 6N HCl (40 mg, 0.082 mmol) was heated at 130 °C for 1.5 h. The reaction mixture was concentrated to afford 23.5 mg of 4-NO_2Tup **82a** as a white solid. ^{1}H NMR (D_2O, 400 MHz): δ 8.04 (d, 2H, J = 8.4 Hz), 7.33 (d, 2H, J = 8.4 Hz), 3.50 (m, 1H), 3.03 (dd, 1H, J = 6.8, 14.4 Hz), 2.89 (dd, 1H, J = 7.6 Hz, 14.4 Hz), 2.45-2.39 (m, 1H), 1.92-1.84 (m, 1H), 1.62-1.55 (m, 1H), and 0.98 (d, 3H, J = 7.2 Hz); MS: (+) m/z 253.1 (M+1). 4-NO_2Tup **82b** was obtained by the same procedure on the same scale as a white solid (23.5 mg). ^{1}H NMR (D_2O, 400 MHz): δ 8.03 (d, 2H, J = 8.4 Hz), 7.33 (d, 2H, J = 8.4 Hz), 3.50 (m, 1H), 2.93 (dd, 2H, J = 2.0, 7.6 Hz), 2.54-2.48 (m, 1H), 1.86-1.78 (m, 1H), 1.60-1.53 (m, 1H), and 0.98 (d, 3H, J = 6.8 Hz); MS: (+) m/z 253.1 (M+1).

Example 18 – Scheme 14

[00245] Scheme 14 (**Fig. 16**) depicts the conversion of 4-NO_2Tup **82a** and **82b** into compounds of this invention.

[00246] *Nitro acid **83a**.* Compound **34a** of Scheme 3 (10 mg, 0.019 mmol) was added to a 0.2 M solution of pentafluorophenol (5.1 mg, 0.028 mmol) and *N*, *N*'-diisopropyl-carbodiimide ("DIC," 0.0032 mL, 0.021 mmol) in DCM (0.2 mL) at 0 °C. The reaction mixture was warmed to RT and stirred at such temperature overnight. The solvent was evaporated. EtOAc (1.8 mL) was added, and the crude product was filtered, with rinsing of the reaction vessel with EtOAc. The filtrate was concentrated under reduced pressure,

and the crude pentafluorophenyl was used without further purification. DMF (0.2 mL) was added to the crude ester, followed by 4-NO_2Tup **82a** (10.7 mg, 0.037 mmol), and DIEA (0.013 mL, 0.074 mmol). The reaction mixture was stirred at RT overnight, and the solvent was then evaporated off. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-20% MeOH containing 1% NH_4OH in DCM to afford 12.9 mg of nitro acid **83a** as a white solid. MS: (+) m/z 773.4 (M+1).

[00247] *Alternative route nitro acid **83a**:* DIEAwas added to a solution of compound 34a (10 mg, 0.019mmol), and HATU (7.8 mg, 0.020 mmol) in DMF (0.3 mL), maintaining the pH at 8-9. The reaction mixture was stirred at RT for 5 min. Then DIEA and nitro amine **82a** (5.4 mg, 0.019 mmol) in DMF (1 mL) were added, maintaining the pH at 8-9. After the reaction mixture was stirred at RT for 15 min, the crude product was purified by preparative HPLC to afford 13.4 mg of nitro acid **83a** as a white solid.

[00248] Nitro acid **83b** was prepared by the same alternative route, on the same scale and was obtained as a white solide (13.4 mg). MS: (+) m/z 773.4 (M+1).

[00249] *Amine **84a**.* A mixture of nitro acid **83a** (7.5 mg, 0.0097 mmol) and palladium on carbon (10%, 1.1 mg) in MeOH (0.37 mL) was stirred under a hydrogen atmosphere for 4 h. The catalyst was filtered off, and the filtrate was concentrated. The crude product was purified by preparative HPLC to afford 6.2 mg of amine **84a** as a white solid. ^{1}H NMR (CD_3OD, 400 MHz): δ 8.06 (s, 1H), 7.36 (d, 2H, J = 8.4 Hz), 7.17 (d, 2H, J = 8.4 Hz), 5.70 (dd, 1H, J = 2.8, 10.8 Hz), 4.71 (d, 1H, J = 7.2 Hz), 4.44-4.35 (m, 2H), 3.74(d, 1H, J = 9.6 Hz), 3.49-3.45 (m, 1H), 3.36-3.35 (m, 1H), 3.30-3.25 (m, 1H), 3.13 (s, 3H), 3.14-3.04 (m, 1H), 2.93 (d, 2H, J = 8.4 Hz), 2.74 (s, 3H), 2.48-2.28 (m, 3H), 2.15 (s, 3H), 2.19-2.03 (m, 2H), 1.95-1.86 (m, 4H), 1.80-1.71 (m, 2H), 1.71-1.57 (m, 3H), 1.24-1.13 (m, 1H), 1.16 (d, 3H, J = 7.2 Hz), 1.04 (d, 3H, J = 6.4Hz), 1.02 (d, 3H, J = 6.8 Hz), 0.94 (t, 3H, J = 7.2 Hz), and 0.84 (d, 3H, J = 6.8 Hz); MS: (+) m/z 743.4 (M+1).

[00250] Nitro acid **83b** was hydrogenated to amine **84b** using the same procedure, on an 8 mg scale. Amine **84b** was obtained as a white solide (6.7 mg). ^{1}H NMR (CD_3OD, 400 MHz): δ 8.06 (s, 1H), 7.35 (d, 2H, J = 8.4 Hz), 7.16 (d, 2H, J = 8.4 Hz), 5.70 (dd, 1H, J = 2.8, 11.2 Hz), 4.72 (d, 1H, J = 7.2 Hz), 4.49-4.32 (m, 2H), 3.75 (d, 1H, J = 10.0 Hz), 3.49-3.45 (m, 1H), 3.36-3.35 (m, 1H), 3.33-3.31 (m, 1H), 3.12 (s, 3H), 3.12-3.04 (m, 1H), 2.91 (d, 2H, J = 7.6 Hz), 2.74 (s, 3H), 2.57-2.52 (m, 1H), 2.45-2.37 (m, 1H), 2.33-2.28 (m, 1H), 2.15 (s, 3H), 2.19-2.13 (m, 1H), 2.03-1.88 (m, 5H), 1.81-1.57 (m, 5H), 1.24-1.13

(m, 1H), 1.17 (d, 3H, J = 6.8 Hz), 1.04 (d, 3H, J = 6.4Hz), 1.02 (d, 3H, J = 7.2 Hz), 0.94 (t, 3H, J = 7.2 Hz), and 0.84 (d, 3H, J = 6.4 Hz); MS: (+) m/z 743.4 (M+1).

[00251] Compounds **84a** and **84b** are also depicted in this specification by formulae (III-r) and (III-s), respectively.

Example 19 – Scheme 15

[00252] Scheme 15 (**Fig. 17**) depicts the synthesis of conjugation-ready compounds of this invention having a single amino acid (citrulline) linker.

[00253] *Compound **85***. A mixture of compound **62** of Scheme 10 (0.22 g, 0.654 mmol), Fmoc-protected citrulline (0.39 g, 0.981 mmol), and *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride ("EDCI," 0.188 g, 0.981 mmol) in DMF (4 mL) was stirred at RT overnight. The reaction was quenched by addition of saturated NH_4Cl, and the aqueous solution was extracted with EtOAc. The combined organic layers were dried, filtered, and concentrated. The crude product was purified by flash chromatography, eluting from silica gel with a gradient of 0-100% MeOH in DCM to afford 0.42 g of compound **85** as a white solid. MS: (+) m/z 716.4 (M+1).

[00254] *Compound **86***. Piperidine was added to a solution of compound **85** (0.248 g, 0.346 mmol) in DMF, maintaining the pH at 9-10. The reaction mixture was stirred at RT for 20 min, and then concentrated to afford 0.17 g of compound **86**. MS: (+) m/z 494.4 (M+1).

[00255] *Compound **87***. LiOH (26.6 mg, 1.11 mmol) in water (3 mL) was added to a solution of compound **86** (0.17 g, 0.346 mmol) in THF (2 mL). After the reaction mixture was stirred at RT for 2 h, the solvent was partially removed. The aqueous solution was acidified to pH 2-3 with HCl and concentrated. The residue was re-dissolved in DMF (2 mL), and *N*-succinimidyl 6-maleimidohexanoate (0.16 g, 0.519 mmol) and DIEA (0.091 mL, 0.519 mmol) were added. After the reaction mixture was stirred at RT for 10 min, the crude product was purified by preparative HPLC to afford 0.198 g of compound **87** as a white solid. MS: (+) m/z 673.4 (M+1).

[00256] *Compound **88***. TFA (0.5 mL) was added to a solution of compound **87** (12.5 mg, 0.019 mmol) in DCM (0.5 mL) at room temperature. The reaction mixture was stirred at RT for 5 min, and then concentrated to afford 12.8 mg of compound **88** as a white solid. MS: (+) m/z 573.4 (M+1).

[00257] *Compound **89***. DIEA was added to a solution of compound **34a** of Scheme 3 (5 mg, 0.0093 mmol) and HATU (3.9 mg, 0.010 mmol) in DMF (0.3 mL), maintaining the pH at 8-9. The reaction mixture was stirred at RT for 5 min. Then DIEA and compound **88** (12.8 mg, 0.019 mmol) in DMF (1 mL) were added, maintaining the pH at 8-9. After the reaction mixture was stirred at RT for 15 min, the crude product was purified by preparative HPLC to afford 8.6 mg of compound **89** as a white solid. MS: (+) m/z 1093.8 (M+1). Compound **89** is also depicted in this specification by formula (VI-m).

[00258] *Compound **90***. DIEA was added to a solution of compound **49** of Scheme 5 (5 mg, 0.0098 mmol) and HATU (4.1 mg, 0.011 mmol) in DMF (0.3 mL), maintaining the pH at 8-9. The reaction mixture was stirred at RT for 5 min. Then DIEA and compound **88** (13.5 mg, 0.0196 mmol) in DMF (1 mL) were added, maintaining the pH 8-9. After the reaction mixture was stirred at RT for 15 min, the crude product was purified by preparative HPLC to afford 8.9 mg of compound **90** as a white solid. MS: (+) m/z 1065.6 (M+1). Compound **90** is also depicted in this specification by formula (VI-p).

Example 20 – Scheme 16

[00259] Scheme 16 (**Fig. 18**) depicts the preparation of conjugation-ready compounds of this invention, having a dipeptide (citrulline-valine) linker.

[00260] *Compound **91***. DIEA was added to a solution of Fmoc-protected valine (62.3 mg, 0.184 mmol) and HATU (63.6 mg, 0.167 mmol) in DMF (0.5 mL), maintaining the pH at 8-9. The reaction mixture was stirred at RT for 5 min. Then DIEA and compound **86** of Scheme 15 (82.5 mg, 0.167 mmol) in DMF (1 mL) were added, maintaining the pH at 8-9. After the reaction mixture was stirred at RT for 15 min, the reaction was quenched by addition of 0.05% aq. TFA. The aqueous solution was extracted with EtOAcef and the combined organic layers were dried, filtered and concentrated. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-20% MeOH in DCM to afford 0.13 g of compound **91** as a white solid. MS: (+) m/z 815.5 (M+1).

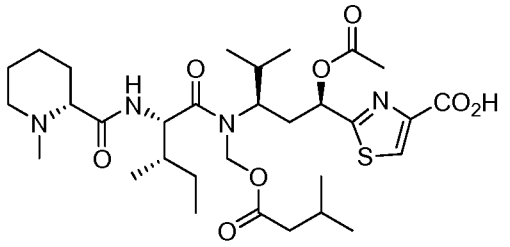
[00261] *Compound **92***. Piperidine was added to a solution of compound **91** (0.144 g, 0.177 mmol) in DMF, maintaining the pH at 9-10. The reaction mixture was stirred at RT for 20 min and then concentrated. The residue was dissolved in THF (2.5 mL) and LiOH (16.3 mg, 0.681 mmol) in water (1.3 mL) was added. After the reaction mixture was stirred at RT for 2 h, the solvent was partially removed. The aqueous solution was

acidified to pH 2-3 with HCl and then concentrated. The residue was re-dissolved in DMF (2.5 mL), and then *N*-succinimidyl 6-maleimidohexanoate (0.105 g, 0.341 mmol) and DIEA (0.060 mL, 0.341 mmol) were added. After the reaction mixture was stirred at RT for 10 min, the crude product was purified by preparative HPLC to afford 0.116 g of compound **92** as a white solid. MS: (+) m/z 772.5 (M+1).

[00262] *Compound **93***. TFA (0.6 mL) was added to a solution of compound **92** (14.4 mg, 0.019 mmol) in DCM (1 mL) at RT. The reaction mixture was stirred at RT for 5 min and then concentrated to afford 14.7 mg of compound **93** as a white solid. ^{1}H NMR (CD_3OD, 400 MHz): δ 7.58 (dd, 2H, J = 1.6, 8.4 Hz), 7.21 (dd, 2H, J = 2.8, 8.8 Hz), 6.79 (s, 2H), 4.48 (m, 1H), 4.13 (d, 1H, J = 7.6 Hz), 3.57-3.46 (m, 3H), 3.33-3.32 (m, 1H), 3.22-3.09 (m, 2H), 2.91-2.80 (m, 1H), 2.27 (t, 2H, J = 7.2 Hz), 2.09-1.85 (m, 3H), 1.81-1.54 (m, 8H), 1.35-1.29 (m, 3H), 1.19 (d, 1.5 H, J = 6.8 Hz), 1.18 (d, 1.5 H, J = 7.2 Hz), 0.98 (d, 3H, J = 2.4 Hz), 0.96 (d, 3H, J = 2.8 Hz); MS: (+) m/z 672.4 (M+1).

[00263] *Compound **94***. DIEA was added to a solution of compound **34a** of Scheme 3 (11 mg, 0.0204 mmol) and HATU (7.8 mg, 0.0204 mmol) in DMF (0.3 mL), maintaining the pH at 8-9. The reaction mixture was stirred at RT for 5 min. Then DIEA and compound **93** (14.7 mg, 0.019 mmol) in DMF (1 mL) were added, maintaining the pH at 8-9. After the reaction mixture was stirred at RT for 15 min, the crude product was purified by preparative HPLC to afford 18.9 mg of compound **94** as a white solid. MS: (+) m/z 1192.6 (M+1). Compound **94** is also depicted in this specification by formula (VI-n).

[00264] *Acetate of compound **27***. Acetic anhydride (0.248 mL) was added to a solution of compound **27** of Scheme 2 (Peltier et al., 2006;, 0.13 g, 0.218 mmol) in pyridine (2.6 mL) at 0 °C. The reaction mixture was then stirred at RT overnight. After the reaction mixture was cooled at 0 °C, a solution of water and 1,4-dioxane (12 mL, v/v 1:1) was added. The reaction mixture was stirred at RT overnight and then concentrated. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 10-20% MeOH in DCM to afford 0.114 g of the acetate of compound **27** as a white solid. MS: (+) m/z 639.4 (M+1).



Acetate of Compound **27**

[00265] *Compound **95***. DIEA was added to a solution of the acetate of compound **27** (3.8 mg, 0.0059 mmol), and HATU (2.5 mg, 0.0065 mmol) in DMF (0.3 mL), maintaining the pH at 8-9. The reaction mixture was stirred at RT for 5 min. Then DIEA and compound **93** (5.6 mg, 0.0071 mmol) in DMF (1 mL) were added, maintaining the pH at 8-9. After the reaction mixture was stirred at RT for 15 min, the crude product was purified by preparative HPLC to afford 6.5 mg of compound **95** as a white solid. MS: (+) m/z 1292.7 (M+1). Compound **95** is also depicted in this specification by formula (VI-o).

Example 21 – Scheme 17

[00266] This example describes the synthesis of acid **108**, an intermediate useful for the preparation of compounds of this invention, with reference to Scheme 17 (**Fig. 19**).

[00267] *Methyl ester **100***. HCl in dioxane (8.3 ml, 4M, 33.2 mmol) was added to a solution of compound **9** of Scheme 1(8 g, 22.1 mmol) in MeOH (10 mL). The reaction mixture was stirred at RT. After 20 min, the solution was evaporated under vacuum to give methyl ester **100** as an oil (6.5 g), which was used for the next reaction step without further purification.

[00268] *Propyl amine **101***. Propanal (700 µL, 7.36 mmol) and $NaBH(OAc)_3$ (2.8 g, 13.2 mmol) were added to a solution of methyl ester **100** (1.96g, 6.6 mmol) in DCM (10 mL). The reaction mixture was stirred at 5 °C. After 1 h the mixture was taken up in EtOAc and washed with 7% K_2CO_3 solution twice and then brine. The EtOAc layer was dried over anhydrous Na_2SO_4 and then evaporated under vacuum to yield a residue which was passed through a column (MeOH:DCM. 0-10%) to give propyl amine **101** (1.12 g, 60%) as an oil. ^1H NMR (400 MHz, $CDCl_3$) δ 8.17 (s, 1H), 5.43 (t, J = 4.6 Hz, 1H), 3.93 (s, 3H), 3.07-2.87 (m, 2H), 2.82-2.70 (m, 1H), 2.54 (s, 1H), 2.45-2.26 (m, 2H), 2.16-2.02 (m, 1H), 1.73 (m, 2H), 1.05-0.94 (m, 9H). MS *m/z* $C_{14}H_{25}N_2O_3S$ $(M+1)^+$ calculated 301.2, found 301.

[00269] *Compound **102**.* (Benzotriazol-1-yloxy)tripyrrolidinophosphonim hexafluorophosphate ("PyBop," 1.28 g, 2.47 mmol), HOBt (0.33 g, 2.47 mmol), Boc-protected isoleucine (430 μL, 2.47 mmol) were added to a solution of propyl amine **101** (570 mg, 1.9 mmol) in DCM (5 mL). The reaction mixture was stirred at RT. After 20 min EtOAc (200mL) was added and the organic layer was washed with 10% citric acid (twice), sat. $NaHCO_3$, and brine. The EtOAc layer was dried over anhydrous Na_2SO_4 and then evaporated under vacuum to yield a residue which was passed through a column to give compound **102** (0.55 g) as an oil. MS *m/z* $C_{25}H_{44}N_3O_6S$ $(M+1)^+$ calculated 514.3, found 514.3.

[00270] *Azido ester **104**.* Acid chloride **103** (2 mmol., Lundquist et al. 2001; see also above in the preparation of azido ester **43** of Scheme 5) in DCM (3 mL) was added to a solution of compound **102** (0.55 g, 1.1 mmol) in DCM (10 mL) and DIEA (871 μL, 5 mmol). The reaction mixture was stirred at 5 °C. After stirring for 10 min the mixture was evaporated under vacuum to give a residue which was passed through a column to give azido ester **104** (300 mg) as an oil. MS *m/z* $C_{31}H_{53}N_6O_7S$ $(M+1)^+$ calculated 653.4, found 653.

[00271] *Compound **106**.* A solution of pentafluorophenyl ester **105** (2.1 mmol, Peltier et al. 2006) in 1 mL of EtOAc was added to a solution of azido ester **104** (300 mg, 0.46 mmol) and Pd/C (10%, 50 mg) in EtOAc (5mL). The reaction flask was filled with H_2 using a balloon and was stirred overnight at RT. After stirring overnight the reaction mixture was filtered, concentrated under vacuum and then passed through a column (MeOH:DCM, 0-10%) to give compound **106** (170 mg) as an oil. MS *m/z* $C_{38}H_{66}N_5O_8S$ $(M+1)^+$ calculated 752.5, found 752.5.

[00272] *Compound **107**.* NaOH (120 uL, 1.2 mmol, 10M) was added to a solution of compound **106** (170 mg, 0.22 mmol) in MeOH (10mL) at RT. After stirring for 2 hr the reaction mixture was acidified to pH 2 with concentrated HCl. The reaction mixture was then evaporated under vacuum and passed through a reverse phase column (ACN:H_2O, 0-100% with 0.1% TFA). After lyophilization, compound **107** (63 mg) was obtained as a white powder. HPLC profile indicated it was a mixture of rotamers. MS *m/z* $C_{26}H_{45}N_4O_5S$ $(M+1)^+$ calculated 525.3, found 525.

[00273] *Acid **108**.* Acetic anhydride (60 uL, 0.64 mmol) was added to a solution of compound **107** (63 mg, 0.12 mmol) in pyridine (1 mL) at 5 °C. The temperature was raised to RT gradually. After allowing reaction to proceed overnight, water (100 uL) was

added. After another 5 hr, the volatile organics were removed under vacuum to give a residue which was passed through a reverse phase column (ACN:H_2O, 0-100% with 0.1% TFA) to give acid **108** (42 mg) as an oil. ^{1}H NMR (400 MHz, CD_3OD) δ 8.35 (s, 1H), 5.71 (dd, *J* = 11.4, 1.4 Hz, 1H), 4.63 (d, *J* = 9.1 Hz, 1H), 3.97 (t, *J* = 16.4 Hz, 1H), 3.65-3.42 (m, 2H), 3.21-3.05 (m, 2H), 2.87 (s, 3H), 2.34-2.14 (m, 4H), 2.13 (s, 3H), 2.03-1.46 (m, 10H), 1.29-1.06 (m, 1H), 1.04-0.85 (m, 15H). MS *m/z* $C_{28}H_{47}N_4O_5S$ $(M+1)^+$ calculated 567.3, found 567.

Example 22 – Schemes 18 and 19

[00274] Scheme 18 (**Figs. 20a** and **20b**) shows the synthesis of compounds of this invention using as acid **108**, prepared in the preceding example.

[00275] *General procedure for HATU-mediated coupling.* HATU (1.2x excess) and DIEA (4x excess) were added into a solution of acid **108** in DMF at 5 °C. After stirring the reaction mixture for 10 min, the corresponding amine was added. The reaction mixture was stirred for another 10 min before diluting it with DMSO and 0.1% TFA solution. The resulting mixture was passed through a reverse phase column (ACN:H_2O, 0-100% with 0.1% TFA). The collected fractions were analyzed and the desired fractions were lyophilized to give the corresponding product.

[00276] *Compound **109***. Obtained from the coupling of acid **108** and phenylalanine methyl ester. MS *m/z* $C_{38}H_{58}N_5O_7S$ $(M+1)^+$ calculated 728.4, found 728.4. Compound **109** is also depicted hereinabove as structure (III-x).

[00277] *Compound **111***. Obtained from the coupling of acid **108** and compound **110** (preparation described below). MS *m/z* $C_{42}H_{63}N_6O_9S$ $(M+1)^+$ calculated 827.4, found 827.5.

[00278] *Compound **112***. To a solution of compound **111** (5 mg, 6 μmol) in 2 mL of MeOH was added Pd/C (10%, 10 mg) . The reaction flask was filled with H_2 using a balloon and was stirred for 2 h at RT. The reaction mixture was then filtered, concentrated under vacuum and passed through a reverse phase column (ACN:H_2O, 0-100% with 0.1% TFA) to give compound **112** (2.1 mg) as a white power. MS *m/z* $C_{42}H_{67}N_6O_7S$ $(M+1)^+$ calculated 799.5, found 799.5. Compound **112** is also depicted hereinabove as structure (III-y).

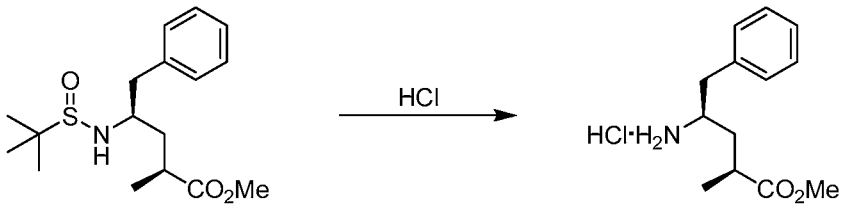
[00279] *Compound 114*. Obtained from the coupling of acid **108** and compound **113** (preparation described below). MS *m/z* $C_{41}H_{64}N_5O_7S$ $(M+1)^+$ calculated 770.4, found 770.

[00280] *Compound 116*. Obtained from the coupling of acid **108** and compound **115** (preparation described below). MS *m/z* $C_{61}H_{95}N_{11}O_{13}S$ $(M+2)^+$ calculated 610.9, found 611. Compound **116** is also depicted hereinabove as structure (VI-t).

[00281] *Compound 117*. Obtained from the coupling of acid **108** and alpha-N-acetyl lysine methyl ester. MS *m/z* $C_{37}H_{63}N_6O_8S$ $(M+1)^+$ calculated 751.4, found 751.5.

[00282] *Compound 110*. Alkene **59** of Scheme 8(as ethyl ester instead of methyl ester, 1 g, 2.6 mmol) was dissolved in DCM (10 mL) containing 5% TFA and the reaction mixture was stirred at 5 °C. After 40 min the mixture was dried under vacuum to give compound **110** (0.3 g, 100%) as a semi-solid. ^{1}H NMR (400 MHz, CD_3OD) δ 8.22-8.17 (m, 2H), 7.50 (dd, J = 9.0, 2.2 Hz, 2H), 6.58 (d, J = 10.0 Hz, 1H), 4.45 (td, J = 9.8, 5.3 Hz, 1H), 4.19 (q, J = 7.1 Hz, 2H), 3.35-3.28 (m, 1H), 3.06 (dd, J = 13.2, 9.6 Hz, 1H), 1.55 (d, J = 0.9 Hz, 3H), 1.27 (t, J = 7.1 Hz, 3H).

[00283] *Compound 113*. HCl (2.5 mL, 10 mmol, 4 M) was added to a solution of compound **118** (2 g, 5.5 mmol, Peltier et al. 2006) in MeOH (10 mL) and the reaction mixture was stirred at RT. After 20 min the reaction mixture was dried under vacuum to give compound compound **113** (2 g, 100%) as a semi-solid. Crude product was used for the next step reaction without further purification. MS *m/z* $C_{13}H_{19}NO_2$ $(M+1)^+$ calculated 222.1, found 222.



118 **113**

[00284] Scheme 19 (**Fig. 21**) shows the synthesis of compound **115**, used in the synthesis of compound **116**, above.

[00285] *Compound 120*. DIEA (697 µL, 12 mmol) and valine t-butyl ester 543 (627 mg, 3 mmol) were added into a 10 mL DCM solution of 6-maleimidohexanoic acid ("6-MHA," 622 mg, 3 mmol) and HATU (1.14 g, 3 mmol). After 20 min EtOAc (200 mL) was added. The organic phase was washed with 10% citric acid, sat. $NaHCO_3$ solution,

and brine. It was then dried with anhydrous Na_2SO_4 and the solvent was removed by evaporation. The resulting residue was passed through a column (Hexane:EtOAc, 0-80%) to give compounn **120** (900 mg) as an oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.66 (s, 2H), 5.94 (d, J = 8.5 Hz, 1H), 4.44 (dd, J = 8.7, 4.5 Hz, 1H), 3.49 (t, J = 7.2 Hz, 2H), 2.31-2.06 (m, 3H), 1.73-1.54 (m, 4H), 1.45 (s, 9H), 1.37-1.25 (m, 2H). MS *m/z* $C_{19}H_{31}N_2O_5$ $(M+1)^+$ calculated 367.2, found 367.

[00286] *Compound **121***. Compound **120** (1g, 2.73 mmol) was dissolved in 20 mL DCM with 3 mL TFA at RT. After 1 h the mixture was dried by evaporation to give compound **121** (1g) as an oil, which was used without further purification. MS *m/z* $C_{15}H_{23}N_2O_5$ $(M+1)^+$ calculated 311.2, found 311.

[00287] *Compound **123***. DIEA (920 µL, 5.28 mmol) and compound **122** (500 mg, 1.32 mmol; see Scheme 22 and Example 25 hereinbelow) were added a 10 mL DMF solution of Fmoc-protected citrulline (524 mg, 1.32 mmol) and HATU (601 mg, 1.58 mmol). After 20 min, 200 mL EtOAc was added. The organic phase was washed with 10% citric acid, sat. $NaHCO_3$ solution, and brine. It was then dried with anhydrous Na_2SO_4 and the EtOAc was evaporated. The resulting residue was passed through a column (MeOH:DCM; 0-10%) to give a solid. This solid was dissolved in DMF (5 mL) with 5% piperidine. After 1h the solution was evaporated and the residue was passed through a reverse phase column (ACN:H_2O; 0-100% with 0.1% TFA) to give compound **123** (212 mg). MS *m/z* $C_{27}H_{46}N_5O_6$ $(M+1)^+$ calculated 536.3, found 536.4.

[00288] *Compound **124***. DIEA (404 µL, 2.4 mmol) and compound **560** (321 mg, 0.6 mmol) were added into a 5 mL DMF solution of compound **550** (180 mg, 0.58 mmol) and HATU (220 mg, 0.58 mmol). After 20 min 200 mL EtOAc was added. The organic phase was washed with 10% citric acid, sat. $NaHCO_3$ solution, and brine. It was then dried with anhydrous Na_2SO_4 and the EtOAc evaporated. The resulting residue was passed through a column (MeOH:DCM; 0-20%) to give compound **124** (240 mg) as an oil. MS *m/z* $C_{42}H_{66}N_7O_{10}$ $(M+1)^+$ calculated 828.5, found 828.5.

[00289] *Compound **115***. Compound **124** (240 mg, 0.29 mmol) was dissolved in a 5 mL solution of TFA and DCM (1:1). After 3 h the mixture was dried by evaporation and the resulting compound **115** was used without further purification. From NMR, a mixture of two (5:1) isomers was obtained. The major isomer is reported: ^{1}H NMR (400 MHz, CD_3OD) δ 8.27 (d, J = 7.5 Hz, 1H), 7.58 (dd, J = 8.5, 1.9 Hz, 2H), 7.21 (d, J = 8.5 Hz,

2H), 6.79 (s, 2H), 4.48 (dd, J = 13.3, 8.1 Hz, 1H), 4.14 (dd, J = 7.5, 4.9 Hz, 1H), 3.62-3.38 (m, 3H), 3.25-2.97 (m, 3H), 2.96-2.78 (m, 2H), 2.70-2.40 (m, 1H), 2.32-2.21 (m, 2H), 2.11-1.92 (m, 2H), 1.94-1.83 (m, 1H), 1.82-1.70 (m, 1H), 1.70-1.49 (m, 7H), 1.19 (d, J = 7.0 Hz, 3H), 0.97 (dd, J = 6.8, 2.8 Hz, 6H). MS *m/z* $C_{33}H_{50}N_7O_8$ $(M+1)^+$ calculated 672.4, found 672.

Example 23 – Scheme 20

[00290] Scheme 20 (**Fig. 22**) shows the synthesis of compound **131**, an intermediate used for making compounds of this invention.

[00291] *Compound **125***. Compound **9** of Scheme 1 (3 g, 8.29 mmol) was dissolved in THF (20mL) and dimethylsufate (1.2 mL, 12.4 mmol). To this solution was added NaH (552 mg, 13.8 mmol) at 5 °C in portions over 1.5 h. The reaction mixture was then poured into sat. NH_4Cl solution. EtOAc was added to the reaction mixture and the organic phase was washed with brine and dried and evaporated under vacuum to give a residue. The resulting residue was passed through a column (Hexane:EtOAc, 0-100%) to give compound **125** (1.2 g) as an oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.12 (s, 1H), 4.95 (dd, J = 10.0, 3.3 Hz, 1H), 3.89 (s, 3H), 3.50 (s, 3H), 3.45-3.40 (m, 2H), 1.93-1.79 (m, 2H), 1.74-1.65 (m, 1H), 1.20 (s, 9H), 0.84 (d, J = 6.8 Hz, 3H), 0.80 (d, J = 6.8 Hz, 3H). MS *m/z* $C_{16}H_{29}N_2O_4S_2$ $(M+1)^+$ calculated 377.1, found 377.2.

[00292] *Compound **126***. HCl in dioxane (1 ml, 4 mmol) was added to a solution of compound **125** (0.7 g, 1.86 mmol) in MeOH (10 mL). The reaction mixture was stirred at RT. After 20 min, the volatiles were evaporated under vacuum to give compound **126** (0.8 g) as an oil which was used for the next reaction step without further purification. MS *m/z* $C_{12}H_{21}N_2O_3S$ $(M+1)^+$ calculated 273.1, found 273.

[00293] *Compound **127***. To a solution of compound **126** (616 mg, 2 mmol) in DCM (10 mL) and DIEA (1.8 mL, 10 mmol) at 5 °C, was added compound **103** (Scheme 17, 6 mmol) in 5 mL of DCM. The reaction mixture was stirred for 3 h at RT. After 3 h the reaction mixture was poured into sat. $NaHCO_3$ solution and EtOAc. The organic phase was washed with brine, dried, and evaporated. The resulting residue was passed through a column (Hexane:EtOAc, 0-50%) to give compound **127** (594 mg, 72%) as a semi oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.18 (s, 1H), 6.47 (d, J = 9.9 Hz, 1H), 4.60-4.52 (m, 1H), 4.23-4.13 (m, 1H), 3.96-3.95 (m, 1H), 3.94 (s, 3H), 3.44 (s, 3H), 2.21-2.08 (m, 1H),

1.94-1.84 (m, 2H), 1.84-1.71 (m, 1H), 1.52-1.38 (m, 1H), 1.35-1.20 (m, 1H), 1.07 (d, J = 6.9 Hz, 3H), 0.95-0.85 (m, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 176.00, 168.63, 161.92, 146.90, 128.24, 78.81, 70.34, 58.97, 52.72, 50.76, 40.48, 38.62, 32.24, 24.32, 19.13, 18.13, 16.25, 11.82. MS *m/z* $C_{18}H_{30}N_5O_4S$ $(M+1)^+$ calculated 412.2, found 412.3.

[00294] *Compound **129**.* Potassium hexamethyldisilazide ("KHMDS," 0.19 mmol, 0.375 mL of toluene solution) was added into a THF (0.5 mL) solution of compound **127** (50 mg, 0.12 mmol) at -43 °C. After 20 min compound **128** (0.36 mmol, 137 uL, Abe et al. 1997) was added. After 2 hr, 100 µL MeOH was added and the reaction mixture was poured into sat. NH_4Cl solution. EtOAc was then added. After separation of the layers, the organic phase was washed with brine, dried with anhydrous Na_2SO_4 and the solvent removed by evaporation. The resulting residue was passed through a column (Hexane:EtOAc, 0-50%) to give compound **129** (51 mg) as a semi solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.16 (s, 1H), 5.70 (s, 1H), 5.43 (d, J = 12.4 Hz, 1H), 5.32 (d, J = 12.3 Hz, 1H), 4.39 (d, J = 10.6 Hz, 1H), 3.92 (d, J = 11.7 Hz, 3H), 3.53-3.44 (m, 1H), 3.37 (d, J = 10.5 Hz, 3H), 2.41 (d, J = 7.2 Hz, 2H), 2.37-2.11 (m, 4H), 1.92 – 1.68 (m, 2H), 1.37-1.21 (m, 1H), 1.12 – 0.85 (m, 18H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.24, 172.79, 171.21, 161.90, 147.09, 128.25, 78.44, 68.69, 63.35, 58.71, 52.48, 43.23, 38.63, 34.91, 31.01, 25.73, 25.25, 22.58, 22.56, 22.48, 20.45, 19.62, 16.14, 10.65. MS *m/z* $C_{24}H_{40}N_5O_6S$ $(M+1)^+$ calculated 526.3, found 424.3 (breaking of the N,O acetal).

[00295] *Compound **130**.* Compounds **129** (200 mg, 0.38 mmol) and **105** (Peltier et al. 2006; 4 mmol) were mixed in 5 mL EtOAc with Pd/C (150 mg, 10%) at rt. The reaction flask was evacuated and refilled with H_2 using a balloon. After stirring overnight at RT, the mixture was filtered and the solvent evaporated. After column chromatography (SiO_2, MeOH:DCM, 0-10%), compound **130** (97 mg) was obtained as a solid. MS *m/z* $C_{31}H_{53}N_4O_7S$ $(M+1)^+$ calculated 625.4, found 625.5.

[00296] *Compound **131**.* Tribuytltin hydroxide (181 mg, 0.59 mmol) was added to a solution of compound **130** (97 mg, 0.16 mmol) in 10 mL 1,2-dichloroethane. After 22 h at 67 °C, the mixture was evaporated and passed through a reverse phase column (ACN:(20 mM $NH_4(HCO_3)$ buffer, pH 7), 5-100%) to give compound **131** (34 mg) as a solid. MS *m/z* $C_{30}H_{51}N_4O_7S$ $(M+1)^+$ calculated 611.3, found 510 (break at the N,O acetal).

Example 24 – Scheme 21

[00297] Scheme 21 (**Fig. 23**) shows the synthesis of compounds of this invention using compound **131** as a precursor.

[00298] *Compound 132.* Compound **60** (Scheme 8, 200 mg, 0.57 mmol) was dissolved in 2 mL DCM with 20% TFA at rt. After 1h the volatiles were evaporated to give compound **132** (200 mg) as a yellow solid, which was used without further purification.

[00299] *Compound 133.* DIEA (43 µL, 0.2 mmol) was added to a DMF (1 mL) solution of compound **131** (30 mg, 0.049 mmol) and HATU (22.3 mg, 0.059 mmol) at -43 °C. After 10 min compound **132** (15 mg, 0.06 mmol) was added. The mixture was then raised to RT. The final mixture was passed through a reverse phase column (ACN:(20 mM $NH_4(HCO_3)$ buffer, pH 7), 5-100%) to give compound **133** (20 mg) as a white powder. MS *m/z* $C_{44}H_{73}N_6O_8S$ $(M+1)^+$ calculated 843.5, found 843.5. Compound **133** is also depicted hereinabove as structure (III-u).

[00300] *Compound 134.* Compound **133** (2 mg, 2.4 µmol) was dissolved in 0.5 mL methanol and the pH of the solution was adjusted to 1 with 1M HCl. After stirring overnight, the volatiles were evaporated and the residue was passed through a reverse phase column (ACN:(20 mM $NH_4(HCO_3)$ buffer, pH 7), 5-100%) to give compound **134** (0.7 mg) as a white powder. MS *m/z* $C_{40}H_{65}N_6O_7S$ $(M+1)^+$ calculated 773.5, found 773.5. Compound **134** is also depicted hereinabove as structure (III-v).

[00301] *Compound 135.* Compound **133** (2 mg, 2.4 µmol) was dissolved in 0.5 mL n-propanol and the pH of the solution was adjusted to 1 with1M HCl. After stirring overnight, the mixture was evaporated and the residue was passed through a reverse phase column (ACN:(20 mM $NH_4(HCO_3)$ buffer, pH 7), 5-100%) to give compound **135** (0.4 mg) as a white powder. MS *m/z* $C_{42}H_{69}N_6O_7S$ $(M+1)^+$ calculated 802.5, found 801.5. Compound **135** is also depicted hereinabove as structure (III-w).

Example 25 – Scheme 22

[00302] Scheme 22 (**Fig. 24**) shows a method for making compound **142**, useful as an intermediate for making compounds of this invention.

[00303] *Compound 136.* NaOH (800 uL, 10M, 8 mmol) was added to a 20 mL solution of THF and MeOH (1:1) with compound **59** of Scheme 8 (1.65 g, 4.37 mmol). After stirring overnight the pH of the solution was adjusted to 1 with 3N HCl at 5 °C.

After evaporation of the solvents, 200 mL EtOAc was added. After separation, the organic phase was washed with brine, dried with anhydrous Na_2SO_4 and the EtOAc evaporated. The residue was passed through a column (MeOH:DCM; 0-10%) to give compound **136** (1.2 g) as an oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.21-8.12 (m, 2H), 7.41-7.32 (m, 2H), 6.62 (d, J = 8.8 Hz, 1H), 4.82-4.57 (m, 2H), 3.15-3.02 (m, 1H), 2.90 (dd, J = 13.3, 7.2 Hz, 1H), 1.71 (d, J = 1.2 Hz, 3H), 1.41 (s, 9H).

[00304] *Compound 137.* DMF-di-t-Butylacetal (1 mL, 4 mmol) was added to a 6 mL toluene solution of compound **136** (128 mg, 0.36 mmol) at 133 °C. After 10 min the reaction mixture was cooled and the solvent evaporated. The residue was passed through a column (Hexane:EtOAc; 0-30%) to give compound **137** (133 mg) as an oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.19-8.10 (m, 2H), 7.39-7.30 (m, 2H), 6.39 (dd, J = 9.1, 1.5 Hz, 1H), 4.63 (d, J = 39.1 Hz, 2H), 3.03 (dd, J = 13.2, 6.2 Hz, 1H), 2.90 (dd, J = 13.3, 7.0 Hz, 1H), 1.67 (d, J = 1.5 Hz, 3H), 1.47 (s, 9H), 1.39 (s, 9H).

[00305] *Compound 122.* Compound **137** (540 mg, 1.22 mmol), Pd/C (136 mg, 10%) and 3N HCl (0.3 mL) were added to a mixture of DCM and MeOH (30 mL:5 mL). The flask was filled with H_2 using a balloon. After stirring overnight at RT, the mixture was filtered and concentrated to give compound **122** (550 mg) as a semi-solid. MS m/z $C_{21}H_{35}N_2O_4$ $(M+1)^+$ calculated 379.3, found 223 (loss of Boc).

[00306] *Compound 138.* Compound **136** (100 mg, 0.28 mmol) and Pd/C (20 mg, 10%) were mixed in a 5 mL mixture of MeOH and DCM (1:1 v:v) under a hydrogen balloon at RT. After stirring overnight the mixture was filtered and the solvents evaporated under vacuum to give compound **138** (95 mg) as oil, which was used for next reaction step without further purification. MS *m/z* $C_{17}H_{27}N_2O_4$ $(M+1)^+$ calculated 323.2, found 223.

[00307] *Compound 139.* Compound **138** (10 mg, 0.03 mmol), tert-butyldimethylsilyl chloride ("TBDMSCl," 4.5 mg, 0.03 mmol) and imidazole (4 mg, 0.06 mmol) were mixed in 1 mL DMF at RT. Fmoc-protected citrulline (24 mg, 0.06 mmol), *N,N'*-disuccinimidyl oxalate ("DSO," 8 mg, 0.06 mmol) and DIEA (20 uL, 0.12 mmol) were mixed in another 1 mL of DMF at RT. After 1 h the two solutions were mixed and the mixture was kept at RT. After stirring overnight, EtOAc was added and the solution was washed with 10% aq. citric acid and brine. The organic phase was then dried with anhydrous Na_2SO_4 and evaporated under vacuum. The resulting residue was passed

through a column (MeOH:DCM, 0-10%) to give compound **139** (7 mg) as an oil. MS *m/z* $C_{38}H_{48}N_5O_8$ $(M+1)^+$ calculated 702, found 702.

[00308] *Compound 141.* Compound **139** (10 mg, 0.014 mmol) was dissolved in 1 mL DMF with 5% piperidine. After 20 min the solvent was evaporated under vacuum and the residue was mixed with N-succinimidyl-4-maleimidobutyrate **140** (5.6 mg, 0.028 mmol) and DIEA (5 uL, 0.028 mmol) in 1 mL DMF. After 10 min the solvents were removed from the reaction mixture under vacuum and passed through a column (MeOH:DCM, 0-20%) to give compound **141** (6 mg) as a solid. MS *m/z* $C_{31}H_{45}N_6O_9$ $(M+1)^+$ calculated 645, found 645.

[00309] *Compound 142.* Compound **141** (6 mg, 0.01 mmol) was dissolved in 1 mL DCM with 10% TFA. After 10 min the solvent was evaporated under vacuum to give compound **142** (6 mg), used for next reaction step without further purification.

Example 26 – Scheme 23

[00310] Scheme 23 (**Fig. 25**) shows the elaboration of compound **142**, made per Scheme 22, into a compound of this invention.

[00311] *Compound 145.* HCl (30 µmmol) in 150 µL MeOH was added to a solution of compound **27** of Scheme 2 (5 mg, 8.3 µmmol) in 0.7 mL MeOH at 5 °C. The temperature was allowed to rise to RT gradually. After stirring overnight the mixture was evaporated and dissolved in 0.7 pyridine. To this solution was added Ac_2O (28 µL, 296 µmmol) at 5 °C. The temperature was allowed to rise to RT gradually and after stirring overnight 50 µL H_2O was added. After 3 h the volatiles was evaporated and the resulting residue was evaporated to give compound **145** (4.7 mg) as a semi solid. MS *m/z* $C_{27}H_{45}N_4O_7S$ $(M+1)^+$ calculated 569.3, found 569.

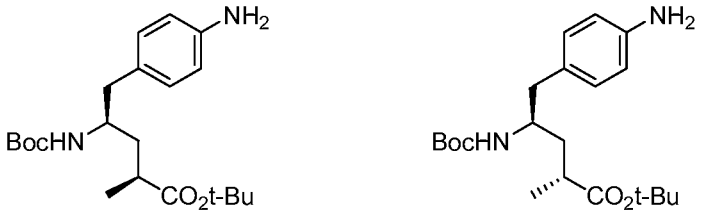
[00312] *Compound 146.* DIEA (6 µL, 34 µmol) and compound **142** (5.5 mg, 8.3 µmol) were added to 0.5 mL DMF solution of compound **530** (4.7 mg, 8.3 µmmol) and HATU (3.2 mg, 8.4 µmol) at 5 °C. After 20 min, the resulting mixture was passed through a reverse phase column (ACN:(20 mM $NH_4(HCO_3)$ buffer, pH 7), 5-100%) to give compound **146** (4.5 mg) as a white solid. MS *m/z* $C_{53}H_{79}N_{10}O_{13}S$ $(M+1)^+$ calculated 1095.5, found 1095.5. Compound **146** is also depicted hereinabove as structure (VI-r).

[00313] *Compound 147.* DIEA (1.4 uL, 8 µmol) and a drop of saturated NH_4Cl solution were added into a 0.5 mL DMF solution of compound **146** (2 mg, 1.8 µmol) and

HATU (1.6 mg, 4.6 μmol). After 10 min the mixture was passed through a reverse phase column (ACN:(20 mM $NH_4(HCO_3)$ buffer, pH 7), 5-100%) to give compound **147** (0.5 mg) as a semi solid. MS *m/z* $C_{53}H_{80}N_{11}O_{12}S$ $(M+1)^+$ calculated 1094.6, found 1094. Compound **147** is also depicted hereinabove as structure (VI-s).

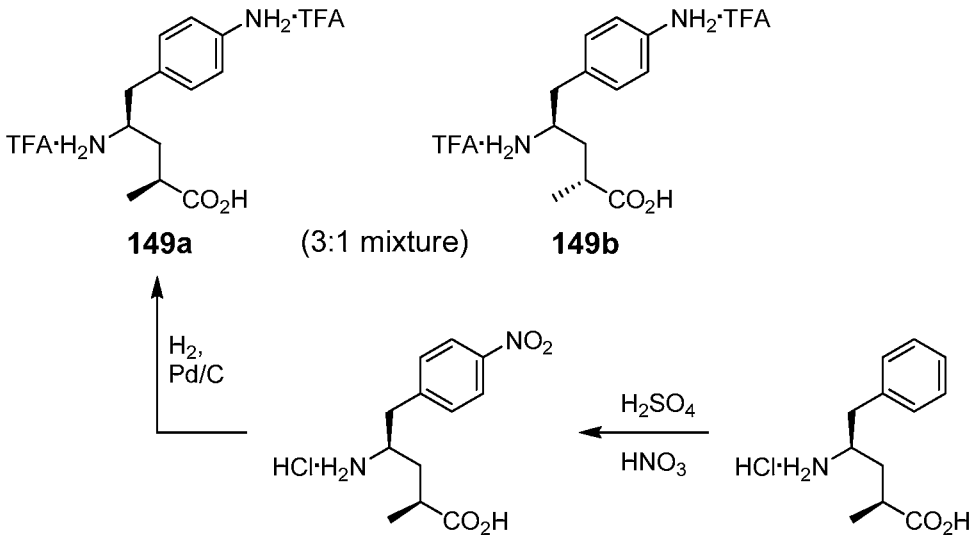
Example 27 – 4-Aminotubuphenylalanine diastereomers

[00314] Compound **122** (Example 25 above) was determined to be a 3:1 mixture of diastereomers **148a** and **148b** as follows.



148a (3:1 mixture) **148b**

[00315] Compound **122** (10 mg, 0.026 mmol) was dissolved in a 2mL mixture of TFA and DCM (1:1) at RT. After 3 h the solvents were evaporated and the residue was passed through a reverse phase column (ACN:H_2O; 0-100% with 0.1% TFA) to give a 3:1 mixture of compounds **149a** and **149b**. The major isomer in this mixture was assigned structure **149a** by comparing the NMR spectrum of the mixture with the NMR of an authentic sample of compound **149a** made from compound **150.**



149a (3:1 mixture) **149b**

151 **150**

[00316] Concentrated HNO_3 (10 μL) was added to a 200 μL concentrated H_2SO_4 solution of tubuphenylalanine **150** (Peltier et al. 2006; 6 mg, 0.025 mmol) at 5 °C. After 20 min the solution was poured onto 2 mL of cooled 7% K_2CO_3 solution. 10 mL EtOAc was then added. After separation, the organic phase was dried by evaporation and the residue was passed through a reverse phase column (ACN:H_2O; 0-100% with 0.1% TFA) to give nitro compound **151** (5 mg). ^{1}H NMR (400 MHz, CD_3OD) δ 8.27-8.21 (m, 2H), 7.56-7.49 (m, 2H), 3.69-3.58 (m, 1H), 3.07 (d, J = 7.2 Hz, 2H), 2.73-2.61 (m, 1H), 2.04-1.91 (m, 1H), 1.64 (ddd, J = 14.7, 8.2, 4.9 Hz, 1H), 1.20 (d, J = 7.1 Hz, 3H). MS m/z $C_{12}H_{17}N_2O_4$ $(M+1)^+$ calculated 253.1, found 253.

[00317] Nitro compound **151** then converted to compound **149a** as follows: Nitro compound **151** (5 mg, 0.01 mmol) was mixed with Pd/C (10 mg, 10%) in 5 mL MeOH at RT. The flask was filled with H_2 using a balloon. After 1 h the mixture was filtered and evaporated to give compound **149a** (4.5 mg). ^{1}H NMR (400 MHz, CD_3OD) δ 7.08-7.03 (m, 2H), 6.83-6.79 (m, 2H), 3.51-3.41 (m, 1H), 2.84-2.78 (m, 2H), 2.68-2.58 (m, 1H), 2.04-1.92 (m, 1H), 1.60 (d, J = 7.8 Hz, 1H), 1.18 (d, J = 7.0, Hz, 3H). MS m/z $C_{12}H_{19}N_2O_2$ $(M+1)^+$ calculated 223.1, found 223. This NMR spectrum was the basis for assigning the structures of the major component of the **148a/148b** and **149a/149b** mixtures.

[00318] A sample of compound **122** was passed through a reverse phase column (ACN:H_2O; 0-100% with 0.1% TFA) and the fractions with the minor isomer were collected and lyophilized. The resulting product was then treated with TFA and DCM to remove the Boc group. After 1 h the solvents were evaporated to give a product, which was assigned to be compound **149b**, after comparing its NMR spectrum with that of compound **149a**. Compound **149b**: ^{1}H NMR (400 MHz, CD_3OD) 7.36-7.23 (m, 2H), 7.22-7.09 (m, 2H), 3.59-3.40 (m, 1H), 3.04-2.84 (m, 2H), 2.61-2.45 (m, 1H) 2.07-1.87 (m, 1H), 1.73-1.58 (m, 1H), 1.21-1.09 (d, J = 7.2 Hz, 3H).

[00319] Compounds **148a, 148b**, **149a**, and **149b** can be used to prepare compounds of this invention having a 4-aminotubuphenylalanine subunit, where the stereochemistry of the alpha-methyl group is defined, utilizing the synthetic approaches exemplified above, *mutatis mutandis*. Compounds **82a** and **82b** (Example 17) can also be put to similar use.

[00320] The foregoing detailed description of the invention includes passages that are chiefly or exclusively concerned with particular parts or aspects of the invention. It is to

be understood that this is for clarity and convenience, that a particular feature may be relevant in more than just the passage in which it is disclosed, and that the disclosure herein includes all the appropriate combinations of information found in the different passages. Similarly, although the various figures and descriptions herein relate to specific embodiments of the invention, it is to be understood that where a specific feature is disclosed in the context of a particular figure or embodiment, such feature can also be used, to the extent appropriate, in the context of another figure or embodiment, in combination with another feature, or in the invention in general.

[00321] Further, while the present invention has been particularly described in terms of certain preferred embodiments, the invention is not limited to such preferred embodiments. Rather, the scope of the invention is defined by the appended claims.

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[00322] Full citations for the following references cited in abbreviated fashion by first author (or inventor) and date earlier in this specification are provided below. Each of these references is incorporated herein by reference for all purposes.

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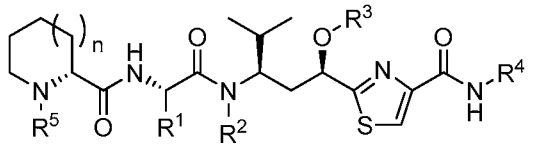
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CLAIMS

What is claimed is:

1. A compound having a structure represented by formula (II)



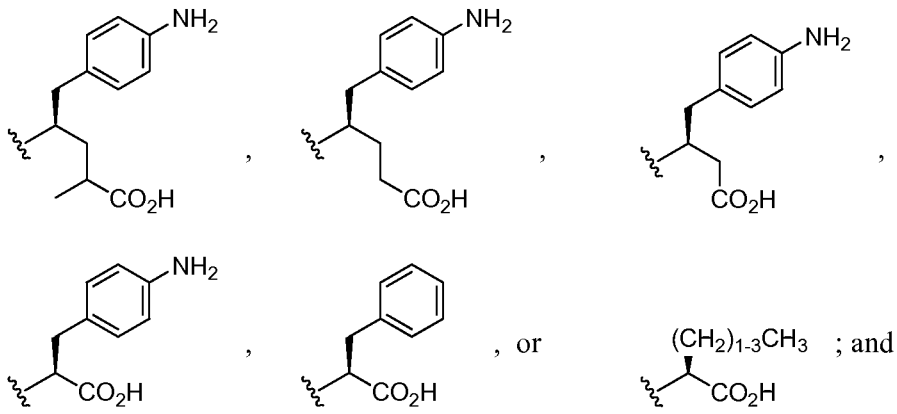
(II)

wherein

n is 0, 1, or 2;

R^1, R^2 and R^3 are independently H, unsubstituted or substituted C_1-C_{10} alkyl, unsubstituted or substituted C_2-C_{10} alkenyl, unsubstituted or substituted C_2-C_{10} alkynyl, unsubstituted or substituted aryl, unsubstituted or substituted heteroaryl, unsubstituted or substituted $(CH_2)_{1-2}O(C_1$-C_{10} alkyl), unsubstituted or substituted $(CH_2)_{1-2}O(C_2$-C_{10} alkenyl), unsubstituted or substituted $(CH_2)_{1-2}O(C_2$-C_{10} alkynyl), $(CH_2)_{1-2}OC(=O)(C_1$-C_{10} alkyl), unsubstituted or substituted $(CH_2)_{1-2}OC(=O)(C_2$-C_{10} alkenyl), unsubstituted or substituted $(CH_2)_{1-2}OC(=O)(C_2$-C_{10} alkynyl), unsubstituted or substituted $C(=O)(C_1$-C_{10} alkyl), unsubstituted or substituted $C(=O)(C_2$-C_{10} alkenyl), unsubstituted or substituted $C(=O)(C_2$-C_{10} alkynyl), unsubstituted or substituted cycloaliphatic, unsubstituted or substituted heterocycloaliphatic, unsubstituted or substituted arylalkyl, or unsubstituted or substituted alkylaryl;

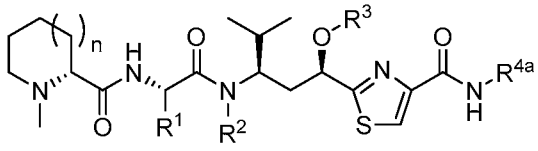
R^4 is



R^5 is H, C_1-C_5 alkyl, C_2-C_5 alkenyl, C_2-C_5 alkynyl, CO(C_1-C_5 alkyl), CO(C_2-C_5 alkenyl), or CO(C_2-C_5 alkynyl);

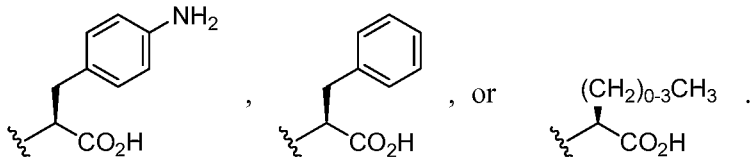
or a pharmaceutically acceptable ester thereof, a pharmaceutically acceptable amide thereof at the carboxyl group of R^4 with the α-amino group of an α-amino acid, or a pharmaceutically acceptable salt thereof.

2. A compound according to claim 1, having a structure represented by formula (II-a)

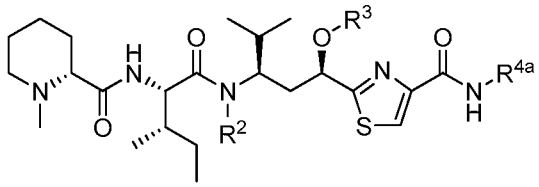


(II-a)

wherein R^{4a} is



3. A compound according to claim 2, having a structure represented by formula (II-a'):



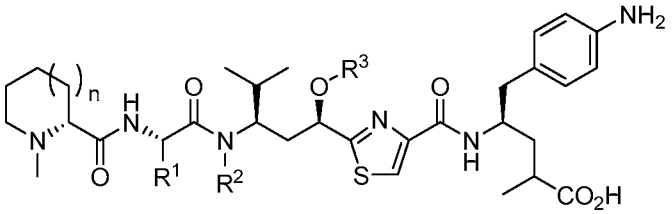
(II-a')

wherein

R^2 is H, C_1-C_5 alkyl, C_2-C_5 alkenyl, CH_2O(C_1-C_5 alkyl), CH_2O(C_2-C_5 alkenyl), CH_2O(C=O)(C_1-C_5 alkyl), or CH_2OC(=O)(C_2-C_5 alkenyl); and

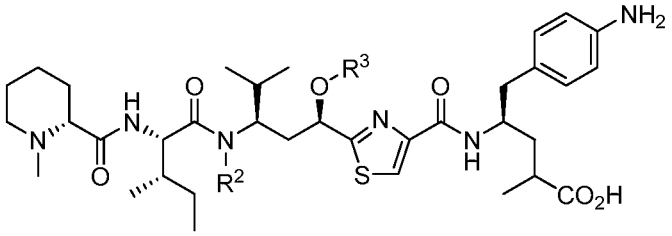
R^3 is H, C_1-C_5 alkyl, C_2-C_5 alkenyl, C(=O)C_1-C_5 alkyl, or C(=O)C_2-C_5 alkenyl.

4. A compound according to claim 1, having a structure represented by formula (II-b):



(II-b)

5. A compound according to claim 4, having a structure represented by formula (II-b'):



(II-b')

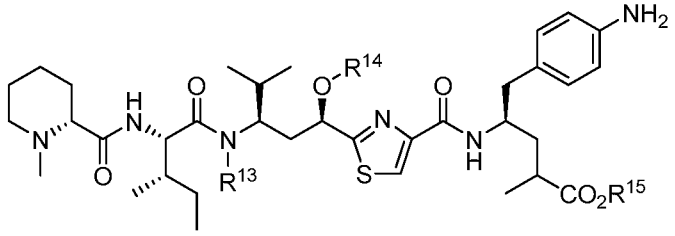
wherein

R^2 is H, C_1-C_5 alkyl, C_2-C_5 alkenyl, $CH_2O(C_1$-C_5 alkyl), $CH_2O(C_2$-C_5 alkenyl), $CH_2O(C{=}O)(C_1$-C_5 alkyl), or $CH_2OC(=O)(C_2$-C_5 alkenyl); and

R^3 is H, C_1-C_5 alkyl, C_2-C_5 alkenyl, $C(=O)C_1$-C_5 alkyl, or $C(=O)C_2$-C_5 alkenyl.

6. A compound according to claim 1, having a structure according to formula (III-a), III-b), (III-c), (III-d), (III-e), (III-f), (III-g), (III-h), (III-i), (III-j), (III-k), (III-l), (III-m), (III-n), (III-o), (III-p), (III-q), (III-r), (III-s), (III-t), (III-u), (III-v), (III-w), or (III-y).

7. A compound according to claim 1, having a structure represented by formula (II-c)



(II-c)

where R^{13} is Me, n-Pr, CH_2OMe, or $CH_2OC(=O)CH_2CH(Me)_2$; R^{14} is Me or C(=O)Me; and R^{15} is H or C_1-C_5 alkyl.

8. A conjugate comprising a compound according to claim 1 conjugated to an antibody.

9. A conjugate having a structure represented by formula (IV):

$$[D(X^D)_aC(X^Z)_b]_mZ \qquad (IV)$$

wherein

D is a compound according to claim 1;

Z is an antibody;

X^D and X^Z are spacer groups;

C is a group cleavable at the site of intended biological action of D;

each of a and b is independently 0 or 1; and

m is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

10. A composition of matter having a structure represented by formula (V-a)

$$D\text{-}(X^D)_aC(X^Z)_b\text{-}R^{31} \qquad (V\text{-}a)$$

wherein

R^{31} is a functional group suitable for reacting with a functional group on an antibody;

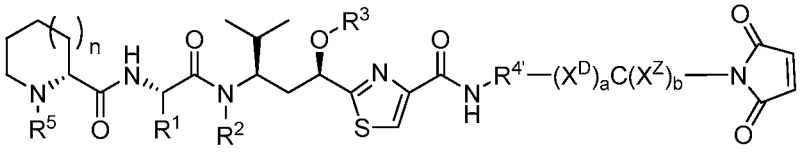
D is a compound according to claim 1;

X^D and X^Z are spacer groups;

C is a group cleavable at the site of intended biological action of D; and

each of a and b is independently 0 or 1.

11. A composition of matter having a structure represented by formula (V-b)



(V-b)

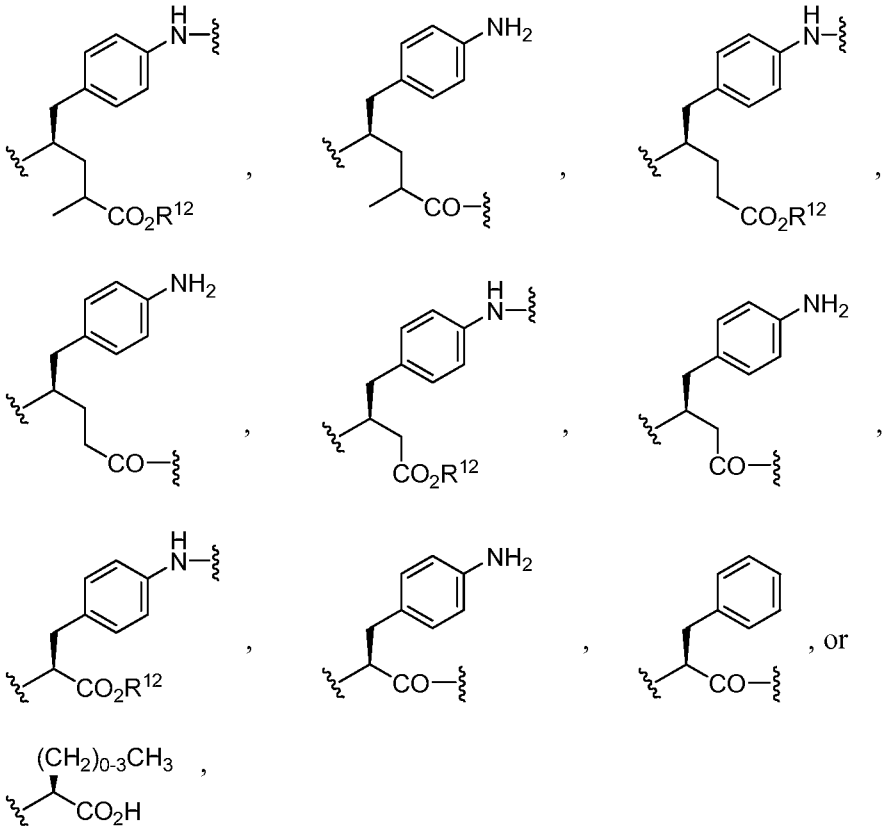
wherein

n is 0, 1, or 2;

R^1, R^2 and R^3 are independently H, unsubstituted or substituted C_1-C_{10} alkyl, unsubstituted or substituted C_2-C_{10} alkenyl, unsubstituted or substituted C_2-C_{10} alkynyl, unsubstituted or substituted aryl, unsubstituted or substituted heteroaryl, unsubstituted or substituted $(CH_2)_{1\text{-}2}O(C_1\text{-}C_{10}$ alkyl), unsubstituted or substituted $(CH_2)_{1\text{-}2}O(C_2\text{-}C_{10}$ alkenyl), unsubstituted or substituted $(CH_2)_{1\text{-}2}O(C_2\text{-}C_{10}$ alkynyl), $(CH_2)_{1\text{-}2}OC(=O)(C_1\text{-}C_{10}$ alkyl),

unsubstituted or substituted $(CH_2)_{1-2}OC(=O)(C_2-C_{10}$ alkenyl), unsubstituted or substituted $(CH_2)_{1-2}OC(=O)(C_2-C_{10}$ alkynyl), unsubstituted or substituted $C(=O)(C_1-C_{10}$ alkyl), unsubstituted or substituted $C(=O)(C_2-C_{10}$ alkenyl), unsubstituted or substituted $C(=O)(C_2-C_{10}$ alkynyl), unsubstituted or substituted cycloaliphatic, unsubstituted or substituted heterocycloaliphatic, unsubstituted or substituted arylalkyl, or unsubstituted or substituted alkylaryl;

$R^{4'}$ is



wherein R^{12} is H, C_1-C_5 alkyl, C_2-C_5 alkenyl, or C_2-C_5 alkynyl; and

R^5 is H, C_1-C_5 alkyl, C_2-C_5 alkenyl, C_2-C_5 alkynyl, $CO(C_1-C_5$ alkyl), $CO(C_2-C_5$ alkenyl), or $CO(C_2-C_5$ alkynyl);

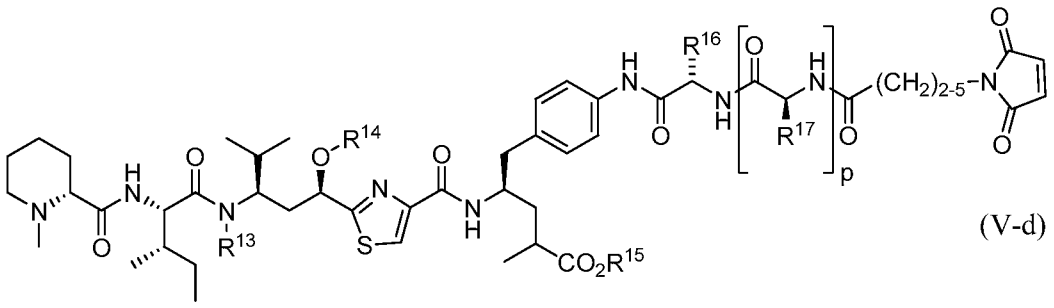
X^D and X^Z are spacer groups;

C is a cleavable group; and

a and b are independently 0 or 1;

wherein group $R^{4'}$ is linked via a carboxyl or amine group therein to either group X^D in the event a is 1 or to group C in the event a is 0.

12. A composition of matter having a structure represented by formula (V-d):

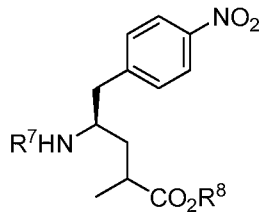


(V-d)

where R^{13} is Me, n-Pr, R^{14} is Me or C(=O)Me; and R^{15} is (preferably H, Me, or Et). where R^{13} is Me, n-Pr, CH_2OMe, or $CH_2OC(=O)CH_2CH(Me)_2$; R^{14} is Me or C(=O)Me; R^{15} is H or C_1-C_5 alkyl; R^{16} is $(CH_2)_4NH_2$ or $(CH_2)_3NHC(=O)NH_2$; R^{17} is $C(Me)_2$ or Me; and p is 0 or 1.

13. A method for treating a cancer in a subject suffering from such cancer, comprising administering to the subject a therapeutically effective amount of a compound according to claim 1, or a conjugate thereof with a ligand, in particular an antibody.

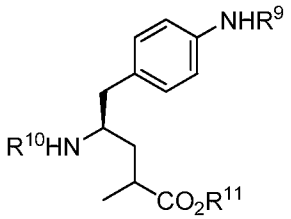
14. A compound having a structure according to formula (VIII-a)



(VIII-a)

wherein R^7 is H or an amine protecting group and R^8 is H, C_1-C_{10} alkyl, C_2-C_{10} alkenyl, C_2-C_{10} alkynyl, aryl, cycloaliphatic, alkylcycloaliphatic, arylalkyl, or alkylaryl.

15. A compound having a structure according to formula (VIII-b)



(VIII-b)

wherein R^9 and R^{10} are independently H or an amine protecting group and R^{11} is H, C_1-C_{10} alkyl, C_2-C_{10} alkenyl, C_2-C_{10} alkynyl, aryl, cycloaliphatic, alkylcycloaliphatic, arylalkyl, or alkylaryl.

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