~~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.

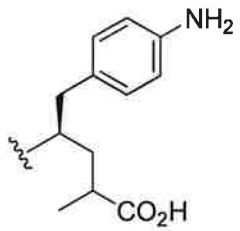
[0002] The tubulysins are cytotoxins originally isolated from cultures of the myxobacteria *Archangium gephyra* or *Angiococcus disciformis*, with each organism producing a different mixture of tubulysins (Sasse et al. 2000; Reichenbach et al. 1998). Their crystal structure and biosynthetic pathway have 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.

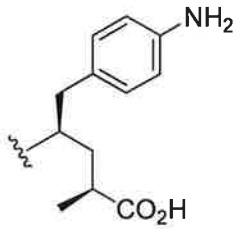
[0004] The tubulysins have a tetrapeptidyl scaffold constructed from one proteinogenic and three non-proteinogenic amino acid subunits: N-methylpipecolinic acid (Mep), isoleucine (Ile), tubuvaline (Tuv), and either tubuphenylalanine (Tup, R^A equals H in formula (I) below) or tubutyrosine (Tut, R^A equals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

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 compounds for use in 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 compound for use in 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 compound for use in 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.

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

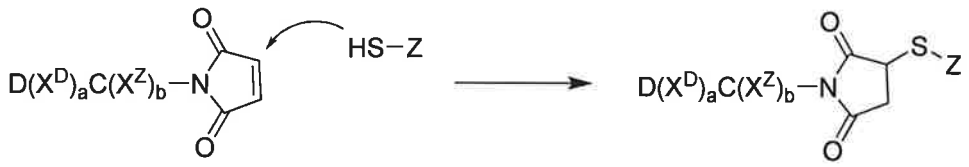
reduction of native disulfide groups. The thiol groups so generated can then be used for conjugation. See, e.g., Packard et al., *Biochemistry* **1986**, *25*, 3548-3552; King et al., *Cancer Res.* 54, 6176-6185 (1994); and Doronina et al., *Nature Biotechnol.* 21(7), 778-784 (2003); ~~the disclosures 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 hetero-robifunctional 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.

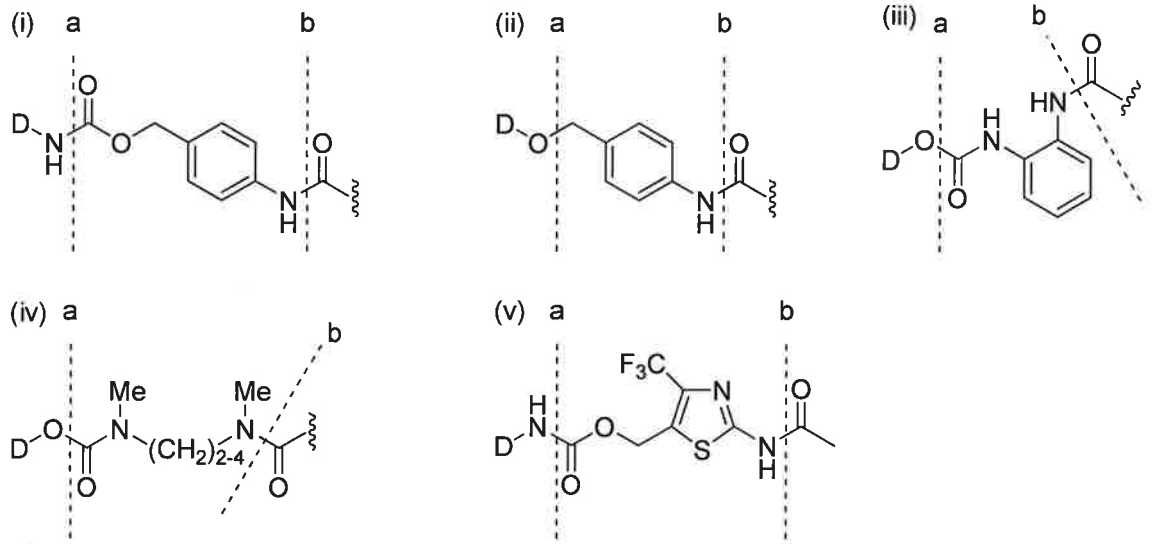
[00110] The preparation and design of cleavable groups C consisting of a single amino acid is disclosed in Chen et al., US 2010/0113476 A1, ~~the disclosure of which is incorporated herein by reference~~.

[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

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:



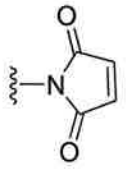
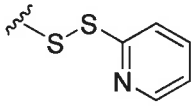
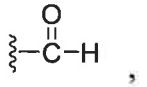
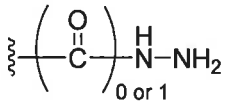
[00117] The self-immolating moiety is the structure between dotted lines a and b, with adjacent structural features shown to provide context. Self-immolating moieties (i) and (v) are bonded to a compound D-NH_2 (i.e., compound D is conjugated via an amino group), while self-immolating moieties (ii), (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):

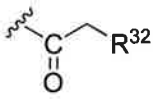
$$D\text{-}(X^D)_aC(X^Z)_b\text{-}R^{31} \qquad \text{(V-a)}$$

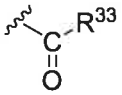
where R^{31} is a functional group suitable for reacting with a functional group on antibody Z to form the conjugate. Examples of suitable groups R^{31} include:


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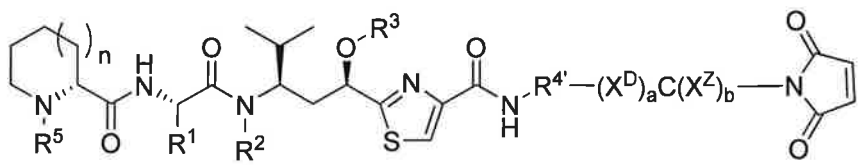
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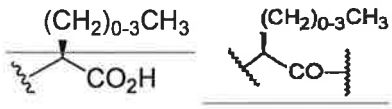
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, and


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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 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;

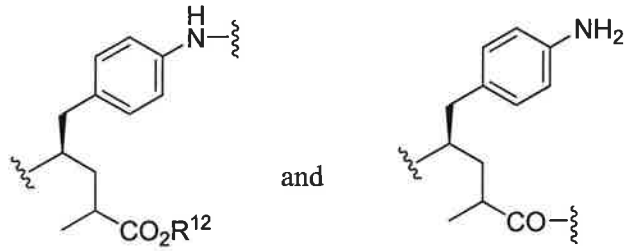
C is a cleavable group; and

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.

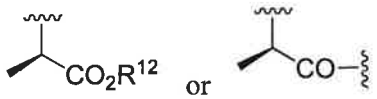
[00120] Preferably, the link between the carboxyl or amine group in $R^{4'}$ and group X^D or C, as the case may be, is via an amide bond.

[00121] In the structures



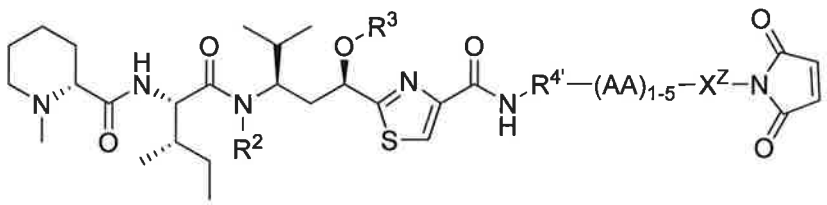
and

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



or

[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)

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

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

[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]~~[00320] 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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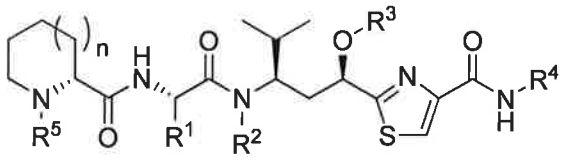
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CLAIMS

~~What is claimed is:~~

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



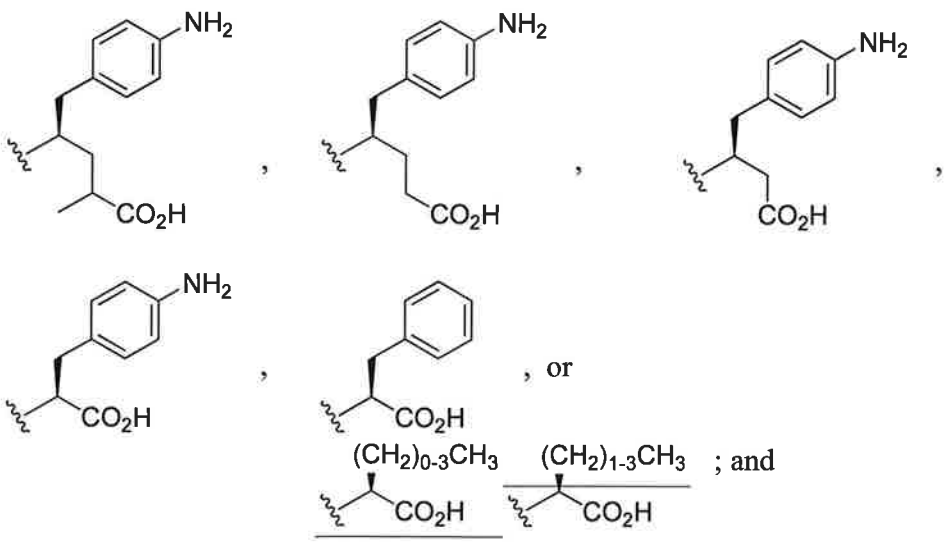
(II)

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 $(CH_2)_{1\text{-}2}O(C_1\text{-}C_{10}$ 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;

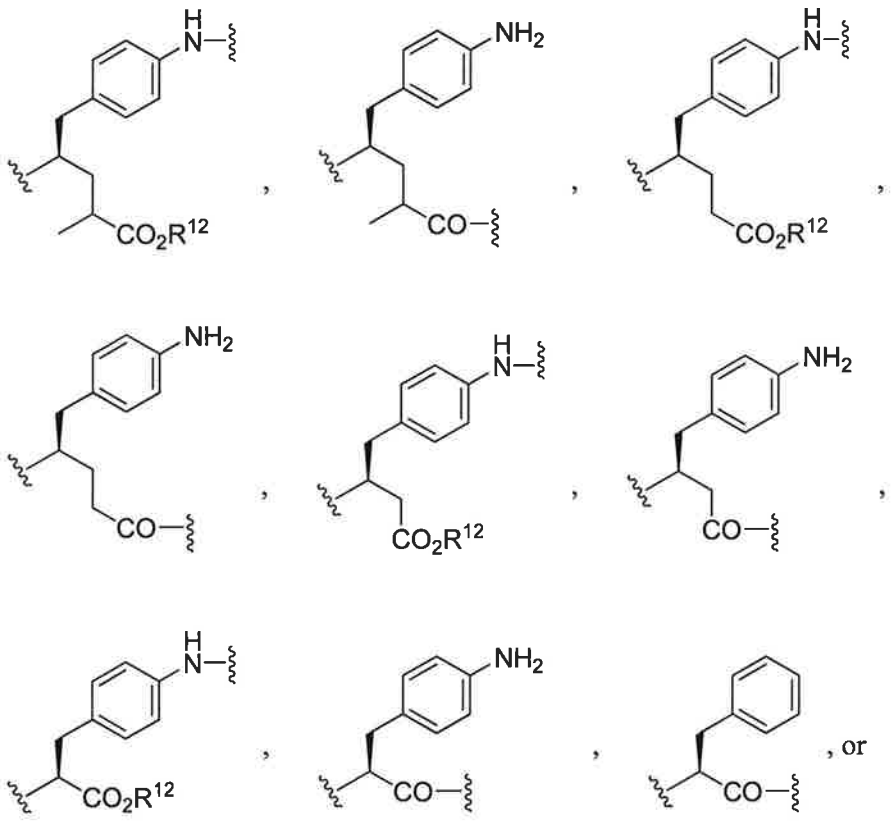
R^4 is



; and

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;

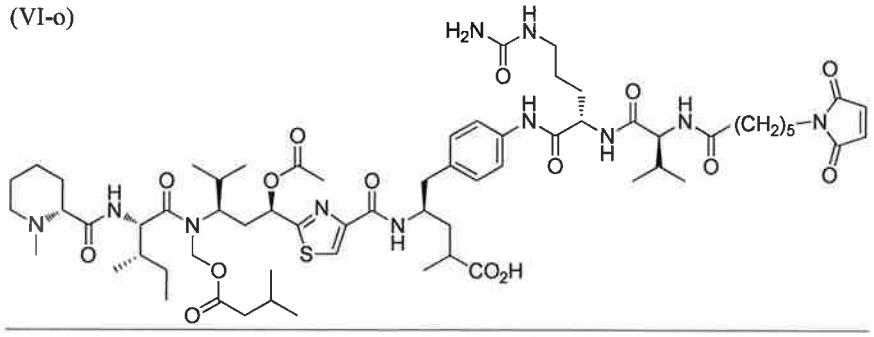
$R^{4'}$ is



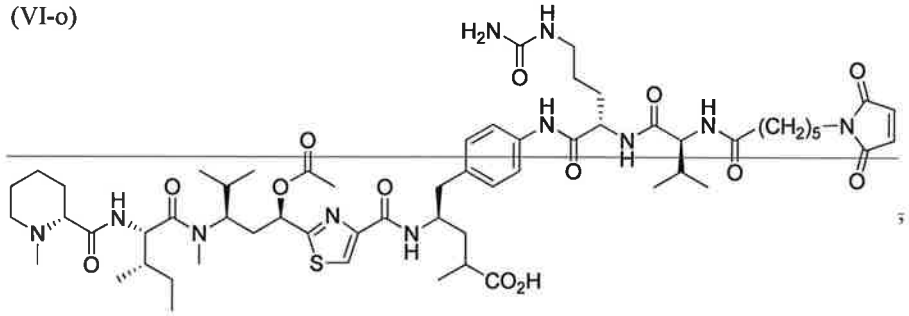
wherein R^{12} is H, C_1-C_5 alkyl, C_2-C_5 alkenyl, or C_2-C_5 alkynyl; and

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);

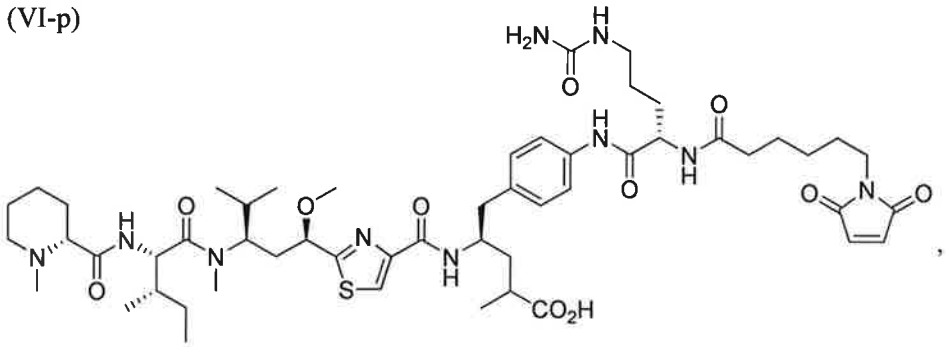
(VI-o)



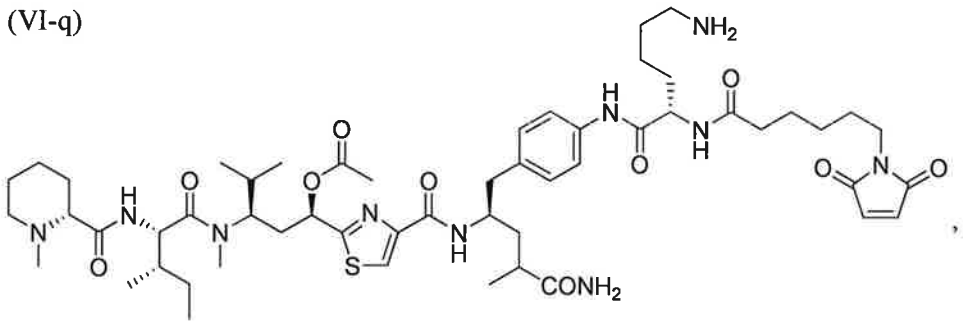
(VI-o)



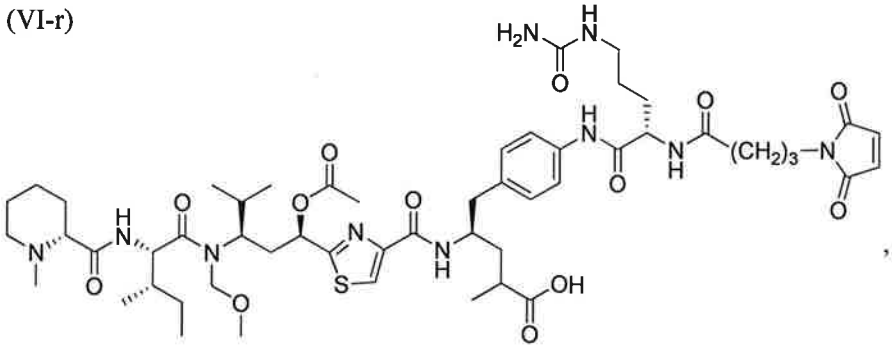
(VI-p)



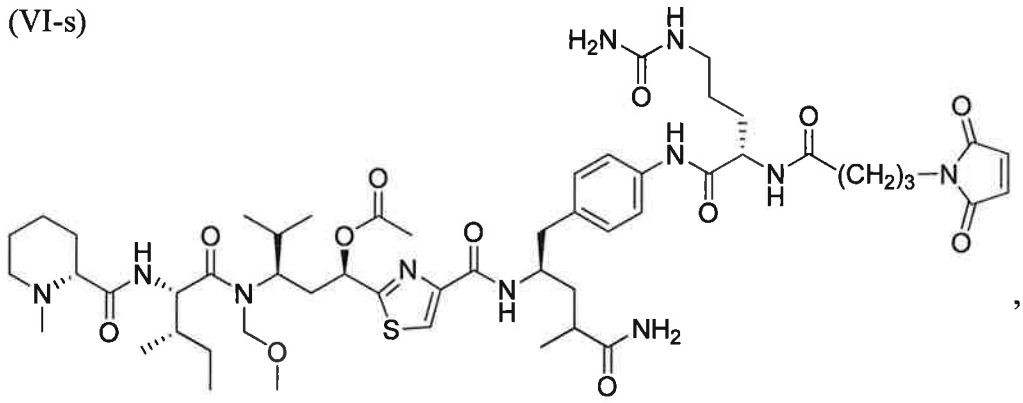
(VI-q)



(VI-r)

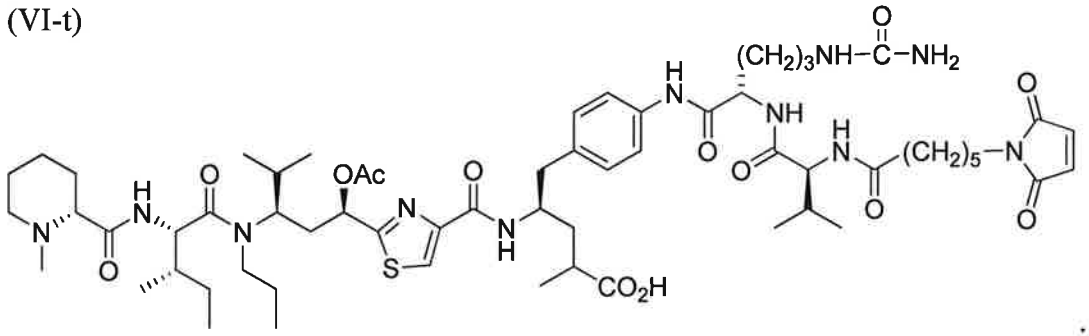


(VI-s)



, and

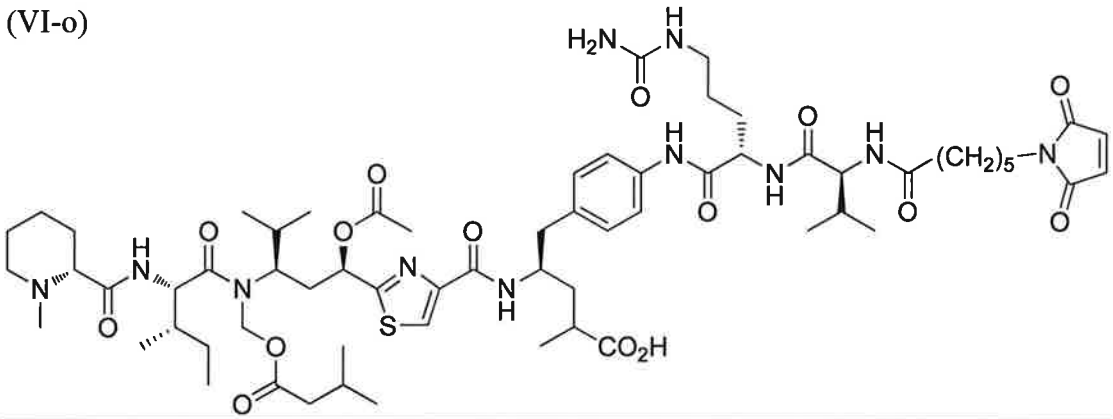
(VI-t)



.

12. The compound-linker molecule according to claim 8 having a structure represented by formula (VI-o)

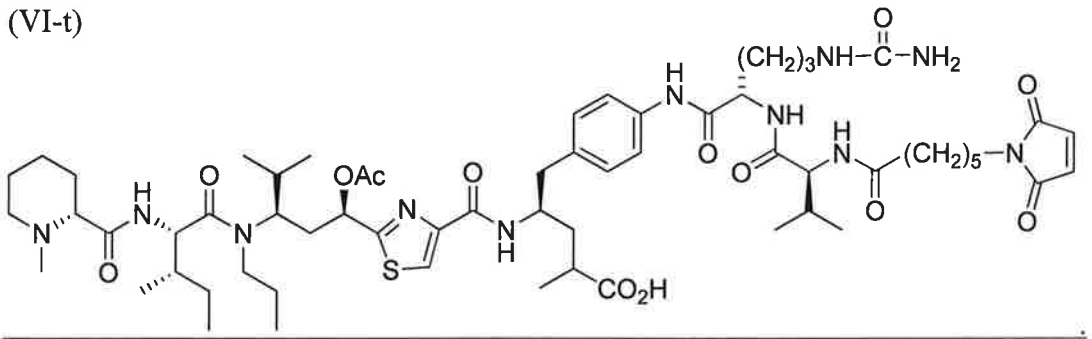
(VI-o)



.

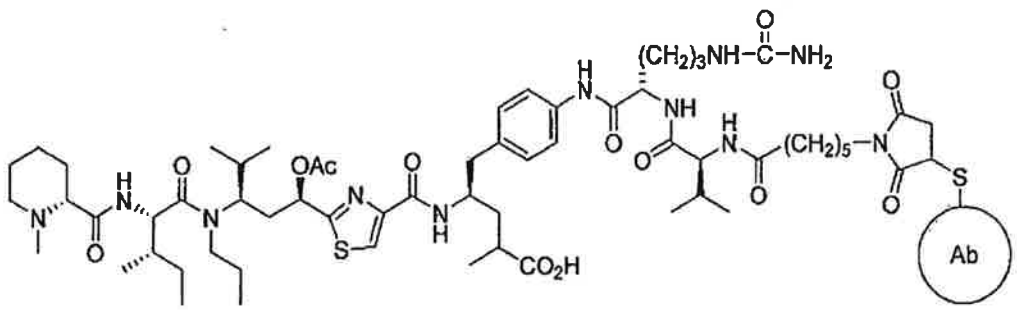
13. The compound-linker molecule according to claim 8 having a structure represented by formula (VI-t)

(VI-t)



~~12~~14. A conjugate comprising the compound-linker molecule according to any one of claims 8 to ~~11~~ 13 conjugated to a ligand via the nucleophilic addition product of a sulfhydryl group of the ligand and the maleimide group of the compound-linker molecule, wherein the ligand is selected from antibodies, antibody fragments and antibody mimetics.

15. The conjugate according to claim 14, wherein the compound-linker molecule has a structure represented by formula:



wherein Ab represents the human monoclonal anti-mesothelin antibody 6A4 having the heavy chain variable region sequence:

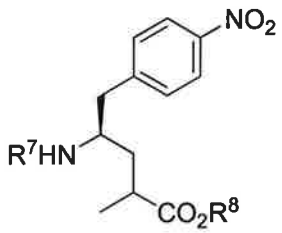
QVHLVESGGGVVQPGRSLRLSCVASGITFRIYGMHWVRQAPGKGLEWVAVLWYDG SHEYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAIYYCARDGDYYDSGSPLDY WGQGTLVTVSS

and the light variable region sequence:

EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRATGI PARFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPLTFGGGTKVEIK.

~~13~~16. ~~Use of a~~ A compound according to any one of claims 1 to 7, or a conjugate according to claim ~~12~~14 or claim 15, ~~in the preparation of a medicament~~ for use in treating a cancer.

~~14~~17. 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~~18. A compound having a structure according to formula (VIII-b)