Office de la Propriété Intellectuelle du Canada
Un organisme d'Industrie Canada

Canadian Intellectual Property Office
An agency of Industry Canada

CA 2900854 C 2017/08/22

(11)(21) 2 900 854

(12) BREVET CANADIEN
CANADIAN PATENT

(13) C

(86) Date de dépôt PCT/PCT Filing Date: 2014/02/10
(87) Date publication PCT/PCT Publication Date: 2014/08/21
(45) Date de délivrance/Issue Date: 2017/08/22
(85) Entrée phase nationale/National Entry: 2015/08/10
(86) N° demande PCT/PCT Application No.: US 2014/015503
(87) N° publication PCT/PCT Publication No.: 2014/126836
(30) Priorité/Priority: 2013/02/14 (US61/764,825)

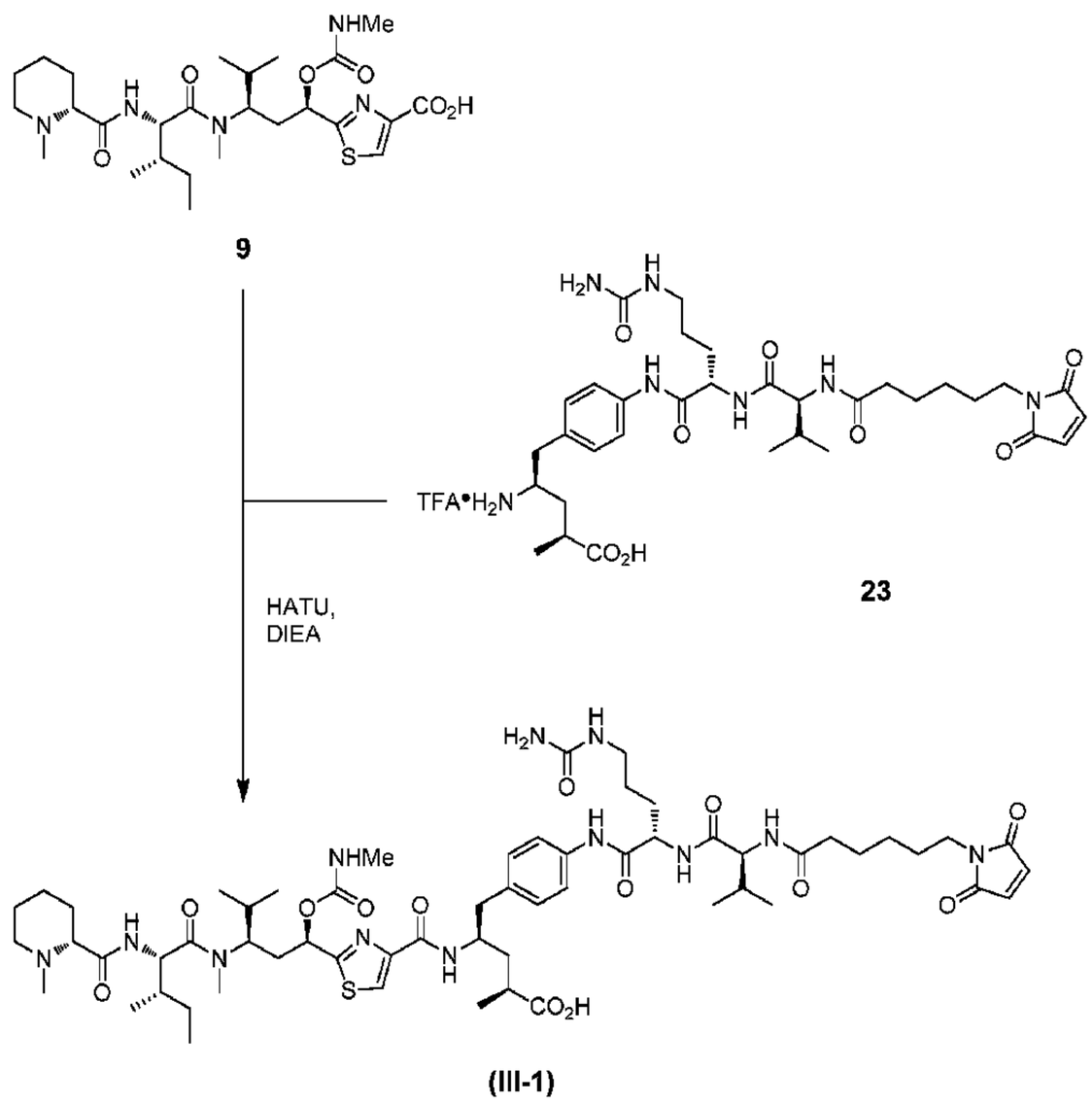
(51) Cl.Int./Int.Cl. *C07K 5/078* (2006.01), *A61P 35/00* (2006.01), *C07K 16/00* (2006.01)

(72) Inventeurs/Inventors:
CHENG, HENG, US;
CONG, QIANG, US;
GANGWAR, SANJEEV, US

(73) Propriétaire/Owner:
BRISTOL-MYERS SQUIBB COMPANY, US

(74) Agent: GOWLING WLG (CANADA) LLP

(54) Titre : COMPOSES DE TUBULYSINE, PROCEDES D'ELABORATION ET D'UTILISATION CORRESPONDANTS
(54) Title: TUBULYSIN COMPOUNDS, METHODS OF MAKING AND USE



(III-1)

(57) Abrégé/Abstract:
Tubulysin compounds of the formula (I) where R^1, R^2 R^{3a}, R^{3b}, R^4, R^5, W, and n are as defined herein, are anti-mitotic agents that can be used in the treatment of cancer, especially when conjugated to a targeting moiety.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
21 August 2014 (21.08.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/126836 A1

(51) **International Patent Classification**:
A61K 47/48 (2006.01) *A61P 35/00* (2006.01)
C07K 5/078 (2006.01)

(21) **International Application Number**:
PCT/US2014/015503

(22) **International Filing Date**:
10 February 2014 (10.02.2014)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:
61/764,825 14 February 2013 (14.02.2013) US

(71) **Applicant**: **BRISTOL-MYERS SQUIBB COMPANY** [US/US]; Route 206 and Province Line Road, Princeton, NJ 08543-4000 (US).

(72) **Inventors**: **CHENG, Heng**; c/o Bristol-Myers Squibb Company, 700 Bay Road, Redwood City, CA 94063 (US). **CONG, Qiang**; c/o Bristol-Myers Squibb Company, 700 Bay Road, Redwood City, CA 94063 (US). **GANGWAR, Sanjeev**; c/o Bristol-Myers Squibb Company, 700 Bay Road, Redwood City, CA 94063 (US).

(74) **Agents**: **CHAO, Yuan** et al.; Bristol-Myers Squibb Company, Route 206 & Province Line Road, Princeton, NJ 08543 (US).

(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,

[Continued on next page]

(54) Title: TUBULYSIN COMPOUNDS, METHODS OF MAKING AND USE

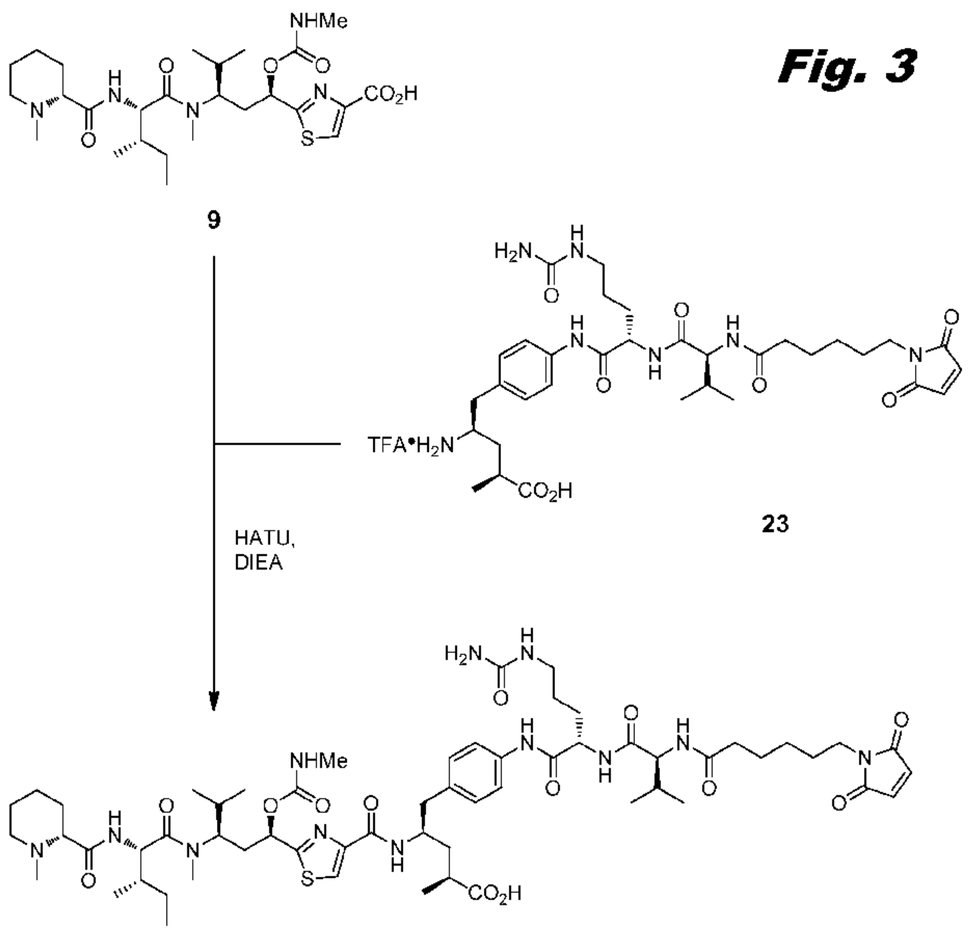
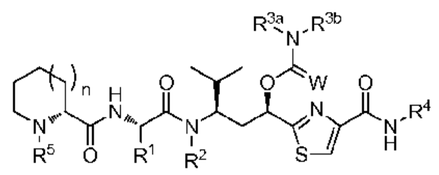


Fig. 3

(III-1)



(I)

(57) Abstract: Tubulysin compounds of the formula (I) where R^1, R^2 R^{3a}, R^{3b}, R^4, R^5, W, and n are as defined herein, are anti-mitotic agents that can be used in the treatment of cancer, especially when conjugated to a targeting moiety.

WO 2014/126836 A1

WO 2014/126836 A1

TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

TUBULYSIN COMPOUNDS, METHODS OF MAKING AND USE

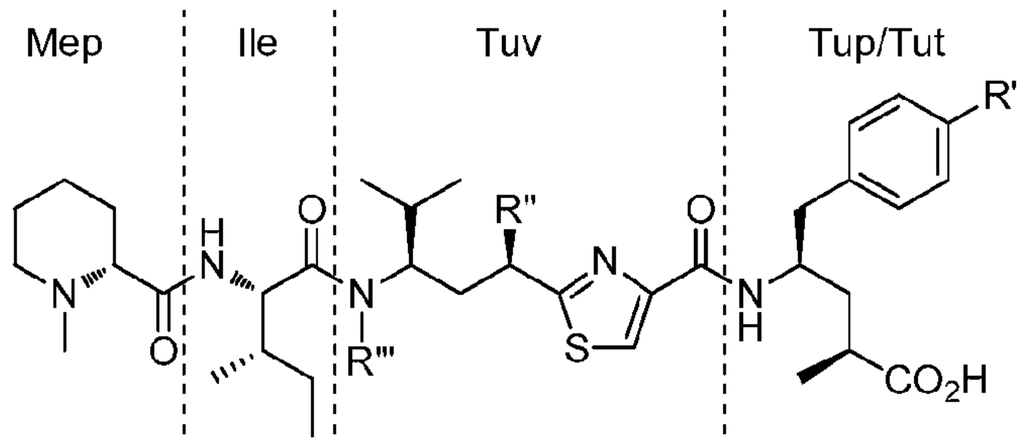
BACKGROUND OF THE INVENTION

[0001] This invention relates to compounds structurally similar 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 some activity (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. At the molecular level the exact blockage mechanism may differ from one anti-mitotic 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). 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 as shown in formula (A): N-methylpipecolinic acid (Mep), isoleucine (Ile), tubuvaline (Tuv), and either tubuphenylalanine (Tup, R' equals H) or tubutyrosine (Tut, R' equals OH). Among the better-known naturally occurring tubulysins (designated A, B, etc.), the sites of structural variation are at residues R', R" and R"' of formula (A), as shown in Table 1:



(A)

Table 1 – Naturally Occurring Tubulysins			
Tubulysin	R’	R”	R”’
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)Mc	$CH_2OC(=O)Mc$
I	OH	OC(=O)Me	$CH_2OC(=O)Me$
U	H	OC(=O)Me	H
V	H	OH	H
Y	OH	OC(=O)Me	H
Z	OH	OH	H
Pretubulysin	H	H	Me

[0005] Additionally, other naturally occurring tubulysins have been identified (Chai *et al.* 2010).

[0006] 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 anti-angiogenic properties in *in vitro* assays. The antimitotic properties of other tubulysins also have 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).

[0007] 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 2010; 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; Chai *et al.* 2011; Domling 2006; Domling *et al.* 2005a; Ellman *et al.* 2013; Hoefle *et al.* 2001 & 2006a; Pando *et al.* 2011; Patterson *et al.* 2007 & 2008; Richter 2012a, 2012b, and 2012c; Shankar *et al.* 2013; Shibue *et al.* 2011; Sreejith *et al.* 2011; Vlahov *et al.* 2010a; Wang *et al.* 2007; Wipf *et al.* 2007 and 2010; and Zanda *et al.* 2013. The SAR studies mainly explored structural variations in the Mep ring, residues R" and R"' of the Tuv subunit, and the aromatic ring or aliphatic carbon chain of the Tup/Tut subunit.

[0008] Domling *et al.* 2005 disclose conjugates of tubulysins with a partner molecule generically described as a polymer or a biomolecule, but with examples limited to polyethylene glycol (PEG) as the partner molecule. Cheng *et al.* 2011 also disclose tubulysin analogs adapted for use in conjugates. Other documents disclosing conjugates of tubulysins are Boyd *et al.* 2008 and 2010; Jackson *et al.* 2013; Vlahov *et al.* 2008a, 2008b and 2010b; Leamon *et al.* 2008 and 2010; Reddy *et al.* 2009; and Low *et al.* 2010. Leung *et al.* 2002 disclose polyanionic polypeptides that can be conjugated to drugs (including tubulysins) to improve their bioactivity and water solubility.

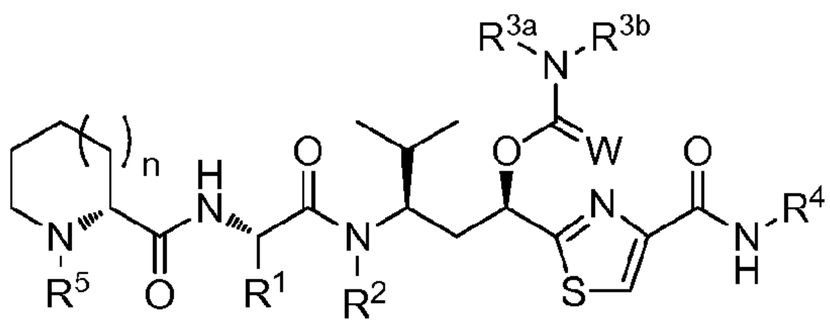
[0009] Davis *et al.* 2008 and Schluep *et al.* 2009 disclose cyclodextrin based formulations in which tubulysins are covalently attached to a cyclodextrin via a hydrazide-disulfide linker moiety bonded to the Tup/Tut carboxyl group.

[0010] The deacetylation of the Tuv subunit (*i.e.*, R" in formula (A) is hydroxyl instead of acetyl) reportedly leads to loss of biological activity (Domling *et al.* 2006). In a study of tubulysins U and V, which differ in the former being acetylated and the latter being deacetylated, tubulysin V was reported to be less potent by about 200X to 600X, depending on the assay (Balasubramanian *et al.* 2009). Because an acetate group is susceptible to hydrolysis, deacetylation at the R" position is a concern, as a potential instability center leading to loss of activity, for the development of tubulysin analogs for pharmaceutical applications.

BRIEF SUMMARY OF THE INVENTION

[0011] We have discovered that it is possible to guard against the loss of biological activity associated with deacetylation as discussed above by replacing an acetate at the R" position with a carbamate group. A carbamate group as described herein does not cause significant loss of biological activity but yet is more stable.

[0012] Accordingly, in one aspect, this invention provides a compound having a structure represented by formula (I)



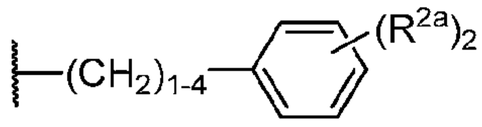
(I)

wherein

R^1 is 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^2 is 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, unsubstituted or substituted alkylaryl, or

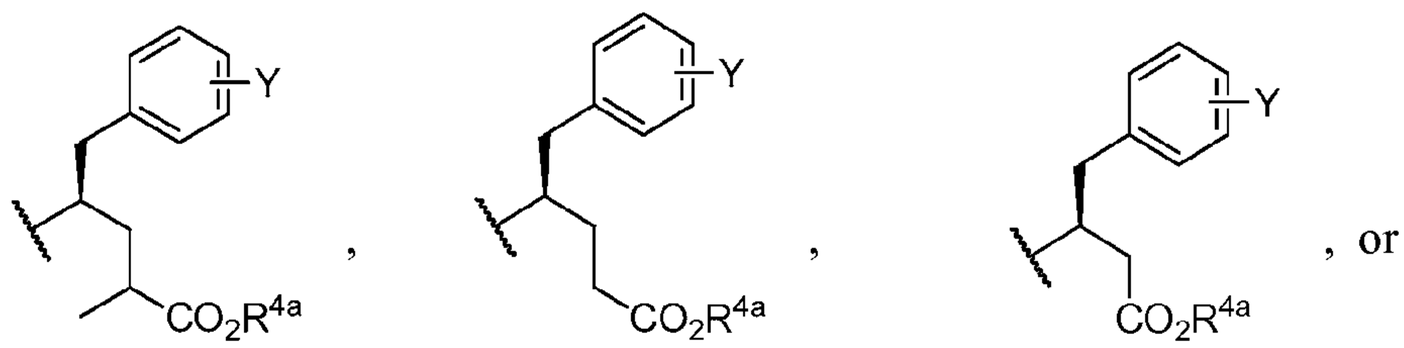


;

wherein each R^{2a} is independently H, NH_2, NHMe, Cl, F, Me, Et, or CN;

R^{3a} and R^{3b} are independently H, C_1-C_5 alkyl, CH_2(C_5-C_6 cycloalkyl), $CH_2C_6H_5$, C_6H_5, or CH_2CH_2OH;

R^4 is



;

wherein R^{4a} is H or C_1-C_3 alkyl; and Y is H, OH, Cl, F, CN, Me, Et, NO_2, or NH_2;

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);

W is O or S (preferably O); and

n is 0, 1, or 2;

or a pharmaceutically acceptable salt thereof.

[0013] In another embodiment, this invention provides a conjugate comprising a compound according to formula (I) covalently linked to a targeting moiety that specifically or preferentially binds to a chemical entity on a target cell, which target cell preferably is a cancer cell. Preferably, the targeting moiety is an antibody – more preferably a monoclonal antibody and even more preferably a human monoclonal antibody – or the antigen-binding portion thereof and the chemical entity is a tumor associated antigen. the tumor associated

antigen can be one that is displayed on the surface of a cancer cell or one that is secreted by a cancer cell into the surrounding extracellular space.

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

[0015] 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 targeting moiety (particularly an antibody). In another embodiment, there is provided the use of a compound of this invention or a conjugate thereof with a targeting moiety (particularly an antibody) for the preparation of a medicament for the treatment of cancer in a subject suffering from such cancer. The cancer can be renal, gastric, lung, or ovarian cancer.

BRIEF DESCRIPTION OF THE DRAWING(S)

[0016] **Figs. 1**, **2a-2b**, and **3** show, in combination, a scheme for the synthesis of compound (III-1).

[0017] **Fig. 4** shows a scheme for the synthesis of compound (III-2).

[0018] **Fig. 5** shows a scheme for the synthesis of compounds (I-2) and (I-3) .

[0019] **Figs. 6a-6c** show in combination a scheme for the synthesis of compounds (III-4) and (III-5).

[0020] **Fig. 7** shows a scheme for the synthesis of compound (I-1).

[0021] **Fig. 8** shows a scheme for the synthesis of compound (I-4).

[0022] **Figs. 9a** and **9b** show in combination a scheme for the synthesis of compound (III-6).

[0023] **Figs. 10** and **11** show schemes for the synthesis of intermediates useful for making compounds of this invention.

[0024] **Fig. 12** shows schemes for synthesis of additional compounds of this invention.

[0025] **Figs. 13a-13d** show the biological activity of some compounds of this invention.

[0026] **Fig. 14** shows the in vitro activity of a conjugate of this invention.

[0027] **Fig. 15** shows the in vivo activity of a conjugate of this invention.

[0028] **Figs. 16a**, **16b**, **17a**, **17b**, **18a**, **18b**, **19a**, **19b**, and **20** present additional in vivo data on the activity of conjugates of this invention.

[0029] **Figs. 21** and **22** show schemes for the synthesis of additional compounds of this invention, illustrating structural variations at the carbamate group.

[0030] **Fig. 23** shows a scheme for the synthesis of compounds of this invention suitable for conjugation via "click" chemistry.

[0031] **Fig. 24** shows a scheme for the synthesis of compounds of this invention suitable for conjugation via an aliphatic amine group.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

[0032] "Antibody" means whole antibodies and any antigen binding fragment (*i.e.*, "antigen-binding portion") or single chain variants thereof. A whole antibody is a protein 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 5×10^{-8} M or less, more preferably 1×10^{-8} M or less, more preferably 6×10^{-9} M or less, more preferably 3×10^{-9} M or less, even more preferably 2×10^{-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.

[0033] "Antigen binding fragment" and "antigen binding portion" of an antibody (or simply "antibody portion" or "antibody fragment") 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 complementarity determining region (CDR); and (viii) a nanobody, a heavy chain variable region containing a single variable domain and two constant domains. Preferred antigen binding fragments are Fab, $F(ab')_2$, Fab', Fv, and Fd fragments. 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.

[0034] 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.

[0035] "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.

[0036] "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.

[0037] "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.

[0038] "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).

[0039] "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. "Alkylene" means a divalent counterpart of an alkyl group, such as CH_2CH_2, $CH_2CH_2CH_2$, and $CH_2CH_2CH_2CH_2$.

[0040] "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.

[0041] "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.

[0042] "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. "Cycloalkylene" means a divalent counterpart of a cycloalkyl group.

[0043] "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. "Heterocycloalkylene" means a divalent counterpart of a heterocycloalkyl group.

[0044] "Alkoxy," "aryloxy," "alkylthio," and "arylthio" mean –O(alkyl), -O(aryl), -S(alkyl), and -S(aryl), respectively. Examples are methoxy, phenoxy, methylthio, and phenylthio, respectively.

[0045] "Halogen" or "halo" means fluorine, chlorine, bromine or iodine.

[0046] "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.

"Arylene" means a divalent counterpart of an aryl group, for example 1,2-phenylene, 1,3-phenylene, or 1,4-phenylene.

[0047] "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. "Heteroarylene" means a divalent counterpart of an aryl group.

[0048] Where it is indicated that a moiety may be substituted, such as by use of "unsubstituted or substituted" or "optionally substituted" phrasing as in "unsubstituted or substituted 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.

[0049] "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).

[0050] For example, 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, $-SO_2(alkyl)$, $-SO_2NH_2$, $-SO_2NH(alkyl)$, $-SO_2N(alkyl)_2$, and the like.

[0051] 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), $-CO_2H$, -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(=O)alkyl, -S(cycloalkyl), $-SO_2(alkyl)$, $-SO_2NH_2$, $-SO_2NH(alkyl)$, and $-SO_2N(alkyl)_2$. 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 $-NHC(=NH)NH_2$. Especially preferred are phenyl, cyano, halo, hydroxyl, nitro, C_1-C_4alkyoxy, O(C_2-C_4 alkylene)OH, and O(C_2-C_4 alkylene)halo.

[0052] 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, $-CO_2H$,

-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_2NH_2, -SO_2NH(alkyl), and -SO_2N(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. Especially preferred are C_1-C_4 alkyl, cyano, nitro, halo, and C_1-C_4alkoxy.

[0053] 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.

[0054] 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 this invention.

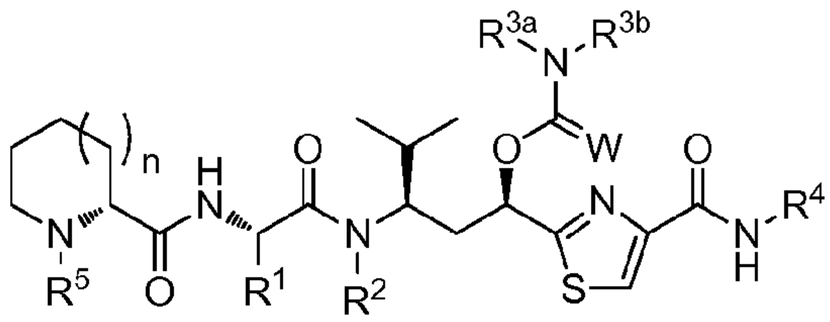
[0055] Those skilled in the art will appreciate that compounds may have tautomeric forms (*e.g.*, keto and enol forms), resonance forms, and zwitterionic forms that are equivalent to those depicted in the structural formulae used herein and that the structural formulae encompass such tautomeric, resonance, or zwitterionic forms.

[0056] "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.

[0057] "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, methyl-sulfate, 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.

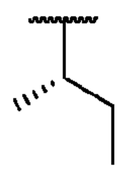
COMPOSITIONS

[0058] In formula (I), repeated below for convenience,



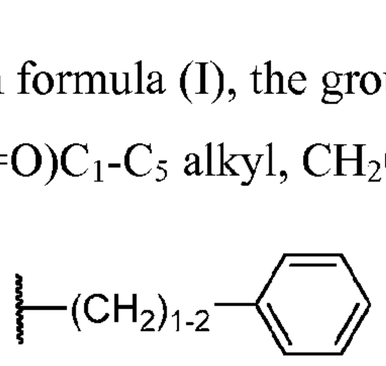
(I)

the group R^1 preferably is Me, Et, n-Pr, i-Pr, or

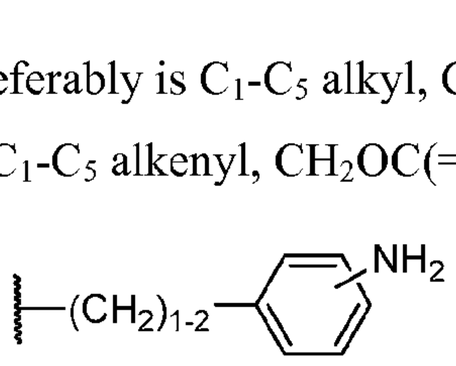


, more preferably the latter.

[0059] Also in formula (I), the group R^2 preferably is C_1-C_5 alkyl, C_1-C_5 alkenyl, C_1-C_5 alkynyl, $CH_2OC(=O)C_1$-C_5 alkyl, $CH_2OC(=O)C_1$-C_5 alkenyl, $CH_2OC(=O)C_1$-C_5 alkynyl,

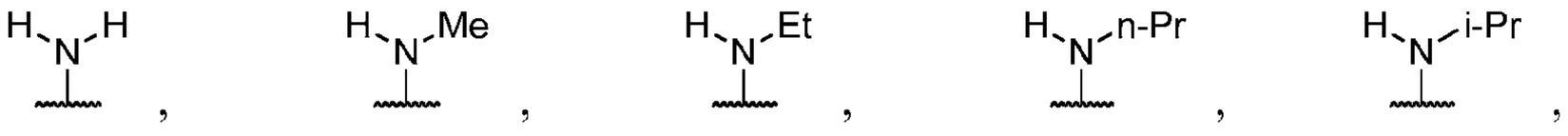


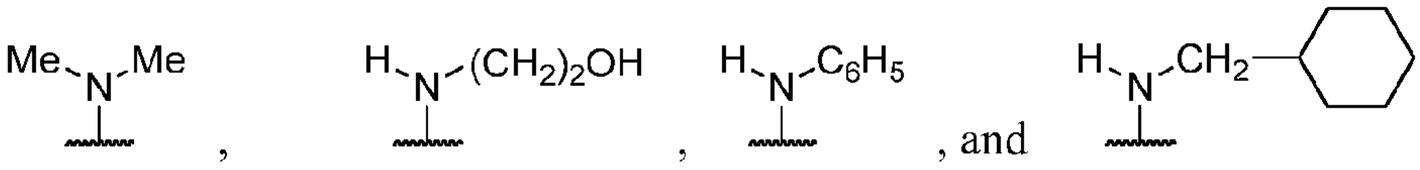
, or



.

[0060] Also in formula (I), preferred groups $N(R^{3a})(R^{3b})$ are:





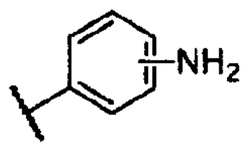
, and

with it being especially preferred that one of R^{3a} and R^{3b} is H and the other is Me. In other preferred embodiments, R^{3a} and R^{3b} are both H or both Me, or one of R^{3a} and R^{3b} is H and the other is C_6H_5.

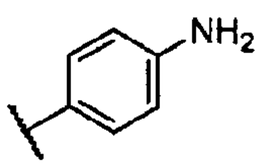
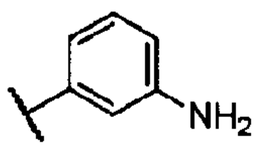
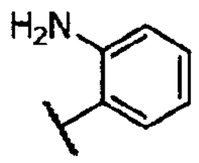
[0061] In another preferred embodiment, R^{3a} and R^{3b} are independently H, C_1-C_5 alkyl, CH_2(C_5-C_6 cycloalkyl), $CH_2C_6H_5$, or CH_2CH_2OH.

[0062] In the definitions of R^1 and R^2 in formula (I), where a group is defined as being either unsubstituted or substituted, it preferably is unsubstituted.

[0063] In the formulae of this specification, a bond traversing a phenyl ring between two carbons of the phenyl ring means that the group attached to the bond may be located at any of the *ortho*, *meta*, or *para* positions of the phenyl ring. By way of illustration, the formula



represents

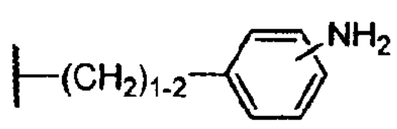
 ,  , or  .

[0064] The synthesis of counterparts of the tubulysin Tuv and Tup subunits with various R^2 and R^4 groups is taught by Cheng *et al.* 2011.

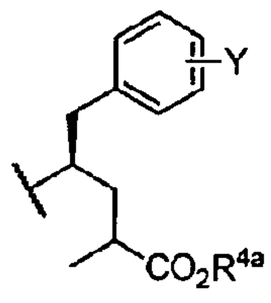
[0065] In a preferred embodiment of compounds according to formula (I), R^1 is

,

R_2 is C_1-C_5 alkyl (especially Me or n-Pr) or

 .

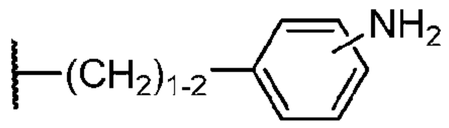
one of R^{3a} and R^{3b} is H and the other is Me; R^4 is

 ,

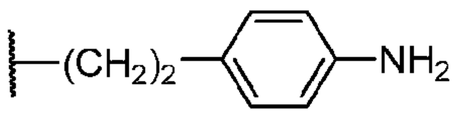
where Y is H or NO_2 and R^{4a} is H, Me, or Et;

R^5 is Me; W is O, and n is 1.

[0066] In another preferred embodiment of compounds according to formula (I), n is 1, W is O, Y in R^4 is H or NO_2 (preferably H), and R^2 is

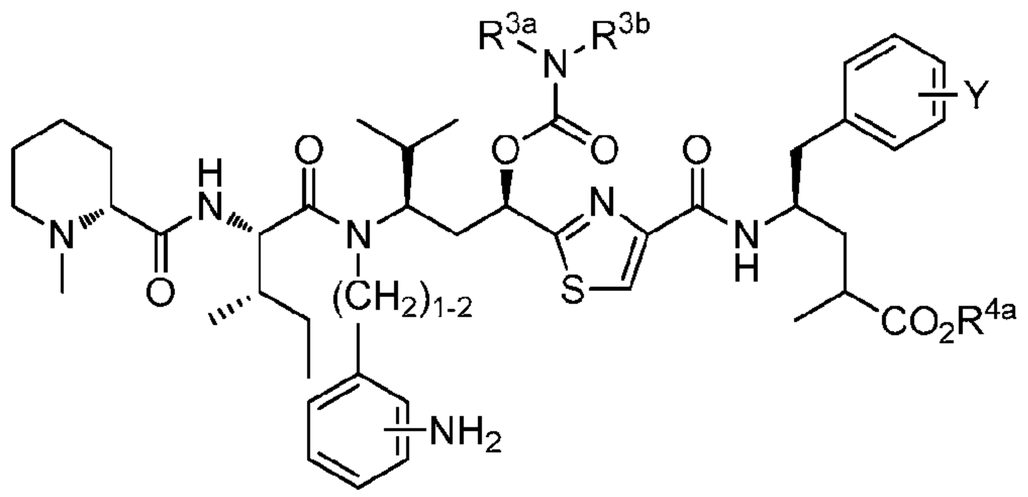


and more preferably



.

[0067] A compound according to this preferred embodiment is represented by formula (Ia)

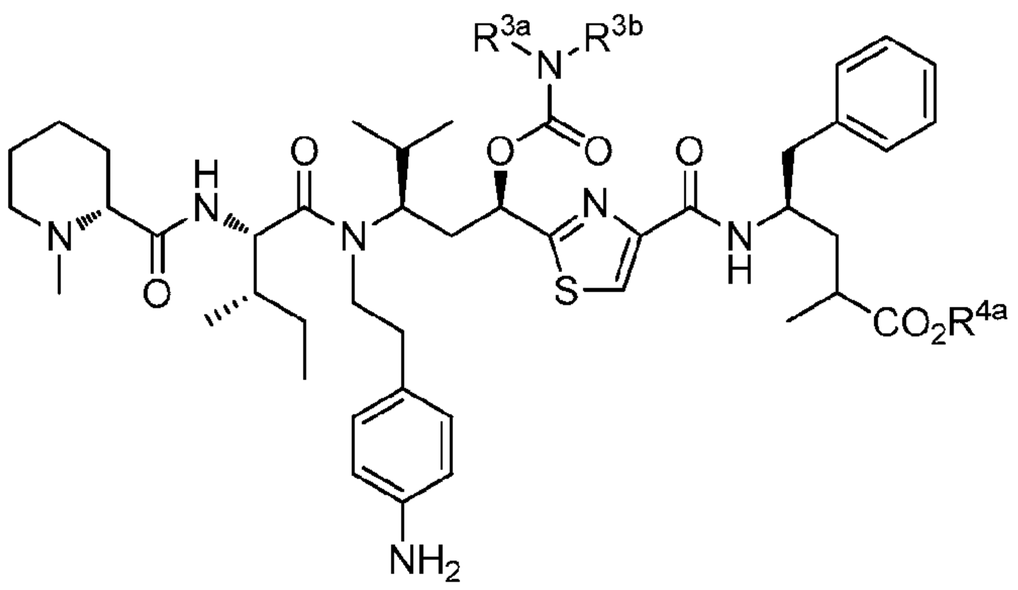


(Ia)

,

wherein Y is H or NO_2; R^{4a} is H, Me, or Et; and R^{3a} and R^{3b} are independently H, C_6H_5, Me, or Et; or a pharmaceutically acceptable salt thereof.

[0068] Even more preferably, the compound has a structure represented by formula (Ia'):

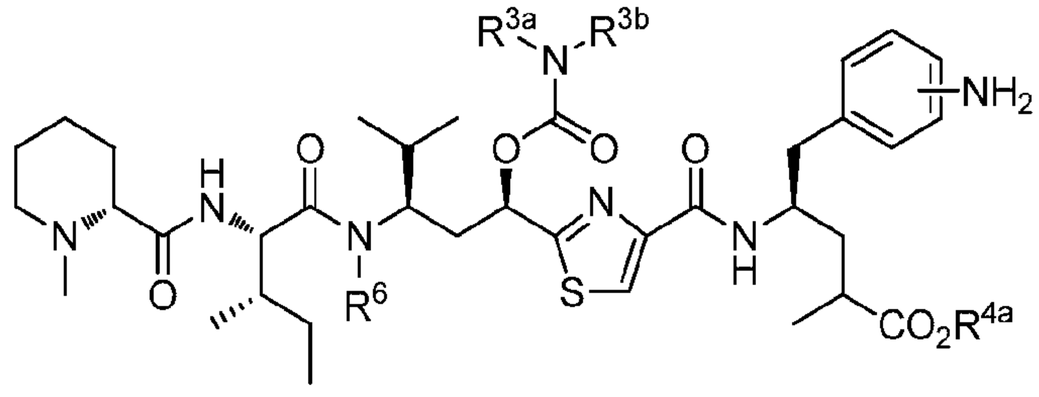


(Ia')

,

where R^{4a} is H, Me, or Et; and R^{3a} and R^{3b} are independently H, C_6H_5, Me, or Et; or a pharmaceutically acceptable salt thereof.

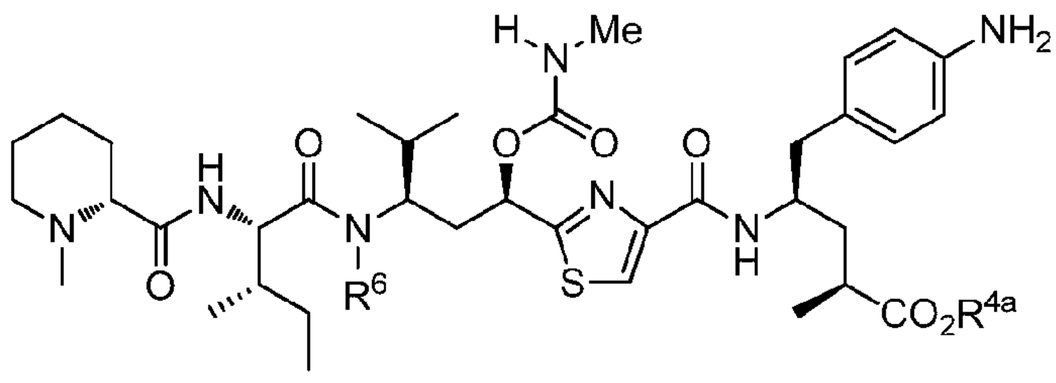
[0069] In yet another preferred embodiment, W is O, Y is NH_2, n is 1, and both groups R^{2a} in R^2 are other than NH_2. A compound according to this embodiment is represented by formula (Ib):



(Ib)

where R^{4a} is H, Me, or Et; R^{3a} and R^{3b} are independently H, C_6H_5, Me, and Et; and R^6 is C_1-C_5 alkyl, $CH_2OC(=O)C_1$-C_5 alkyl, or $(CH_2)_{1-2}C_6H_5$; or a pharmaceutically acceptable salt thereof.

[0070] In yet another preferred embodiment, the compound has a structure represented by formula (Ib'):



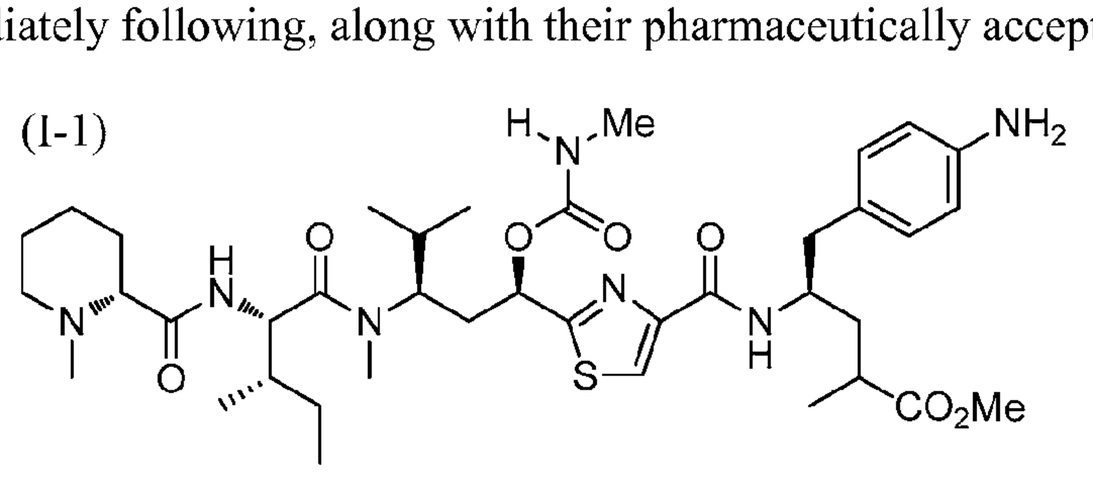
(Ib')

where R^{4a} is H, Me, or Et (preferably H) and R^6 is Me or n-Pr; or a pharmaceutically acceptable salt thereof.

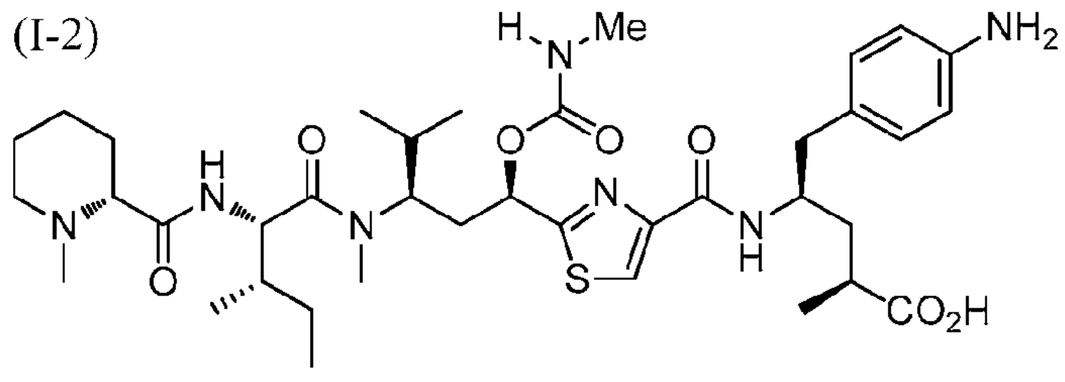
[0071] Preferably, in formulae (Ia), (Ia'), and (Ib), one of R^{3a} and R^{3b} is H and the other is Me. In other preferred embodiments, R^{3a} and R^{3b} are both H or both Me, or one of R^{3a} and R^{3b} is H and the other is C_6H_5. In yet other preferred embodiments, R^{3a} and R^{3b} are independently H, Me, or Et

[0072] Specific examples of compounds of this invention include the compounds shown immediately following, along with their pharmaceutically acceptable salts:

(I-1)

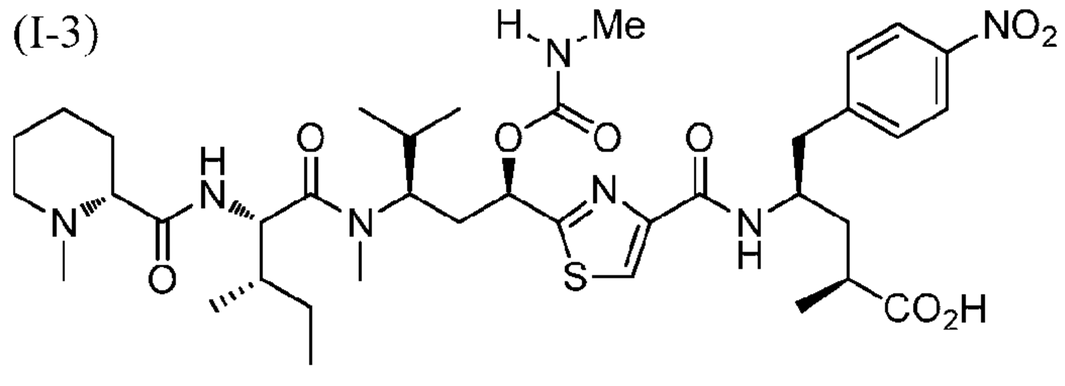


(I-2)



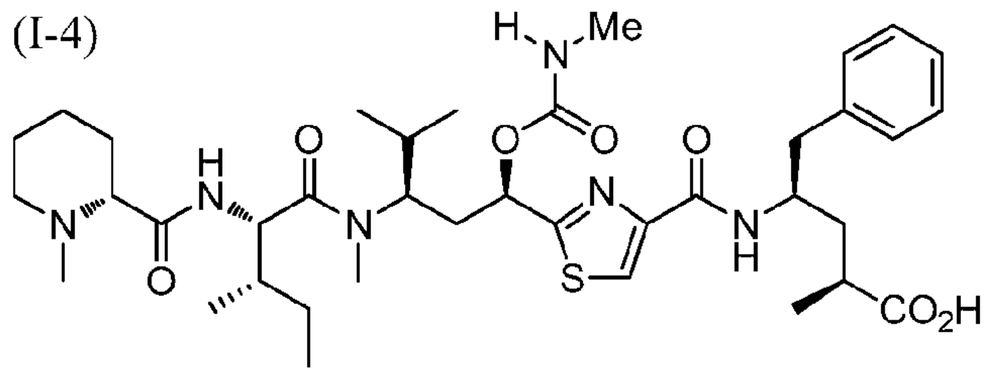
,

(I-3)



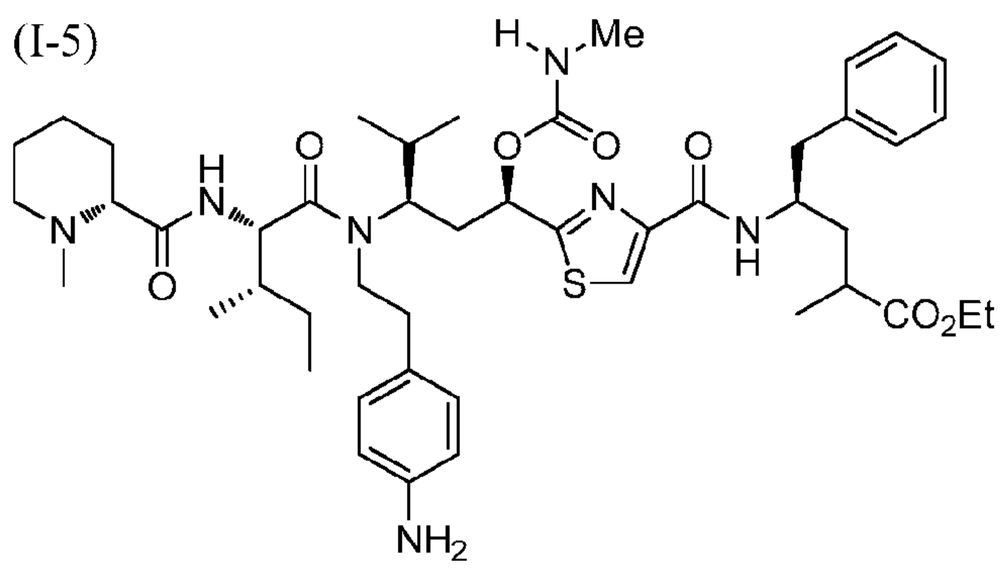
,

(I-4)



,

(I-5)

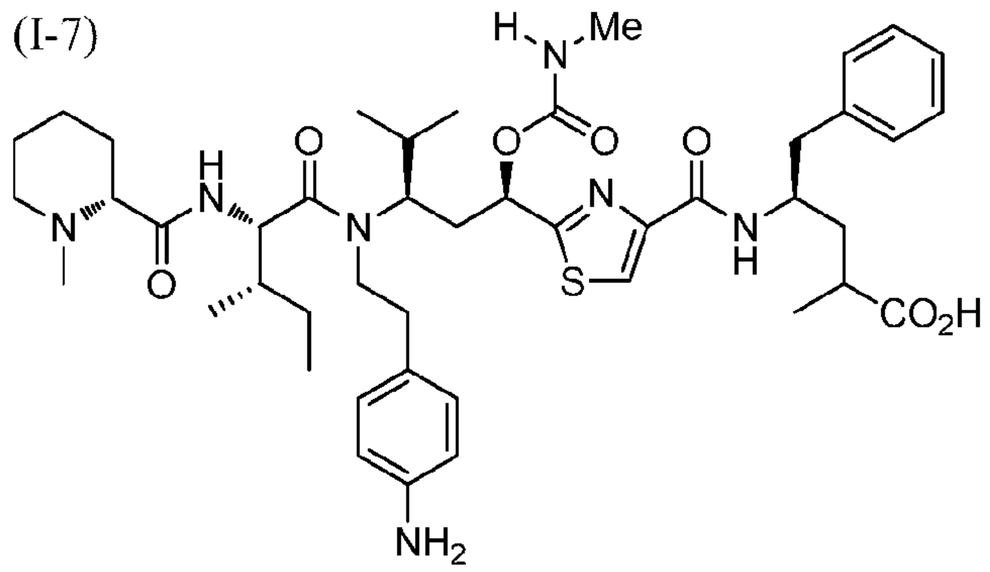


,

(I-6)

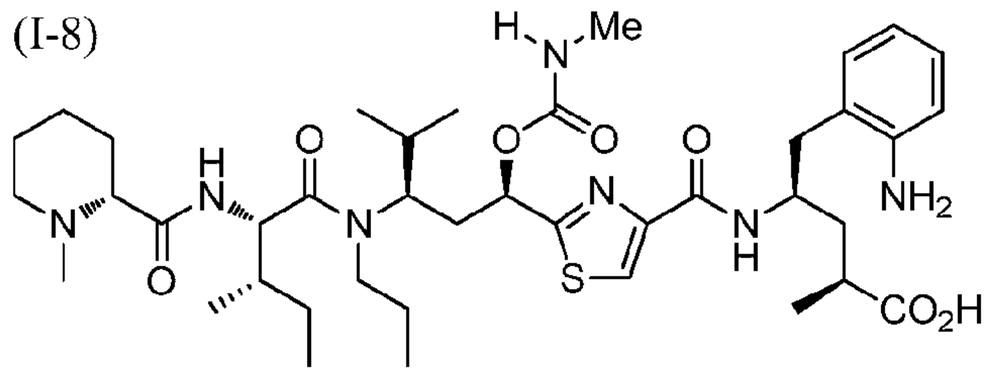
,

(I-7)



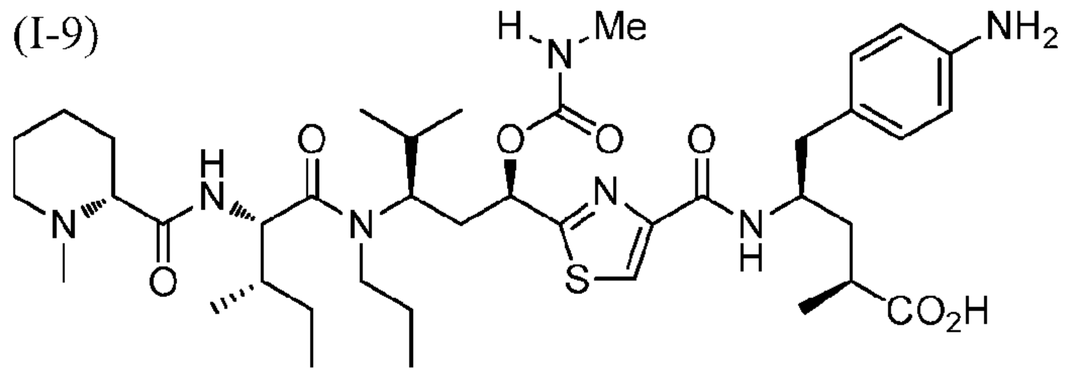
,

(I-8)



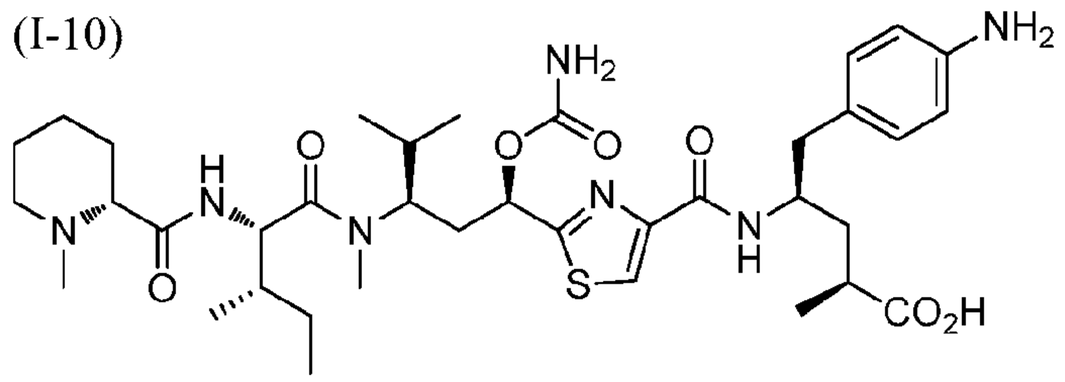
,

(I-9)



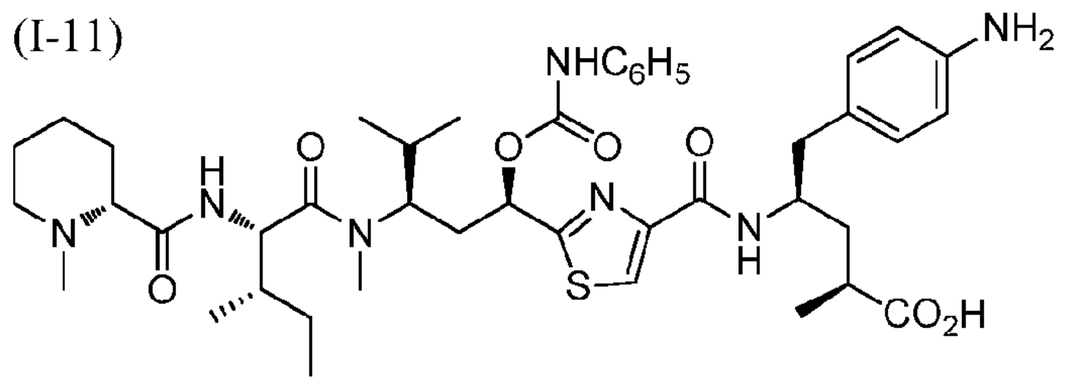
,

(I-10)



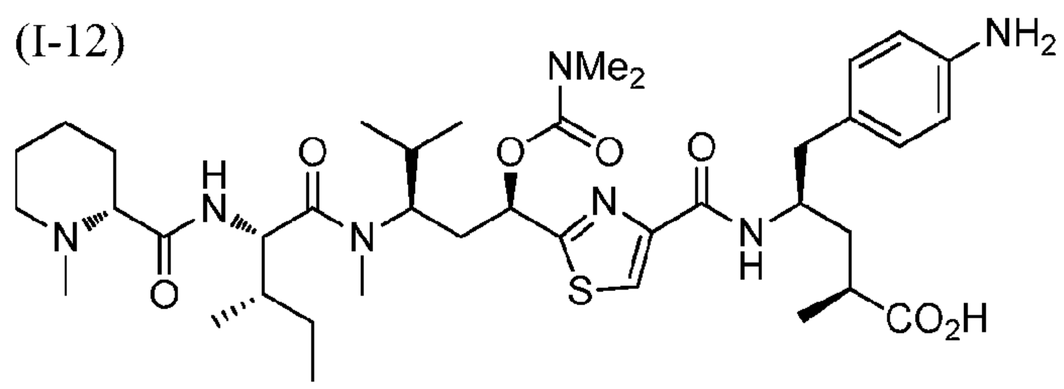
,

(I-11)



, and

(I-12)



[0073] Compounds (I-2), (I-7), (I-8) and (I-9) are preferred.

CONJUGATES

[0074] Optionally, compounds of this invention may be conjugated to a targeting moiety that specifically or preferentially binds to a chemical entity on a cancer cell. Preferably, the targeting moiety is an antibody or antigen binding portion thereof and the chemical entity is a tumor associated antigen. Preferably, the conjugation is effected through a chemical bond to a functional group in the Tuv or Tup subunit, such as an amino group.

[0075] In another embodiment, there is provided a conjugate comprising cytotoxic compound according to this invention and a ligand, represented by formula (II)

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

where Z is a ligand; D is a cytotoxic compound according to this invention (*e.g.*, a compound according to formula (I), (Ia), (Ia'), or (Ib)); 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 or near 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 described hereinbelow.

[0076] 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.

[0077] 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 ligand Z 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. This ratio is referred to as the substitution ratio (SR) or, alternatively, in the case of antibody-drug conjugates, the drug-antibody ratio (DAR).

Ligand Z and Conjugation Thereof

[0078] 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.* 2008; Leamon *et al.* 2008). For the same reason, the detailed discussion below is primarily written in terms of a 1:1 ratio of antibody Z to compound D (m = 1).

[0079] 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), glypican-3, 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.*, 8,097,703 B2 (CD19); King *et al.*, US 2010/0143368 A1 (CD22); Keler *et al.*, US 7,387,776 B2 (2008) (CD30); Terrett *et al.*, US 8,124,738 B2 (CD70); Korman *et al.*, US 6,984,720 B1 (2006) (CTLA-4); Korman *et al.*, US 8,008,449 B2 (2011) (PD-1); Huang *et al.*, US 2009/0297438 A1 and Cardarelli *et al.*, US 7,875,278 B2 (PSMA); Terrett *et al.*, US 2010/0034826 A1 (PTK7); Terrett *et al.*, US 2010/0209432 (A1) (glypican-3); Harkins *et al.*,

US 7,335,748 B2(2008) (RG1); Terrett *et al.*, US 8,268,970 B2 (2012) (mesothelin); and Xu *et al.*, US 2010/0092484 A1 (CD44).

[0080] 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.

[0081] 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, carboxylic 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.

[0082] 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.

[0083] 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).

As with lysine ε-amino groups, there are concerns regarding reproducibility of the location of the conjugation site(s) and stoichiometry.

[0084] 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.

[0085] 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).

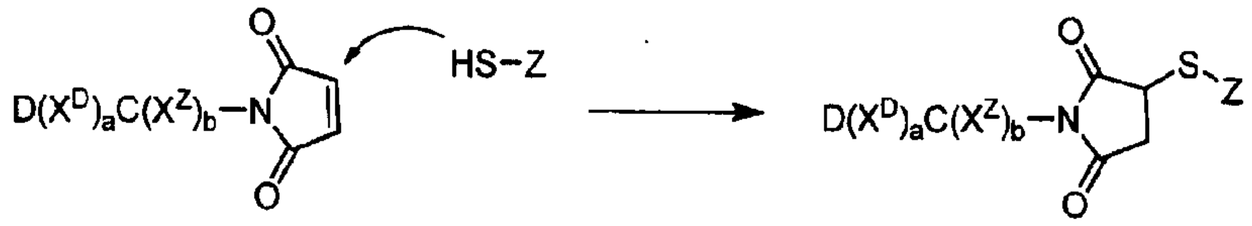
Again, there are concerns regarding conjugation site location and stoichiometry and the possible disruption of antibody native conformation.

[0086] 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 7,521,541 B2 (2009); 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 awat from the antigen binding site.

[0087] 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.

[0088] In yet another preferred embodiment, ligand Z is conjugated via the nucleophi-lic 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 illus-trated below. The thiol group can be a native one, or one introduced as described above.



[0089] Ligand Z can also be conjugated via a functional group adapted for use with "click" chemistry, as discussed hereinbelow.

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

[0090] 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.

[0091] Cleavable group C is a group cleavable under physiological conditions, preferably 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).

[0092] 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).

[0093] 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 7,541,530 B2 (2009); 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.*, US 2010/0145036 A1.

[0094] 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.

[0095] 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).

[0096] 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.

[0097] 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).

Another enzyme that can be utilized for cleaving peptidyl linkers is legumain, a lysosomal cysteine protease that preferentially cleaves at Ala-Ala-Asn.

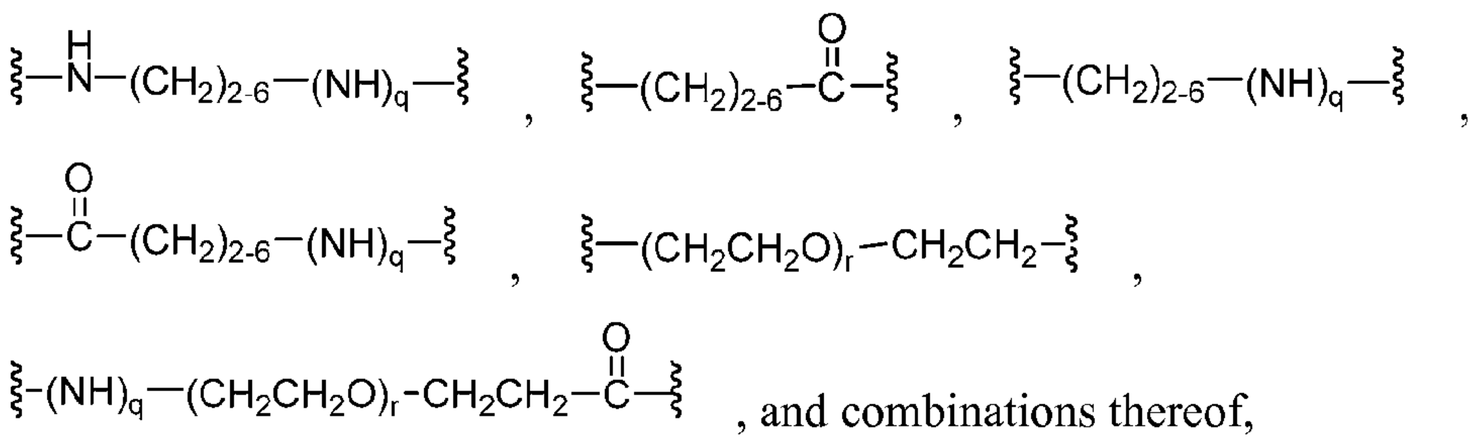
[0098] In one embodiment, Group C is a peptide comprising the two-amino acid sequence $-AA^2-AA^1-$ wherein AA^1 is lysine, arginine, or citrulline and AA^2 is phenylalanine, valine, alanine, leucine or isoleucine. In another embodiment, C consists of a sequence of one to five amino acids, selected from 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.

[0099] The preparation and design of cleavable groups C consisting of a single amino acid is disclosed in Chen *et al.*, US 2010/0113476 A1.

[00100] Group C can also be a photocleavable one, for example a nitrobenzyl ether that is cleaved upon exposure to light.

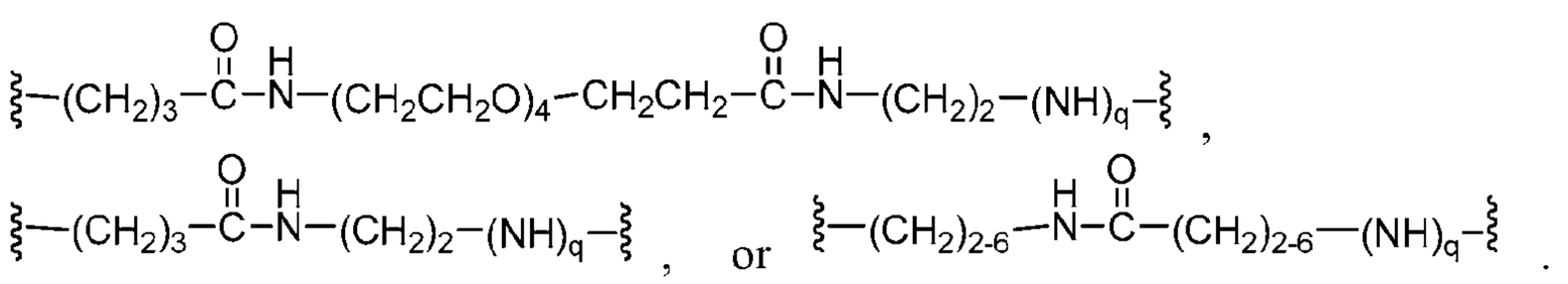
[00101] Group C can be bonded directly to antibody Z or compound D; that is, spacers X^Z and X^D, as the case may be, can be absent. For example, if group C is a disulfide, one of the two sulfurs can be a cysteine residue or its surrogate on antibody Z. Or, group C can be a hydrazone bonded to an aldehyde 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.

[00102] 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:



, and combinations thereof,

where the subscript q is 0 or 1 and the subscript r is 1 to 24, preferably 2 to 4. These segments can be combined, such as shown below:



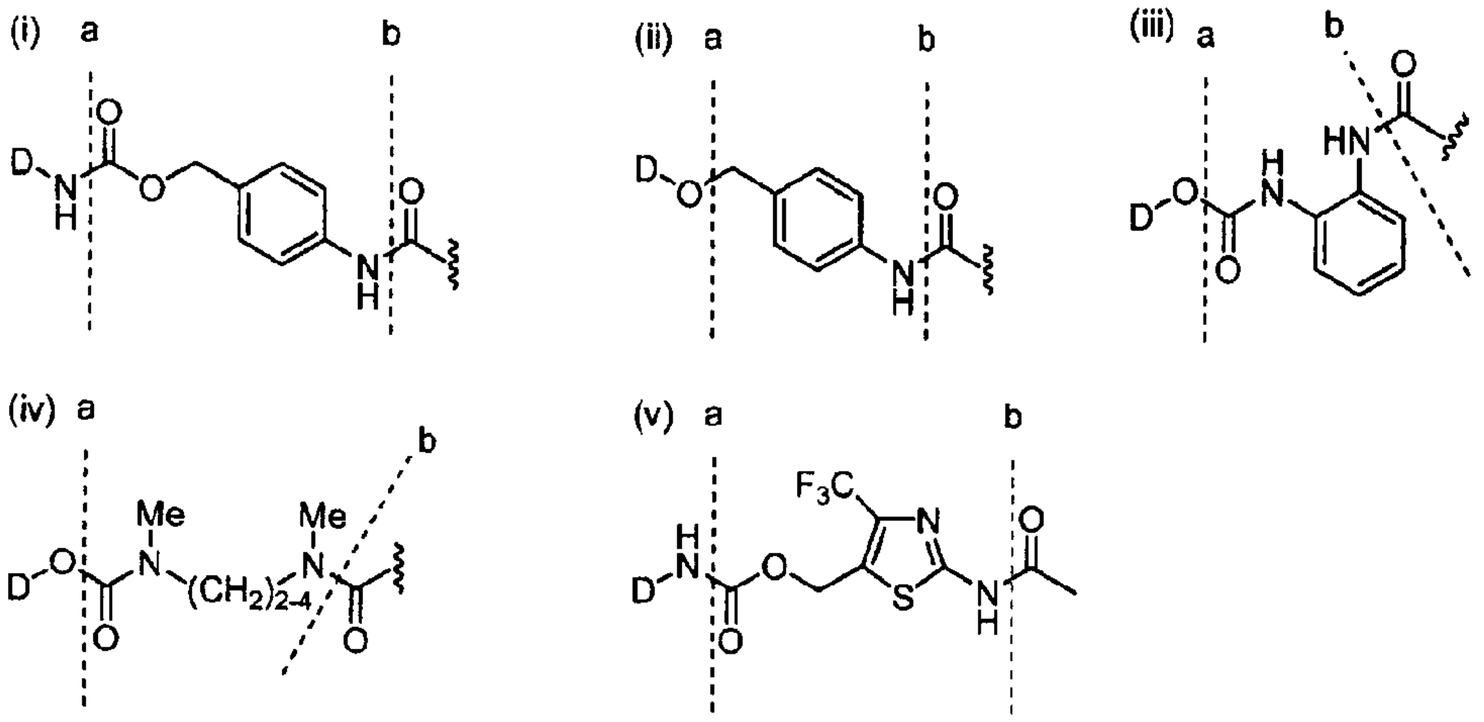
[00103] Spacer X^D, if present, provides spatial separation between group C and compound D, lest the latter interfere sterically or electronically with cleavage of the former. Spacer X^D also can serve to introduce additional molecular mass and chemical functionality into a conjugate. Generally, the 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.

[00104] Spacers X^Z and/or X^D, where present, preferably provide a linear separation of from 4 to 25 atoms, more preferably from 4 to 20 atoms, between Z and C or D and C, respectively.

[00105] 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.

[00106] Exemplary self-immolating moieties (i)-(v) bonded to a hydroxyl or amino group on a partner molecule D are shown below:



[00107] 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.

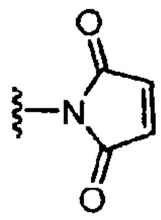
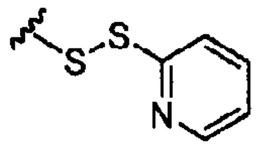
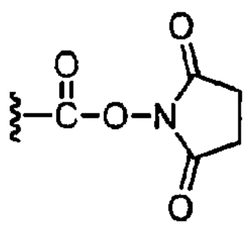
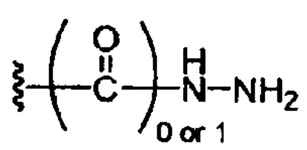
[00108] In another embodiment, an antibody targeting moiety and the cytotoxic compound D are linked by a non-cleavable linker. Degradation of the antibody eventually reduces the linker to a small appended moiety that does not interfere with the biological activity of cytotoxic compound D.

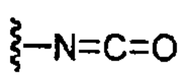
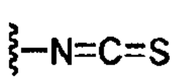
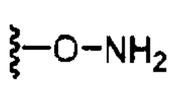
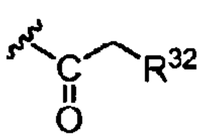
Compound D – Linker Compositions

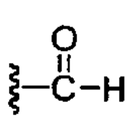
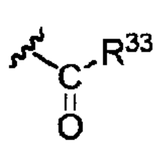
[00109] Conjugates of this invention preferably are prepared by first joining a compound D and linker $(X^D)_aC(X^Z)_b$ (where X^D, C, X^Z, a, and b are as defined for formula (II)) to form a drug-linker composition represented by formula (III):

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

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 amino, azide, cyclooctyne,

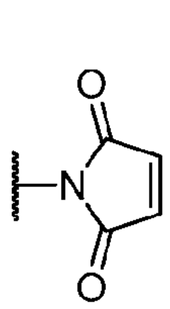
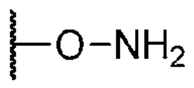
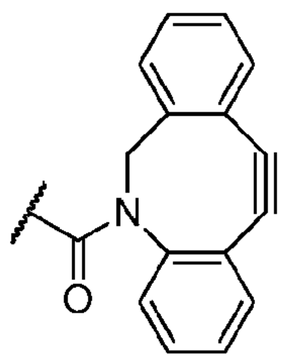
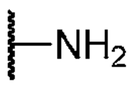

,

,


,


,

,

,

,


, and

.

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 7,517,903 B2 (2009); Gangwar *et al.*, US 7,714,016 B2 (2010); Boyd *et al.*, US 2008/0279868 A1; Gangwar *et al.*, US 7,847,105 B2 (2010); Gangwar *et al.*, US 7,968,586 B2 (2011); Sufi *et al.*, US 2010/0145036 A1; and Chen *et al.*, US 2010/0113476 A1.

[00110] Preferably reactive functional group $-R^{31}$ is $-NH_2$, -OH, $-CO_2H$, -SH, maleimido, cyclooctyne, azido ($-N_3$), hydroxylamino ($-ONH_2$) or N-hydroxysuccinimido. Especially preferred functional groups $-R^{31}$ are selected from the group consisting of:


,

,

, and

.

[00111] An –OH group can be esterified with a carboxy group on the antibody, for example, on an aspartic or glutamic acid side chain.

[00112] A $-CO_2H$ group can be esterified with a –OH group or amidated with an amino group (for example on a lysine side chain) on the antibody.

[00113] An N-hydroxysuccinimidc group is functionally an activatcd carboxyl group and can conveniently be amidated by reaction with an amino group (*e.g.*, from lysine).

[00114] A maleimide group can be conjugated with an -SH group on the antibody (*e.g.*, from cysteine or from the chemical modification of the antibody to introduce a sulfhydryl functionality), in a Michael addition reaction.

[00115] An –SH group is particularly useful for conjugation where the antibody has been modified to introduce a maleimide group thereto, in a Michael addition reaction that is the "mirror image" of that described above. Antibodies can be modified to have maleimide groups with N-succinimidyl 4-(maleimidomethyl)-cyclohexanecarboxylate (SMCC) or its sulfonated variant sulfo-SMCC, both reagents being available from Sigma-Aldrich.

[00116] Azide and cyclooctyne are complementary functional groups that can effect conjugation via so-called copper-free "click chemistry," in which the azide adds across the strained alkyne bond of the cyclooctyne to form an 1,2,3-triazole ring. See, *e.g.*, Agard *et al.*, *J. Amer. Chem. Soc.* **2004**, *126*, 15046-15047; Best, *Biochemistry* **2009**, *48*, 6571-6584. The azide can be the reactive functional group R^{31} in formula (III) and the cyclooctyne can be situated on the antibody or antigen binding portion thereof, or vice-versa. A cyclooctyne group can be provided by a DIBO reagent (available from Invitrogen/Molecular Probes, Eugene, Oregon).

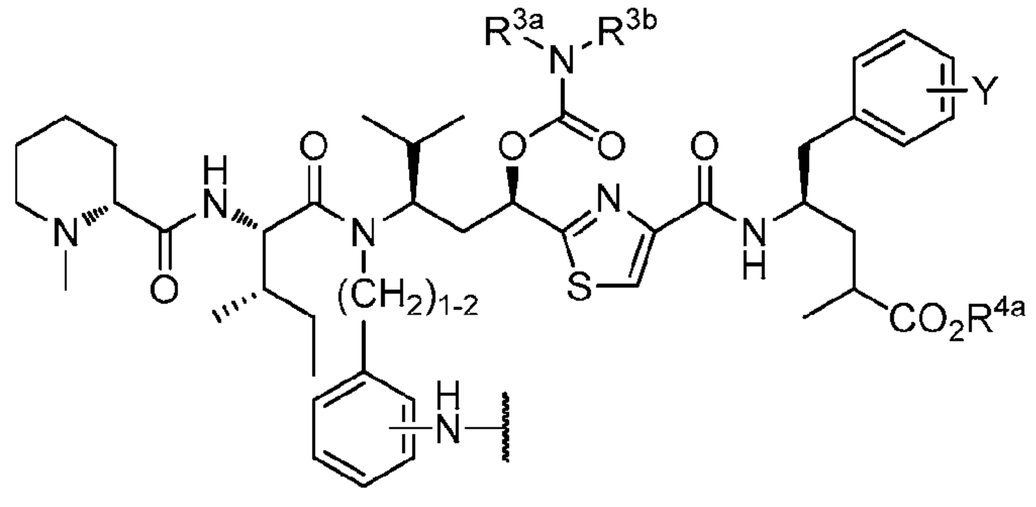
[00117] Techniques for introducing non-natural amino acids into antibodies can be utilized, with the non-natural amino acid providing a functionality for conjugation with the reactive functional group. For instance, the non-natural amino acid p-acetylphenylalanine can be incorporated into an antibody or other polypeptide, as taught in Tian *et al.*, WO 2008/030612 A2 (2008). The ketone group in p-acetylphenyalanine can be a conjugation site by the formation of an oxime with a hydroxylamino reactive functional group. Alternatively,

the non-natural amino acid p-azidophenylalanine can be incorporated into an antibody to provide an azide functional group for conjugation via click chemistry. Non-natural amino acids can also be incorporated into an antibody or other polypeptide using cell-free methods, as taught in Goerke *et al.*, US 2010/0093024 A1 (2010) and Goerke *et al.*, *Biotechnol. Bioeng.* **2009**, 102 (2), 400-416.

[00118] An amine (NH_2) group can be used for conjugation using the enzyme transglutaminase, as taught in Jeger *et al.*, *Angew. Chem. Int. Ed.* **2010**, *49*, 9995 –9997.

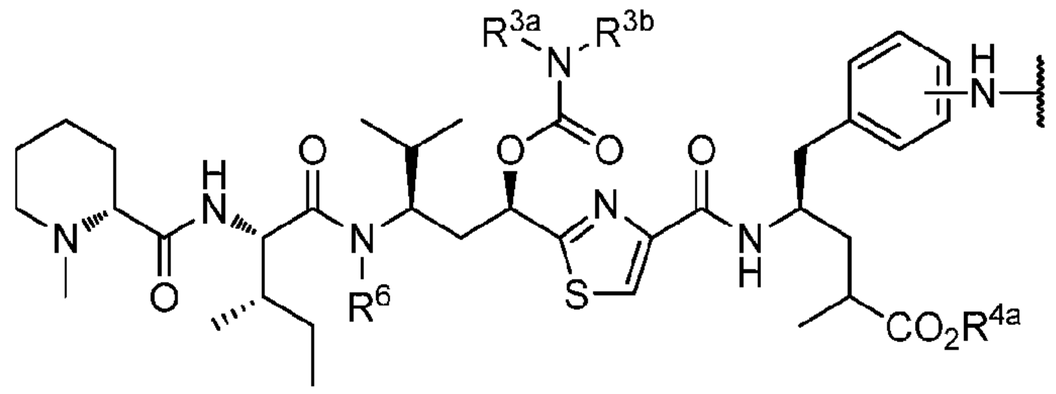
[00119] Conjugation can also be effected using the enzyme Sortase A, as taught in Levary *et al.*, *PLoS One* **2011**, 6(4), e18342; Proft, *Biotechnol. Lett.* **2010**, 32, 1-10; Ploegh *et al.*, WO 2010/087994 A2 (2010); and Mao *et al.*, WO 2005/051976 A2 (2005). The Sortase A recognition motif (typically LPXTG, where X is any natural amino acid) may be located on the ligand Z and the nucleophilic acceptor motif (typically GGG) may be the group R^{31} in formula (III), or vice-versa.

[00120] The group D in the formulae $[D(X^D)_aC(X^Z)_b]_mZ$ and $D\text{-}(X^D)_aC(X^Z)_b\text{-}R^{31}$ preferably has a structure according to formula (D-a)



(D-a)

or formula (D-b)

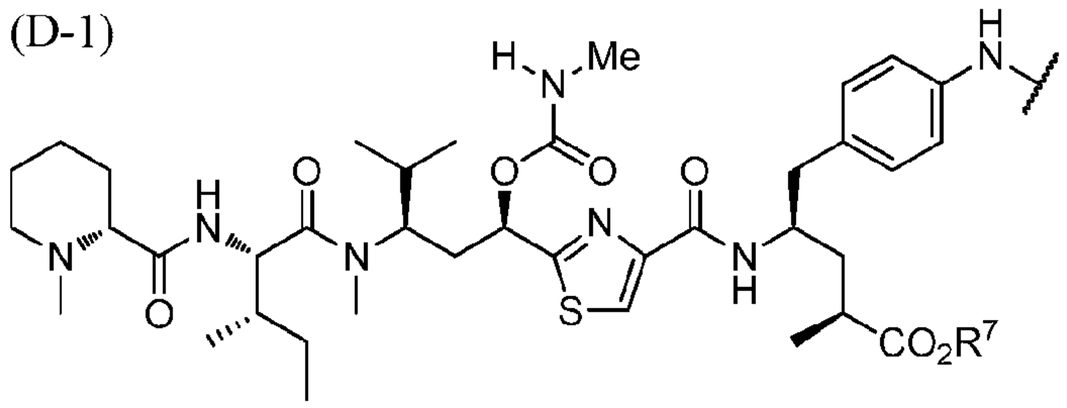


(D-b)

wherein Y is H or NO_2; R^{4a} is H, Me, or Et; R^{3a} and R^{3b} are independently H, Me, or Et; and R^6 is C_1-C_5 alkyl, $CH_2OC(=O)C_1$-C_5 alkyl, or $(CH_2)_{1\text{-}2}C_6H_5$.

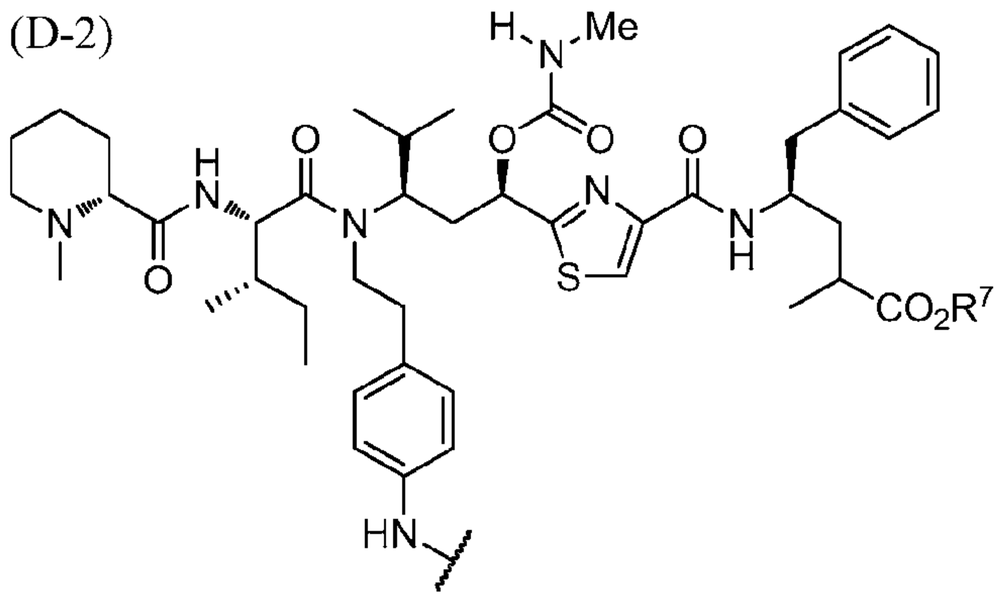
[00121] Examples of such groups D include:

(D-1)



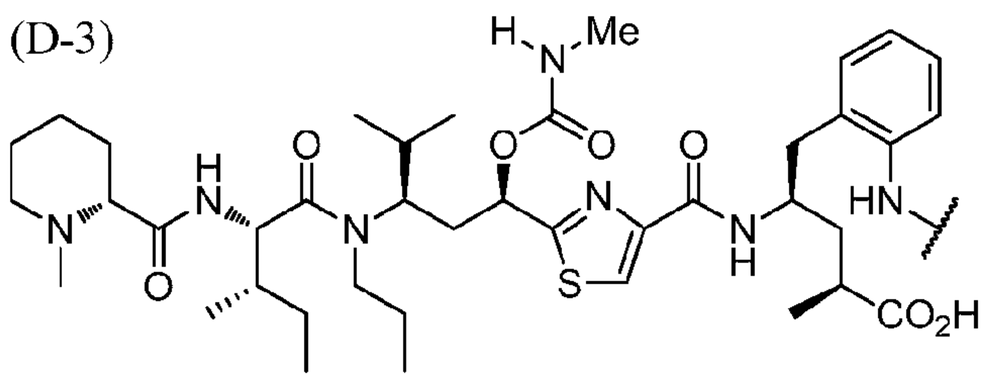
,

(D-2)



, and

(D-3)

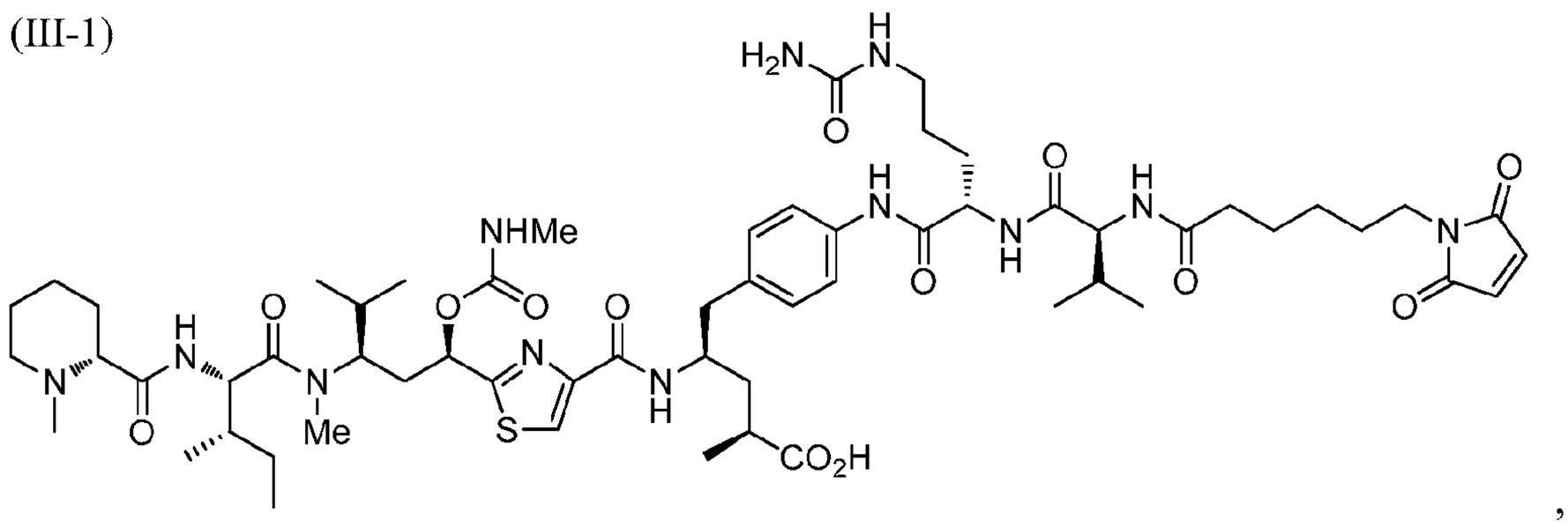


,

wherein R^7 is H, Me, or Et.

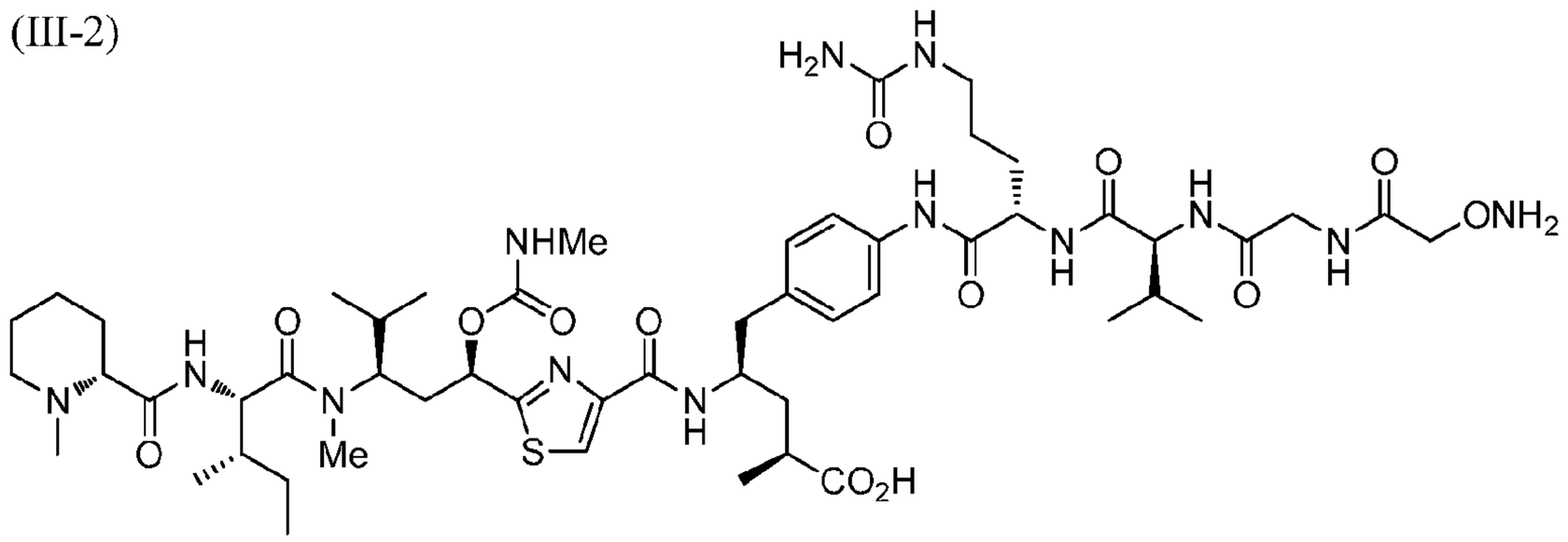
[00122] Examples of compositions according to formula D-$(X^D)_aC(X^Z)_b$-R^{31} include the ones shown immediately following; along with their pharmaceutically acceptable salts:

(III-1)



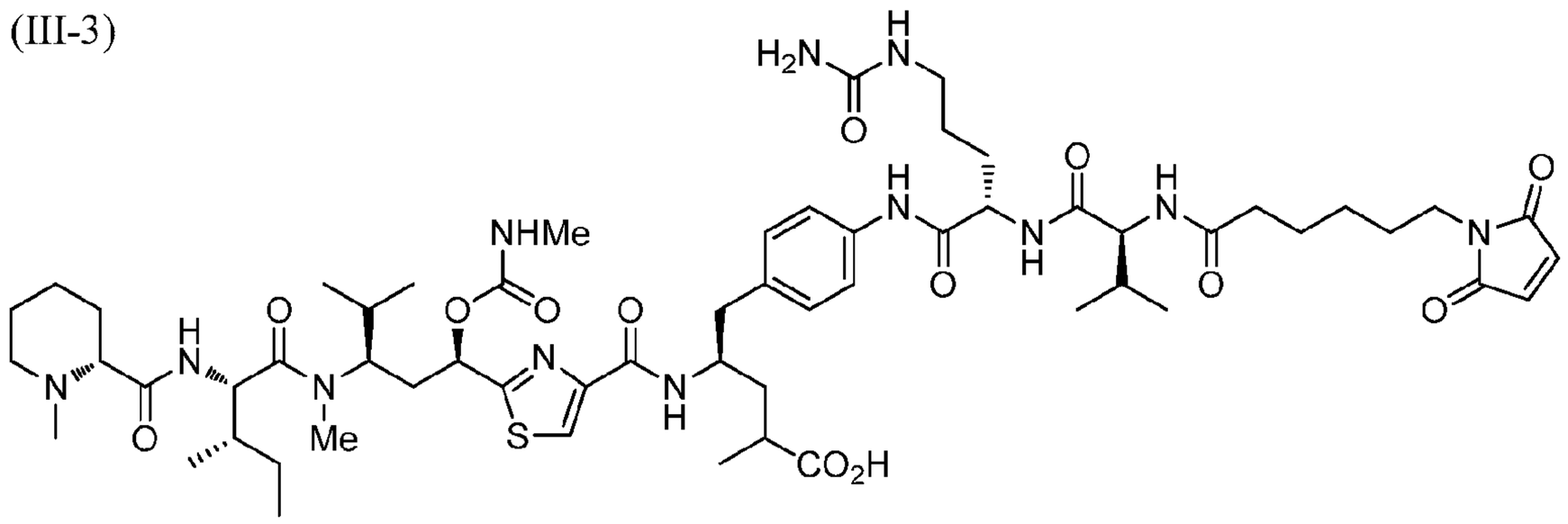
,

(III-2)



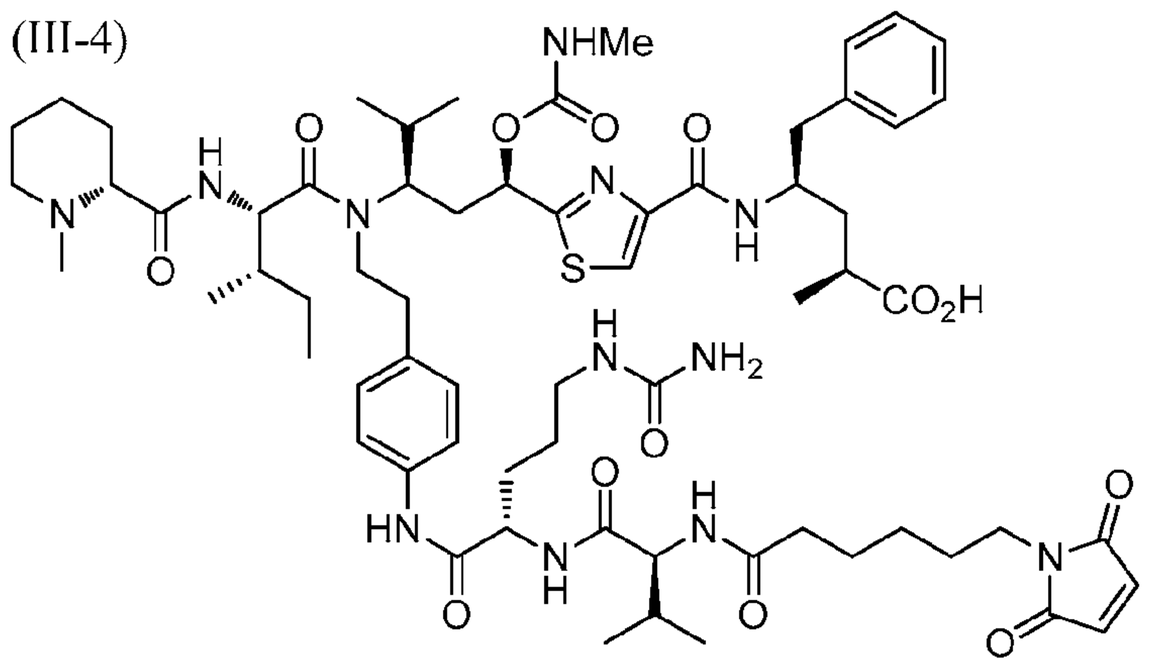
,

(III-3)



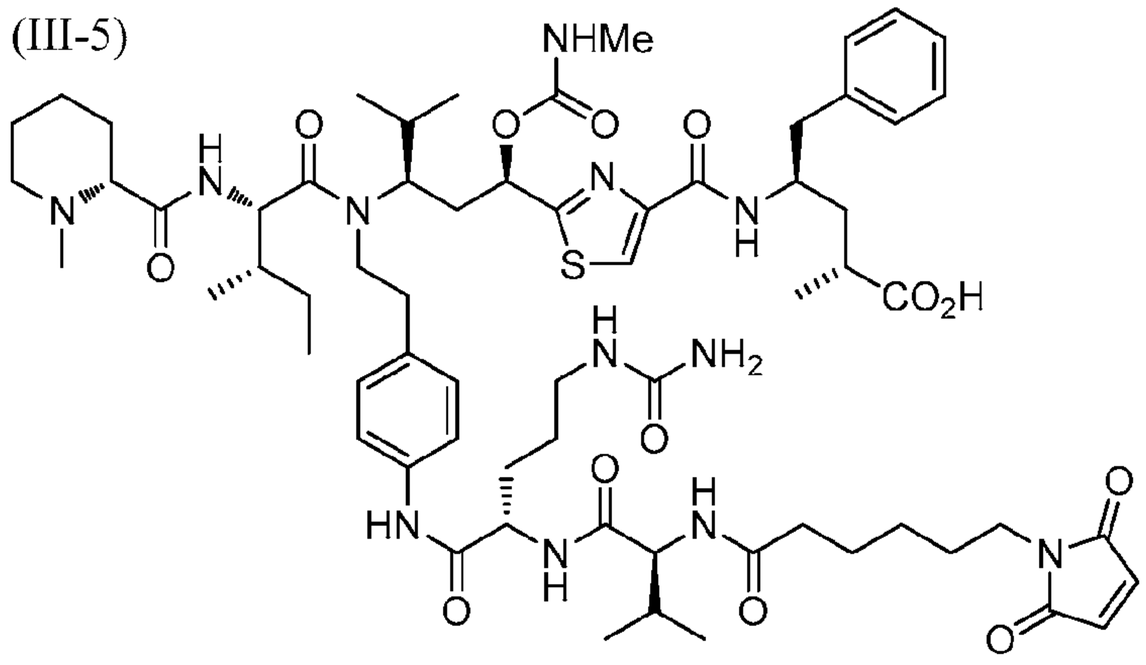
,

(III-4)



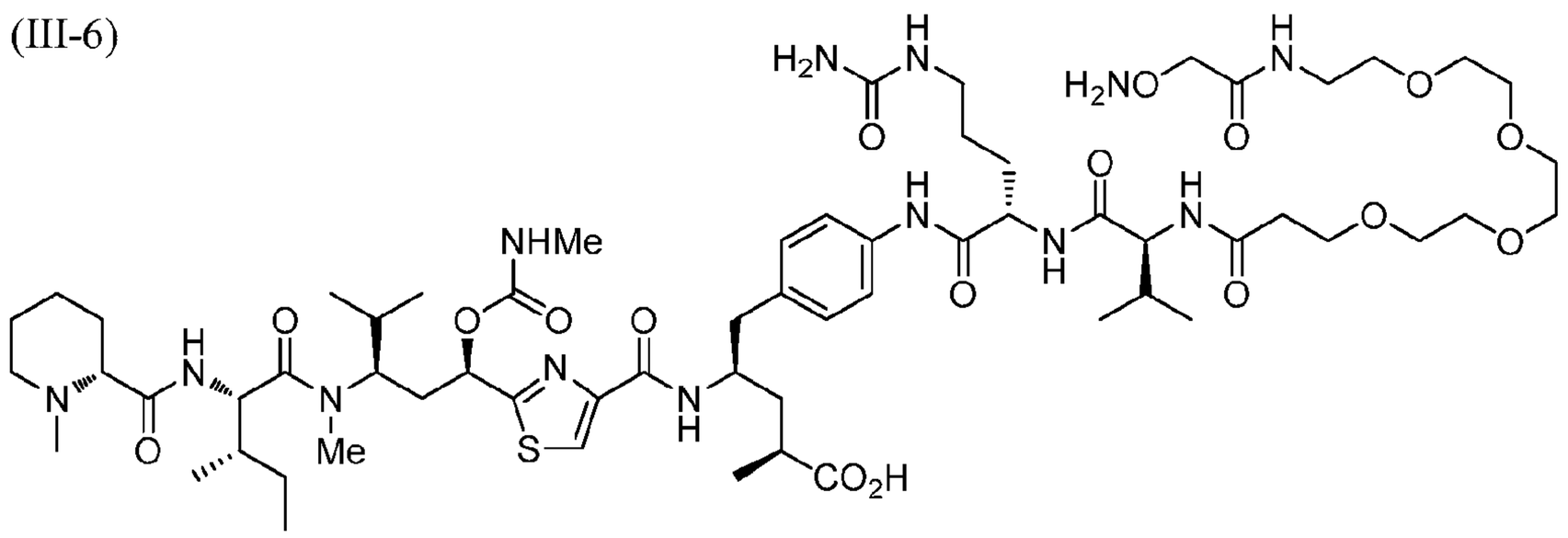
,

(III-5)

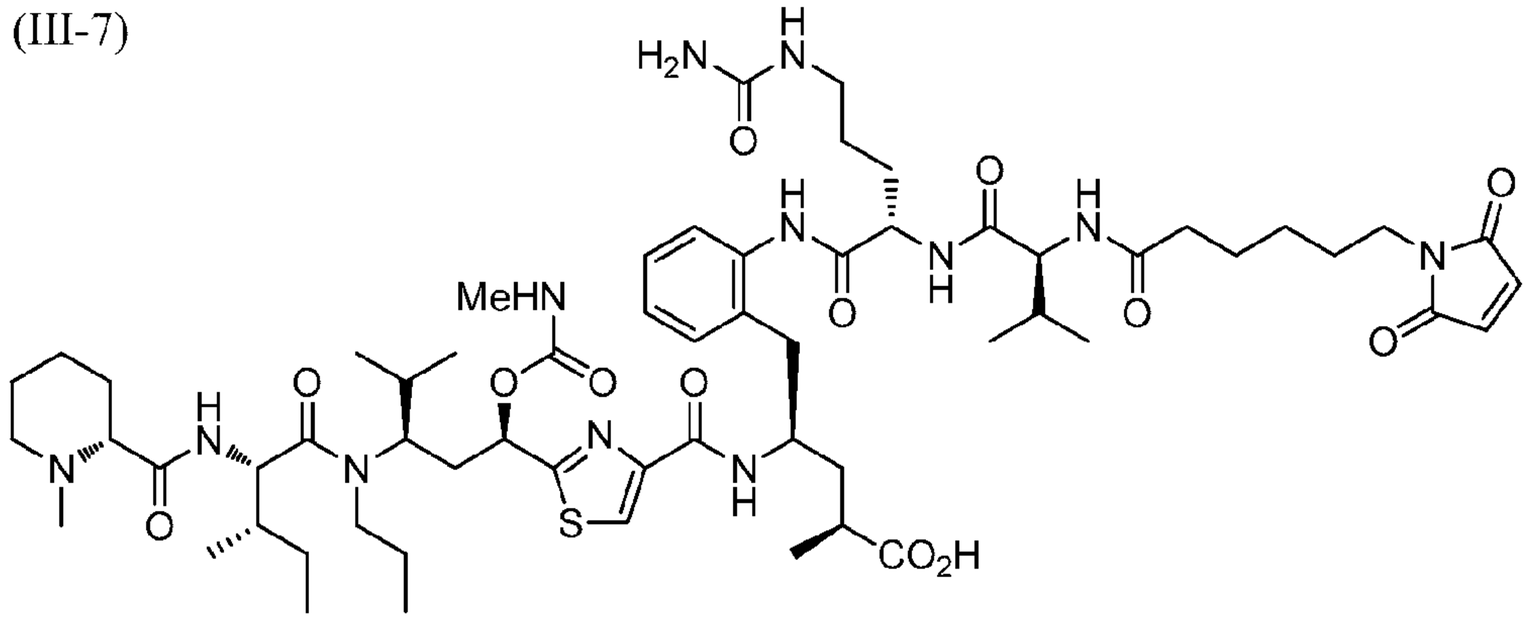


,

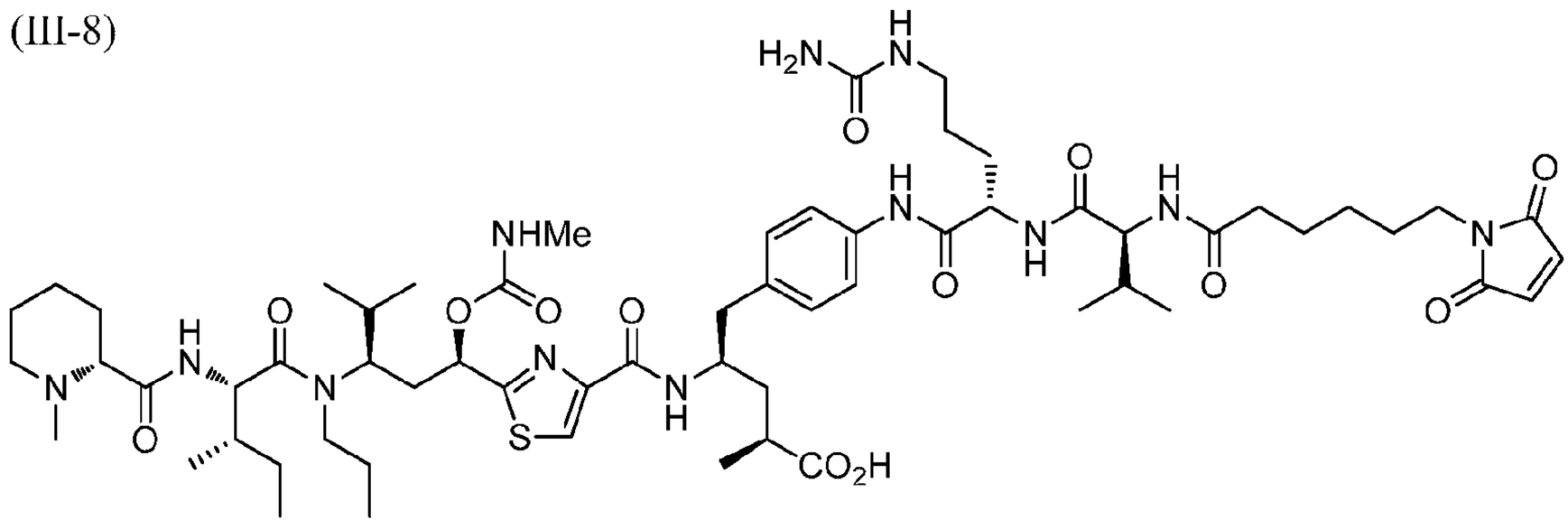
(III-6)



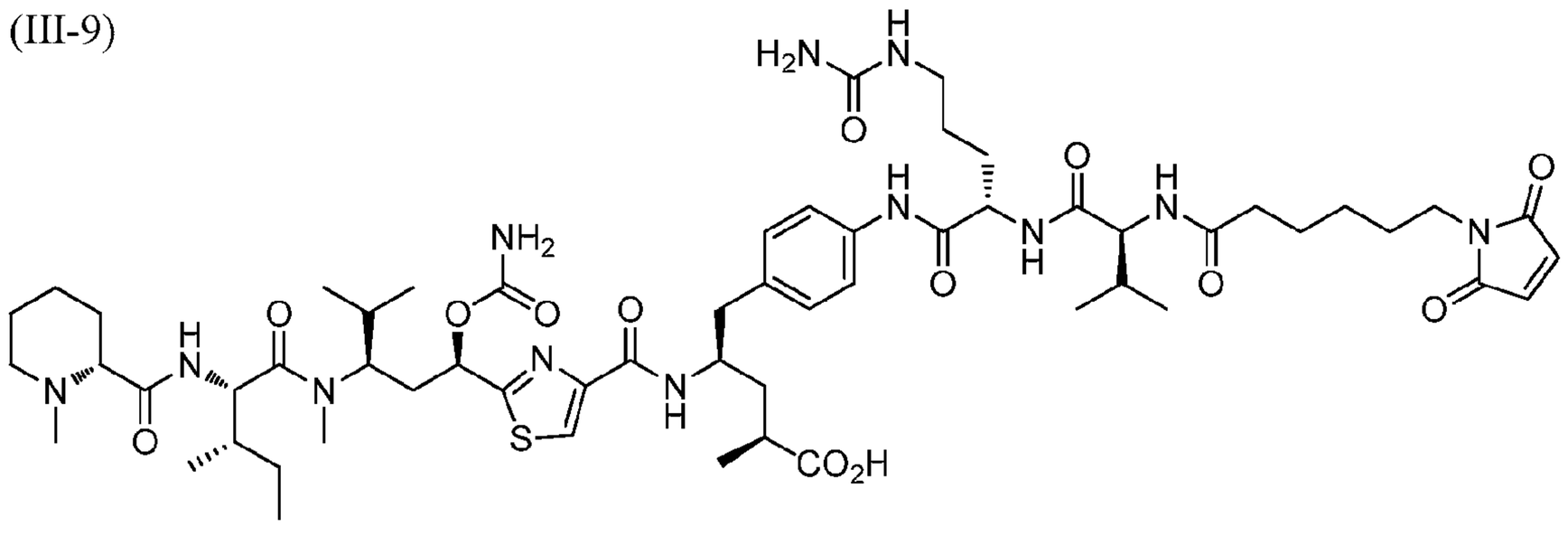
(III-7)



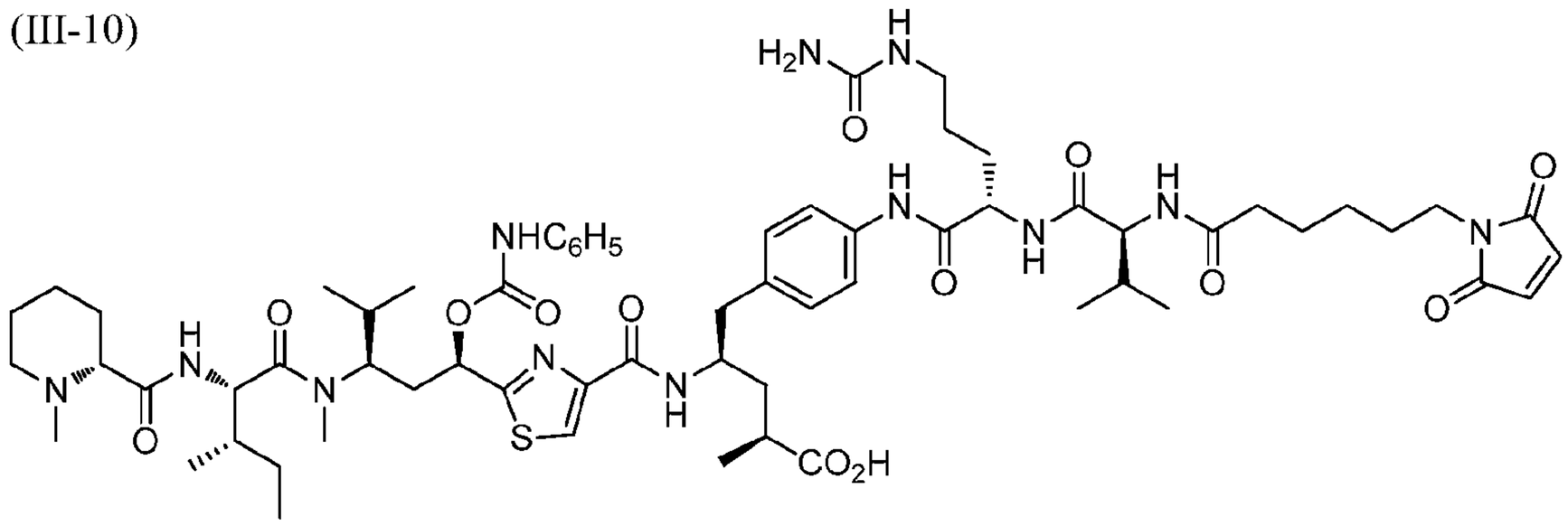
(III-8)



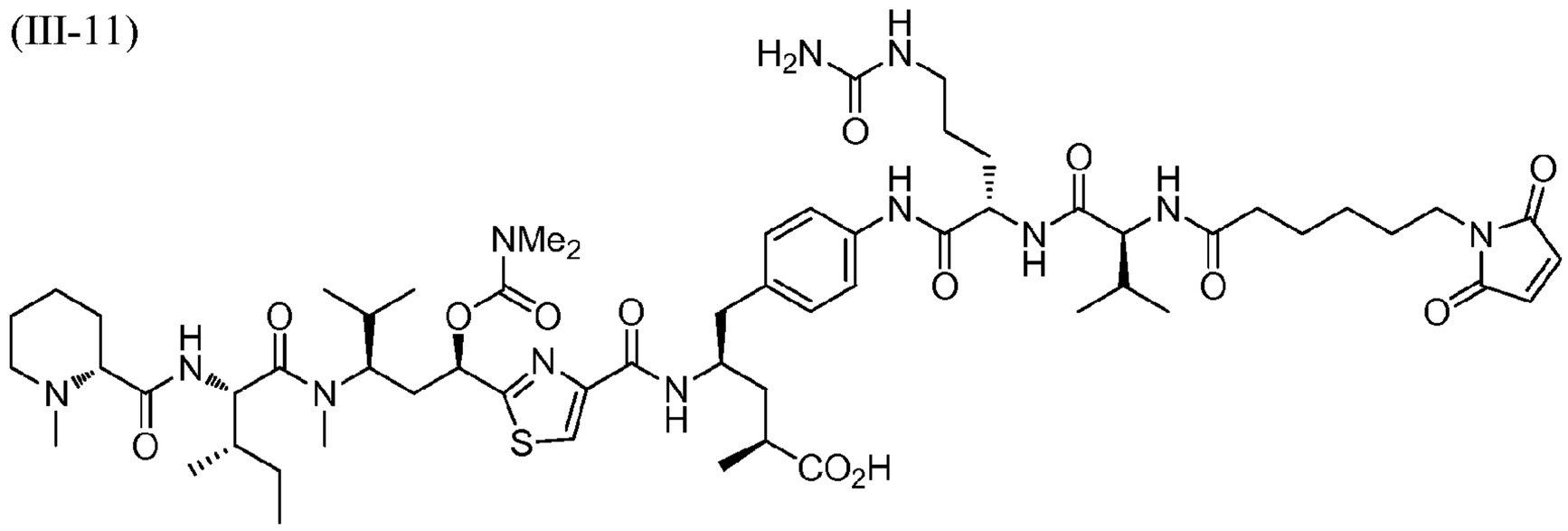
(III-9)



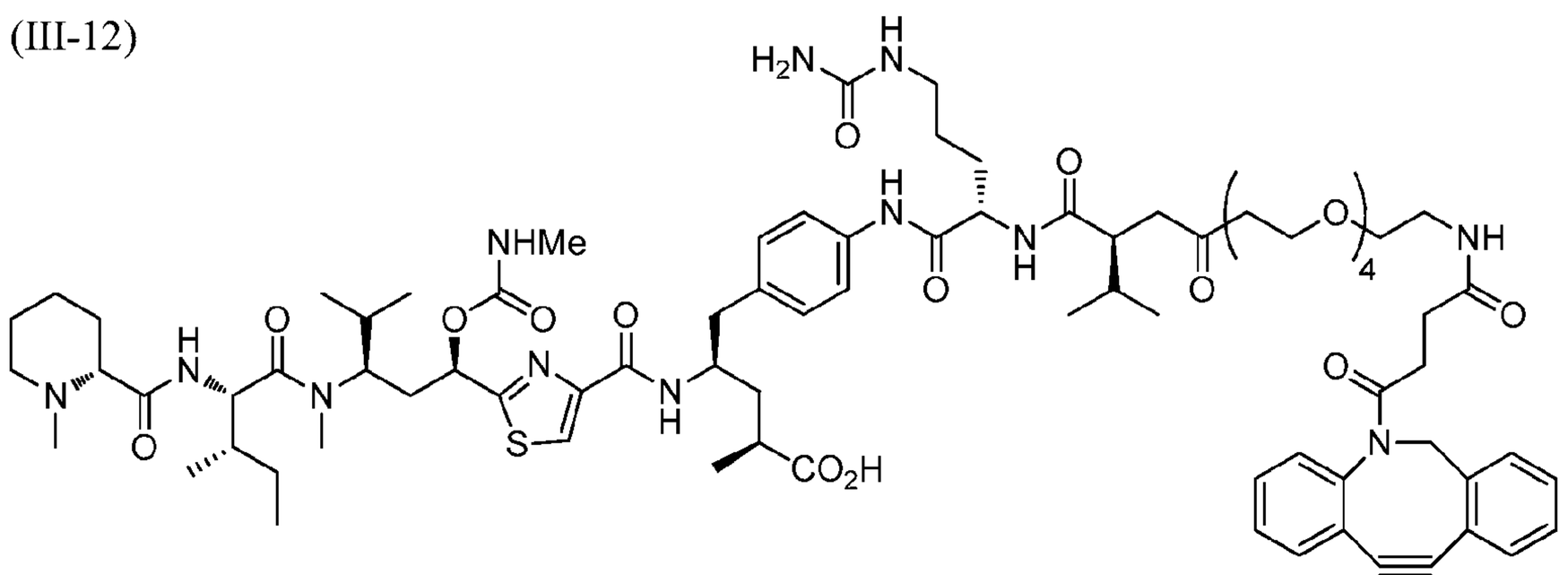
(III-10)



(III-11)

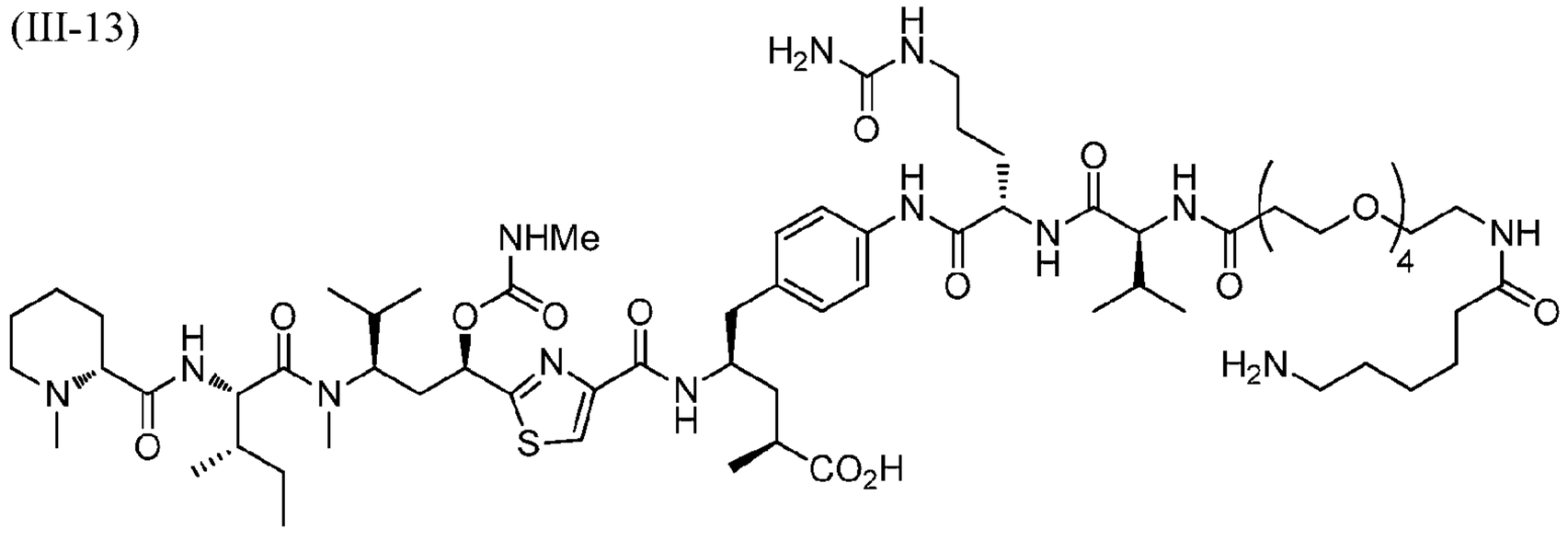


(III-12)



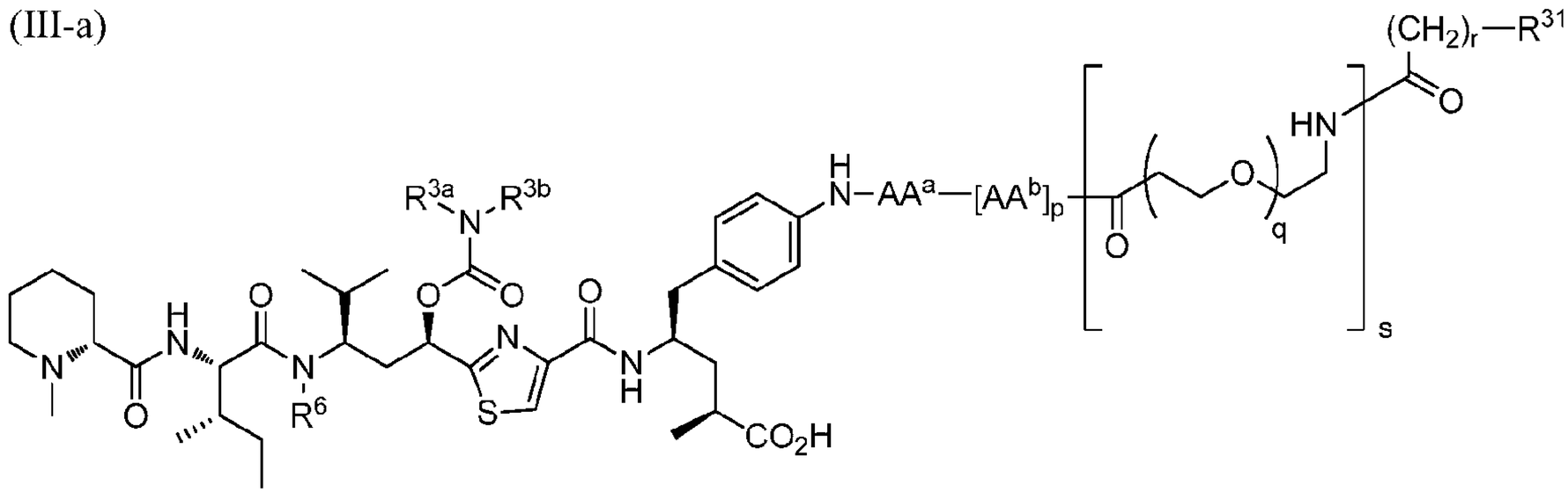
and

(III-13)



[00123] A preferred drug-linker compound has a structure represented by formula (III-a):

(III-a)



where

R^{3a} and R^{3b} are independently H, Me, or Et;

R^6 is Me, Et, or n-Pr;

AA^a and each AA^b are independently selected from the group consisting of alanine, β-alanine, γ-aminobutyric acid, arginine, asparagine, aspartic acid, γ-carboxyglutamic acid, citrulline, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, norleucine, norvaline, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine;

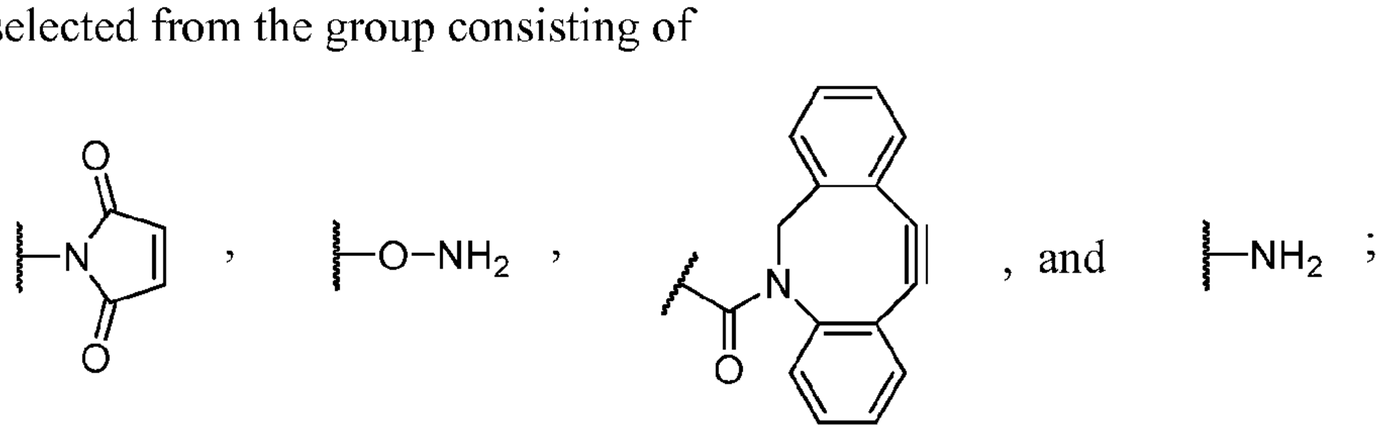
p is 1, 2, 3, or 4;

q is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 (preferably 2, 3, or 4);

r is 1, 2, 3, 4, or 5;

s is 0 or 1; and

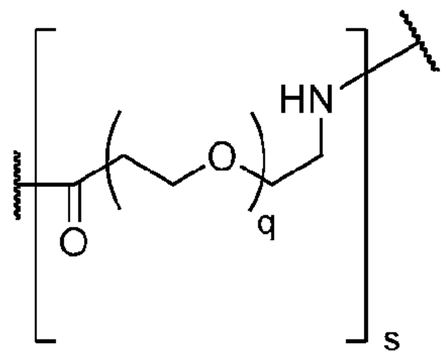
R^{31} is selected from the group consisting of



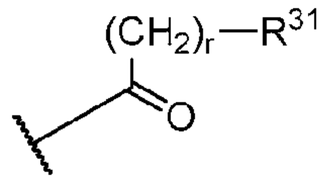
or a pharmaceutically acceptable salt thereof.

[00124] In formula (III-a), $-AA^a-[AA^b]_p-$ represents a polypeptide whose length is determined by the value of p (*e.g.*, dipeptide if p is 1, tetrapeptide if p is 3, etc.). AA^a is at the carboxy terminus of the polypeptide and its carboxyl group forms a peptide (amide) bond

with the anilino nitrogen of the drug. Conversely, the last AA^b is at the amino terminus of the polypeptide and its alpha-amino group forms a peptide bond with



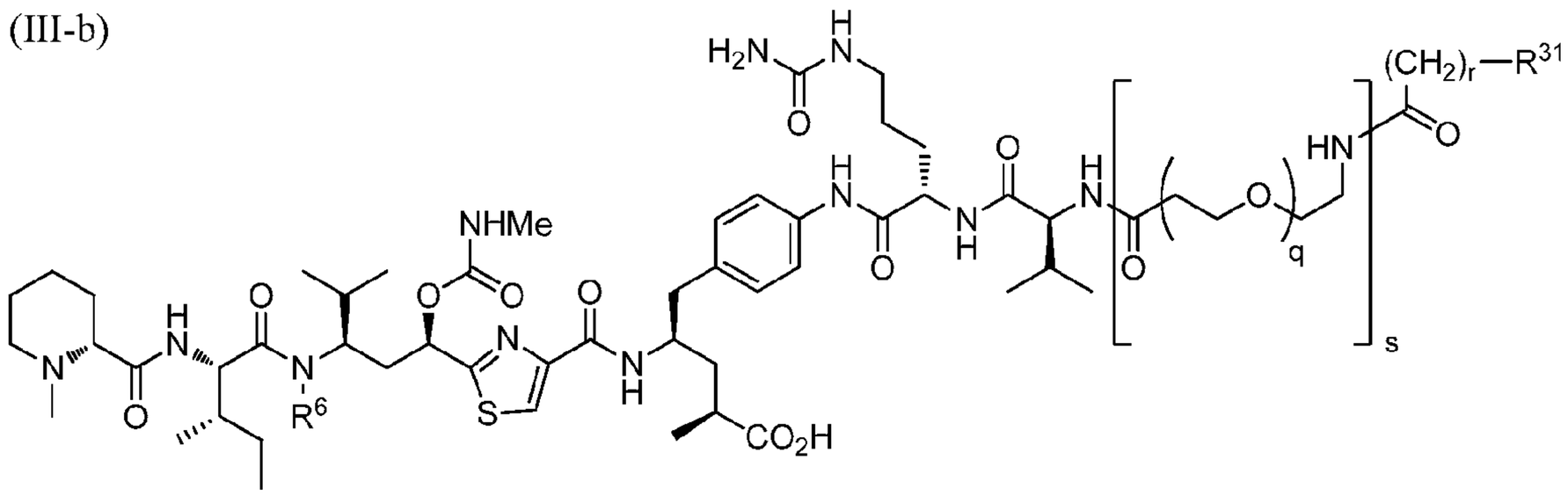
if s is 1 and with



if s is 0.

[00125] A more preferred drug-linker compound has a structure represented by formula (III-b):

(III-b)



where

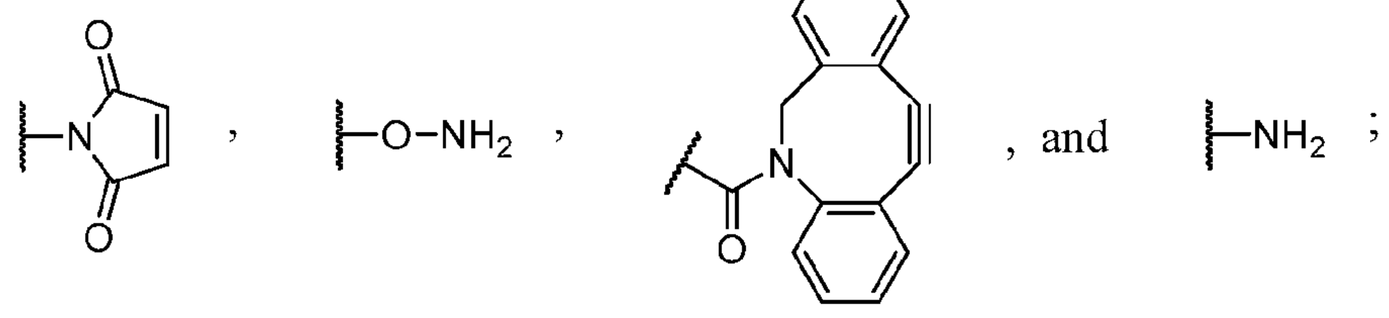
R^6 is Me or n-Pr;

q is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 (preferably 2, 3, or 4);

r is 1, 2, 3, 4, or 5;

s is 0 or 1; and

R^{31} is selected from the group consisting of



or a pharmaceutically acceptable salt thereof.

Preparation of Conjugates

[00126] 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}.

[00127] 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.

[00128] 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%

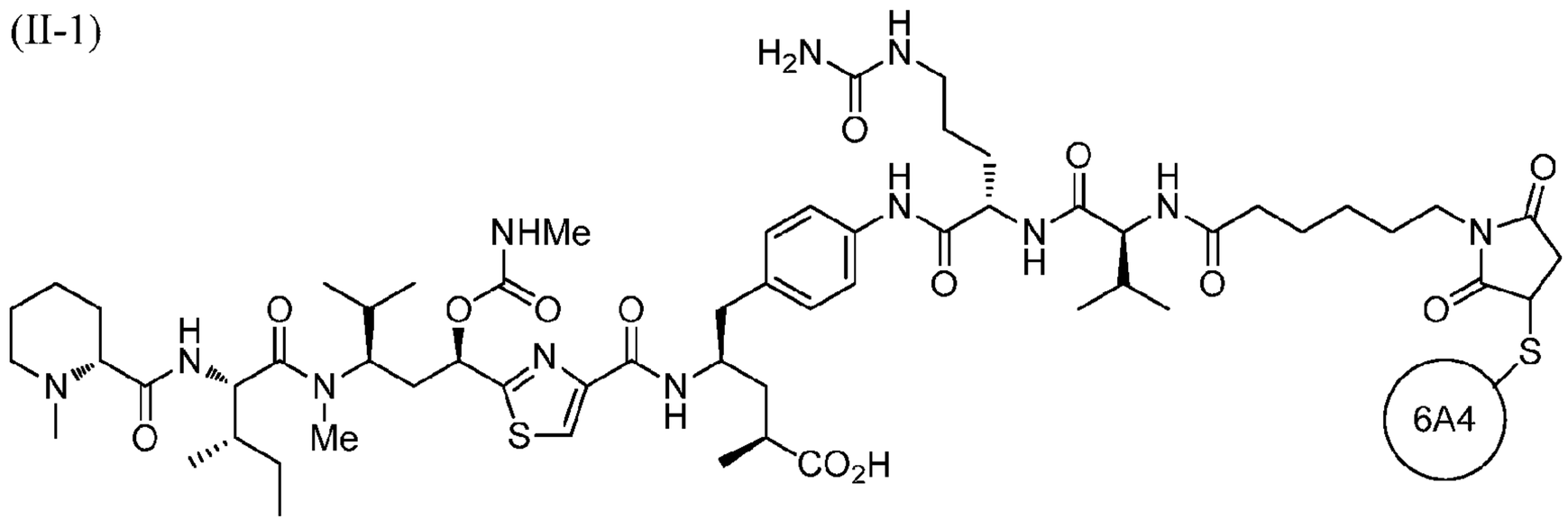
DMSO. The stock solution is added directly to the thiolated antibody, which has enough DMSO added to bring the final concentration to 10%, or pre-diluted in conjugation buffer containing a final concentration of 10% DMSO, followed by addition to an equal volume of thiolated antibody.

[00129] 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 150 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).

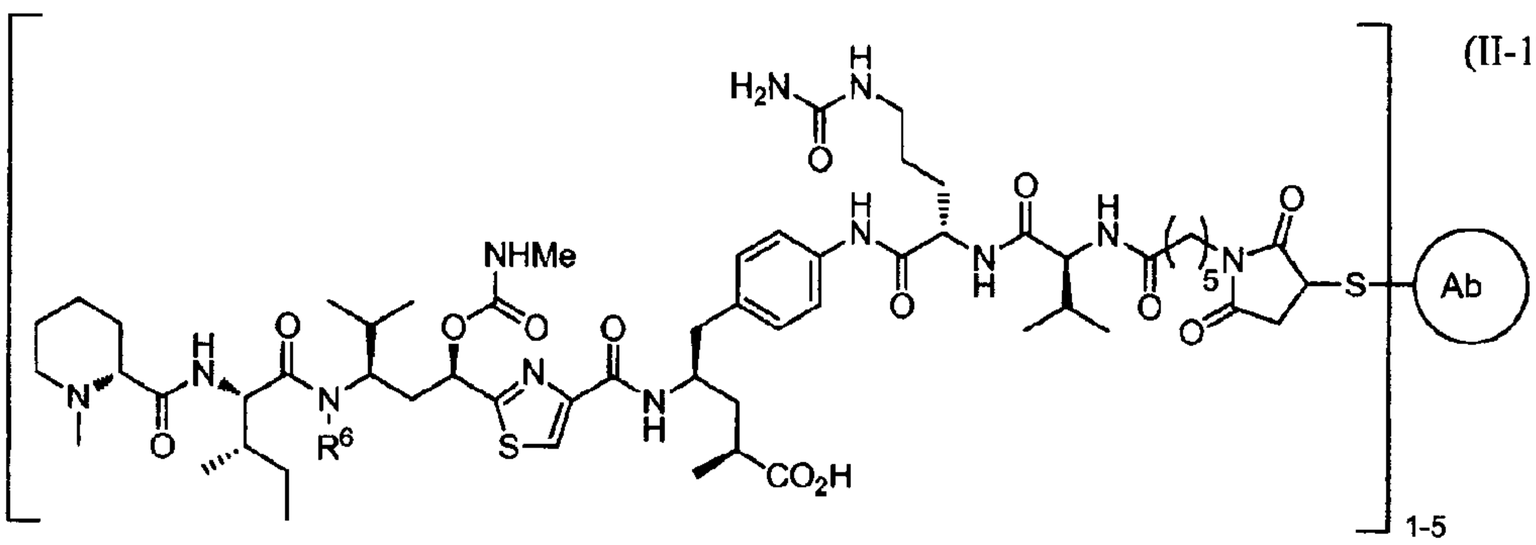
[00130] Those skilled in the art will understand that the above-described conditions and methodology are exemplary and non-limiting and that other approaches for conjugation are known in the art and usable in the present invention.

[00131] A conjugate prepared by the procedure described above is represented by formula (II-1). It is a conjugate of compound (III-1) and the anti-mesothelin antibody 6A4 (Terrett *et al.* 2012):

(II-1)



[00132] Those skilled in the art will appreciate that such a conjugate preparation may have moieties with different substitution ratios, typically ranging from 1 to 5, and that such preparation can be represented by formula (II-1'):



where R^6 is Me or n-Pr and Ab is an antibody. The antibody preferably is an anti-CD70, anti-mesothelin, or anti-glypican-3 antibody.

PHARMACEUTICAL COMPOSITIONS

[00133] In another aspect, the present disclosure provides a pharmaceutical composition comprising a compound of the present invention, or of a conjugate thereof, formulated together with a pharmaceutically acceptable carrier or 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.

[00134] 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).

[00135] Preferably, a pharmaceutical composition is suitable for intravenous, intra-muscular, 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.

[00136] 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. The compositions can also be provided in the form of lyophilates, for reconstitution in water prior to administration.

[00137] 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.

[00138] 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.

[00139] 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.

[00140] 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.

[00141] 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.

[00142] 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).

[00143] 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

[00144] Compounds of this invention or their conjugates can 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 renal, lung, gastric, or ovarian cancer.

[00145] Compounds of this invention or their conjugates can be administered in combination with other therapeutic 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 therapeutic agents include adalimumab, ansamitocin P3, auristatin, bendamustine, bevacizumab, bicalutamide, bleomycin, bortezomib, busulfan, callistatin A, camptothecin, capecitabine, carboplatin, carmustine, cetuximab, cisplatin, cladribin, cytarabin, cryptophycins, dacarbazine, dasatinib, daunorubicin, docetaxel, doxorubicin, duocarmycin, dynemycin A, epothilones, etoposide, floxuridine, fludarabine, 5-fluorouracil, gefitinib, gemcitabine, ipilimumab, hydroxyurea, imatinib, infliximab, interferons, interleukins, β-lapachone, lenalidomide, irinotecan, maytansine, mechlorethamine, melphalan, 6-mercaptopurine, methotrexate, mitomycin C, nilotinib, oxaliplatin, paclitaxel, procarbazine, suberoylanilide hydroxamic acid (SAHA), 6-thioguanidine, thiotepa, teniposide, topotecan, trastuzumab, trichostatin A, vinblastine, vincristine, and vindesine.

EXAMPLES

[00146] 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 – Compound (III-1)

[00147] This example describes the synthesis of compound (III-1), the corresponding scheme being shown in combined **Figs. 1**, **2a-2b**, and **3**.

[00148] *Compound 2.* A mixture of compound **1** (6 g, 16.6 mmol; prepared according to Peltier *et al.* 2006) and paraformaldehyde (9.94 g, 331 mmol) in toluene (150 mL) was heated in a sealed vessel at 70 °C for 24 h. Thin layer chromatography (TLC) showed that the reaction was complete. The reaction mixture was filtered through CELITE™ filter media and the filter cake was washed thoroughly with toluene. After evaporation of the solvent, the crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-70% ethyl acetate (EtOAc) in dichloromethane (DCM) to afford 4.76 g of compound **2** as a light yellow oil. MS: (+) m/z 375.2 (M+1).

[00149] *Compound 3.* Hydrochloric acid (4.0 M in 1,4-dioxane, 12.24 mL, 50.8 mmol) was added drop-wise to a solution of compound **2** (4.76 g, 12.7 mmol) in acetonitrile (62 mL) and methanol (6.8 mL), in the presence of support-bound cyanoborohydride (MP-BH_3CN) resin (4.85 g, 12.7 mmol). The reaction mixture was stirred at room temperature (RT) for 3 h. LCMS showed the reaction went to completion. The resin was filtered off and washed with acetonitrile-methanol mixture. After evaporation of the solvent, the crude

product was purified by flash chromatography eluting from silica gel with a gradient of 0-10% methanol in DCM containing 1% NH_4OH to afford crude compound **3**.

[00150] The product fractions were concentrated, diluted with EtOAc, and washed once with saturated aq. $NaHCO_3$ to remove excess ammonium salts. The aqueous fraction was back-extracted once with EtOAc. The combined organic phases were dried and concentrated to afford 2.82 g of compound **3** as a foamy solid. MS: (+) m/z 273.2 (M+1).

[00151] *Compound 4.* Polymer-bound N-benzyl-N-cyclohexylcarbodiimide (Aldrich, 4.5 g, 5.21 mmol) was added to a solution of compound **3** (1.42 g, 5.21 mmol), *t*-butanol (0.72 g, 5.32 mmol) and Boc-protected isoleucine **3a** (1.27 g, 5.47 mmol) in DCM (48 mL) at 0 °C. The reaction mixture was stirred at RT overnight. The resin was filtered off and washed with DCM. The filtrate was concentrated, diluted with EtOAc, and washed once with saturated aq. $NaHCO_3$. The aqueous solution was extracted twice 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 0-10% methanol in DCM containing 1% NH_4OH to afford fractons containing intermediate product **3b**.

[00152] The product-containing fractions were combined, concentrated, diluted with EtOAc, and washed with saturated aq. $NaHCO_3$ to remove excess ammonium salts. The aqueous fraction was back-extracted once with EtOAc. The combined organic phases were dried and concentrated to afford intermediate product **3b** as a white solid.

[00153] Intermediate product **3b** in toluene (50 mL) was heated to 90 °C in a sealed vessel overnight, with stirring. LCMS showed the reaction went to completion. The solvent was evaporated. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-100% EtOAc in hexanes to afford 1.3 g of compound **4** as a light yellow solid. MS: (+) m/z 486.3 (M+1).

[00154] *Compound 5.* Trifluoroacetic acid (TFA, 26 mL) was added to a mixture of compound **4** in DCM (26 mL). After stirring at RT for 30 min, LCMS showed the reaction was complete. The solution was concentrated, diluted with EtOAC, and washed once with saturated aq. $NaHCO_3$. The aqueous solution was back-extracted twice with EtOAc. The combined organic layers were dried, filtered, and concentrated to afford 1.03 g of compound **5** as white solid. MS: (+) m/z 386.3 (M+1).

[00155] *Compound 6.* DCC (0.664 g, 3.22 mmol) was added to a mixture of compound **5** (1.03 g, 2.68 mmol), (*R*)-1-methylpiperidine-2-carboxylic acid **5a** (0.4 g, 2.81 mmol; prepared according to Peltier *et al.* 2006), and *t*-butanol (0.369 g, 2.73 mmol) in DCM at 0

°C. The reaction mixture was allowed to warm to RT and stirred at RT overnight. The solid was filtered off, and the filtrate was concentrated. The residue was dissolved in EtOAc and washed once with saturated aq. $NaHCO_3$. The aqueous solution was back-extracted twice 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 0-20% methanol in DCM to afford 1.23 g of compound **6** as a light yellow solid. MS: (+) m/z 511.4 (M+1).

[00156] *Compound 7.* A mixture of *N,N*-diisopropylethylamine (DIEA, also referred to as DIPEA, 0.972 mL, 5.58 mmol), bis(4-nitrophenyl) carbonate (BNPC, 1.698 g, 5.58 mmol) and compound **6** (0.57 g, 1.116 mmol) in N,N-dimethylformamide (DMF, 10 mL) was stirred at RT overnight. LCMS showed the reaction went to completion. The solvent was evaporated. The crude product was purified by silica gel flash chromatography with a gradient of 0-20% methanol in DCM to afford 0.68 g of compound **7** as a yellow oil. MS: (+) m/z 676.4 (M+1).

[00157] *Compound 8.* Methylamine in methanol (2.0 M, 0.089 mL, 0.178 mmol) was added to compound **7** (0.1 g, 0.148 mmol) in methanol (1 mL). After the reaction mixture was stirred at RT for 10 min, LCMS showed the reaction was complete. The solvent was evaporated to afford 0.084 g of compound **8**. MS: (+) m/z 568.4 (M+1).

[00158] *Compound 9.* Lithium hydroxide (7.09 mg, 0.296 mmol) in water (0.5 mL) was added to a solution of compound **8** (0.084 g, 0.148 mmol) in 1,4-dioxane (0.5 mL) at RT. After the reaction mixture was stirred at RT for 2 h, LCMS showed the reaction was complete. The solvent was evaporated. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-30% methanol in DCM to afford 0.075 g of compound **9** as a white solid. MS: (+) m/z 554.4 (M+1).

[00159] *Compound 10.* Triethylamine (11.73 mL, 84 mmol) was added to a mixture of di-t-butyldicarbonate (BOC_2O, 10.57 mL, 46.0 mmol) and (*S*)-methyl 2-amino-3-(4-nitrophenyl)propanoate hydrochloride **9a** (10 g, 38.4 mmol) in acetonitrile (300 mL) at 0 °C. The reaction mixture was allowed to warm to RT, and stirred at RT overnight. LCMS showed the reaction went to completion. The reaction mixture was concentrated, and the product was re-dissolved in 200 mL of diethyl ether. The solid was filtered off, and the filtrate was concentrated. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-50% EtOAc in hexanes to afford 11.3 g of a Boc protected intermediate as a white solid.

[00160] Pd /C catalyst (10 wt.%, 0.85 g, 7.99 mmol) was added to a solution of the Boc protected intermediate (15 g, 46.2 mmol) in MeOH (200 mL). The reaction mixture was stirred under a hydrogen atmosphere overnight. The Pd/C catalyst was filtered, and the filtrate was concentrated to afford 13.6 g of compound **10** as a white solid. MS: (+) m/z 195.2 (M+1-Boc).

[00161] *Compound 11*. Pyridine (5.77 mL, 71.3 mmol) was added to a solution of benzyl chloroformate (10.18 mL, 71.3 mmol) and compound **10** (17.5 g, 59.5 mmol) in DCM (185 mL) at 0 °C. The reaction mixture was allowed to warm to RT and was stirred at RT overnight. The reaction was quenched by addition of saturated aq. $NaHCO_3$, and washed with brine. The organic layer was dried, filtered, and concentrated. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-50% EtOAc in hexanes to afford 22.6 g of compound **11** as a colorless oil. MS: (+) m/z 329.2 (M+1-Boc).

[00162] *Compound 12*. Diisobutylaluminum hydride (DIBAL-H) in hexanes (1M, 26.5 mL, 26.5 mmol) was added to a solution of compound **11** (5.17 g, 12.07 mmol) in DCM (39 mL) at -78 °C. The reaction mixture was stirred at -78 °C for 2 h. Acetic acid (24 mL) and toluene (36 mL) were added at -78 °C. The reaction mixture was warmed to RT. Tartaric acid (10% aq., 69 mL) was added to the reaction mixture. The aqueous solution was extracted with hexanes and EtOAc (v/v 1:1) mixture. 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 0-50% EtOAc in hexanes to afford 3.12 g of compound **12** as a white solid. MS: (+) m/z 299.2 (M+1-Boc).

[00163] *Compound 13*. Dibutyl(((trifluoromethyl)sulfonyl)oxy)borane (Bu_2BOTf, 1M in DCM, 8.61 mL, 8.61 mmol) and DIEA (1.637 mL, 9.40 mmol) were added to a solution of (*S*)-4-isopropyl-3-propionyloxazolidin-2-one **12a** (1.450 g, 7.83 mmol) in DCM (7.8 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 45 min. A solution of compound **12** (3.12 g, 7.83 mmol) in DCM (7.8 mL) was added to the reaction mixture at -78 °C. The reaction mixture was allowed to warm up to RT overnight. Sodium phosphate buffer (pH 7, 29 mL) was added. The aqueous solution was extracted with DCM. The combined organic layers were washed with brine, dried, filtered, and concentrated.

[00164] The residue was re-dissolved in methanol (130 mL) and cooled to 0 °C. Aqueous H_2O_2 (30%, 39.7 mL) was added to the reaction mixture at 0 °C. The reaction mixture was stirred at 0 °C for 4 h. Water (39 mL) was added. Some of the solvent (MeOH) was evaporated. The aqueous solution was extracted with EtOAc. The combined organic layers

were washed with 5% $NaHCO_3$ solution and brine, dried, filtered, and concentrated. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-50% EtOAc in hexanes to afford 4.23 g of compound **13** as a colorless oil. MS: (+) m/z 484.3 (M+1-Boc).

[00165] *Compound 14*. Di(1*H*-imidazol-1-yl)methanethione (1.5 g, 8.42 mmol) was added to a solution of compound **13** (2.46 g, 4.21 mmol) in THF (20 mL). The reaction mixture was refluxed overnight. LCMS showed the reaction went to completion. The solvent was evaporated. The crude product was purified by silica gel flash chromatography with a gradient of 0-50% EtOAc in hexanes to afford 1.25 g of compound **14** as a white solid. MS: (+) m/z 694.3 (M+1).

[00166] *Compound 15*. (*E*)-2,2'-(diazene-1,2-diyl)bis(2-methylpropanenitrile) (AIBN, 0.016 g, 0.095 mmol) was added to a solution of compound **14** (1.78 g, 2.57 mmol) and tributylstannane (Bu_3SnH, 1.380 mL, 5.13 mmol). The reaction mixture was refluxed for 30 min (oil bath temperature at 142 °C). The solvent was evaporated. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-33% EtOAc in hexanes to afford 0.84 g of compound **15** as a light yellow oil. MS: (+) m/z 468.3 (M+1-Boc).

[00167] *Compound 16*. LiOH (0.071 g, 2.96 mmol) in water (3.7 mL) was added to a solution of compound **15** (0.84 g, 1.480 mmol) in tetrahydrofuran (THF, 11.4 mL), followed by addition of 30% aq. H_2O_2 (0.271 mL, 8.88 mmol) at 0 °C. After the reaction mixture was stirred at 0 °C for 4 h, 20 mL of 1.33 M aq. Na_2SO_3 was added to quench the reaction. Hydrochloric acid (1 M) was added to adjust the pH to 2-3. The resulting aqueous solution was extracted with DCM. 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 0-75% EtOAc in hexanes to afford 0.53 g of compound **16** as a colorless oil. MS: (+) m/z 357.3 (M+1-Boc).

[00168] *Compound 17.* Concentrated hydrochloric acid (4 drops) was added to a solution of 2,2-dimethoxypropane (3.53 mL, 28.7 mmol) and compound **16** (0.53 g, 1.161 mmol) in methanol (17.7 mL). The reaction mixture was stirred at RT overnight. LCMS showed the reaction went to completion. LCMS also showed the formation of some deprotected byproduct **16a**. The solvent was evaporated.

[00169] Triethylamine (2.2 eq., 0.36 mL) was added to a solution of the above residue and BOC_2O (1.2 eq., 304.3 mg) in acetonitrile at RT, to re-protect by-product **16a**. The reaction

mixture was stirred at RT for 2 h. LCMS showed the reaction went to completion. The solvent was evaporated. Water (7 mL) was added, 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 0-50% EtOAc in hexanes to afford 0.3 g of compound **17** as a colorless oil. MS: (+) m/z 371.3 (M+1-Boc).

[00170] *Compound **18***. A mixture of compound **17** (0.223 g, 0.474 mmol) and Pd/C 10 wt% (20 mg, 0.474 mmol) in methanol (6 mL) was stirred under H_2 overnight. The Pd/C catalyst was filtered off, and the filtrate concentrated. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-50% EtOAc in hexanes to afford 0.112 g of compound **18** as a white solid. MS: (+) m/z 237.2 (M+1-Boc).

[00171] *Compound **19***. A mixture of compound **18** (0.204 g, 0.606 mmol), *N*-Ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, 0.174 g, 0.910 mmol), and Fmoc-protected citrulline **18a** (0.361 g, 0.910 mmol) in DMF (12.4 mL) was stirred at RT overnight. Saturated NH_4Cl solution (20 mL) was added to quench the reaction. 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 with a gradient of 0-30% MeOH in DCM to afford 0.25 g of compound **19** as a white solid. MS: (+) m/z 716.4 (M+1).

[00172] *Compound **20***. Piperidine (0.5 mL, 5.06 mmol) was added to a solution of compound **19** (0.25 g, 0.349 mmol) in DMF (5 mL). After the reaction mixture was stirred at RT for 20 min, the solvent was evaporated to afford the Fmoc-deprotected intermediate as a residue.

[00173] DIEA was added to a solution of (S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)-amino)-3-methylbutanoic acid **19a** (0.142 g, 0.418 mmol) and *N,N,N',N'*-tetramethyl-*O*-(7-azabenzotriazol-1-yl)uronium hexafluorophosphate (HATU, 0.146 g, 0.383 mmol) in DMF (2 mL), adjusting the pH to 8-9. After the reaction mixture was stirred at RT for 5 min, the above residue in DMF (1 mL) and DIEA were added to the reaction mixture, adjusting the pH to 8-9. After the reaction mixture was stirred at RT for 15 min, 20 mL of water containing 8 mL of 0.1% TFA water was added. 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 0-20% MeOH in DCM to afford 0.24 g of compound **20** as a white solid. MS: (+) m/z 815.4 (M+1).

[00174] *Compound 21*. Piperidine (0.3 mL) was added to a solution of compound **20** in DMF (3 mL). The reaction mixture was stirred at RT for 1 h. LCMS showed the reaction went to completion. The solvent was evaporated.

[00175] Lithium hydroxide (0.028 g, 1.176 mmol) in water (2 mL) was added to a solution of the above residue in THF (4 mL). After the reaction mixture was stirred at RT for 4 h, aq. HCl (0.1N) was added to acidify the reaction mixture (pH 2-3). The solvent was partially evaporated, and lyophilized to afford compound **21** as a white solid. MS: (+) m/z 579.4 (M+1).

[00176] *Compound 22*. DIEA was added to a mixture of ε-maleimidocaproic acid *N*-hydroxysuccinimide ester **21a** (Tokyo Chemical Industry, 64.7 mg, 0.210 mmol) and compound **21** (81 mg, 0.14 mmol) in DMF (3 mL), adjusting pH 8-9. After the reaction mixture was stirred at RT for 2h, 10 mL of 1:1 (v/v) mixture of acetonitrile and water containing 0.1% TFA was added. The product **22** was purified by preparative high performance liquid chromatography (HPLC). MS: (+) m/z 772.5 (M+1).

[00177] *Compound 23*. 2,2,2-Trifluoroacetic acid (0.7 mL, 0.013 mmol) was added to a mixture of compound **22** (30 mg, 0.039 mmol) in DCM (1 mL) at RT. After the reaction mixture was stirred at RT for 10 min, LCMS showed the reaction went to completion. The solvent was evaporated, affording compound **23**. MS: (+) m/z 672.4 (M+1).

[00178] *Compound **(III-1)***. DIEA was added to a solution of compound **9** (23.66 mg, 0.043 mmol) and HATU (14.77 mg, 0.039 mmol) in DMF (1 mL). The pH of the reaction mixture was adjusted to 8-9. After the reaction mixture was stirred at RT for 10 min, compound **23** (26.1 mg, 0.039 mmol) in DMF (1 mL) and DIEA were added. The pH of the reaction solution was adjusted to 8-9. After the reaction mixture was stirred at RT for 10 min, LCMS showed the reaction was complete. The reaction was quenched by addition of 10 mL 1:1 (v/v) mixture of water containing 0.1% TFA and acetonitrile. The product compound **(III-1)** was purified by prep HPLC. MS: (+) m/z 1207.7 (M+1).

[00179] Compounds such as **(III-1)**, having a maleimido group, can be used to prepare conjugates by reaction with a sulfhydryl group on an antibody or other ligand. The sulfhydryl group can be one from a cysteine residue or one obtained by derivatization of a lysine residue with 2-iminothiolane.

Example 2 – Compound (III-2)

[00180] This example describes a synthesis of compound (III-2), the corresponding scheme being shown in **Fig. 4**.

[00181] *Compound 24*. *N*-Ethyl-*N*-isopropylpropan-2-amine (0.556 mL, 3.19 mmol) was added to a solution of glycine tert-butyl ester hydrochloride **23a** (0.209 g, 1.596 mmol), Fmoc-aminoxyacetic acid **23b** (0.5g, 1.596 mmol) and HATU (0.607 g, 1.596 mmol) in DMF (5 mL) at RT. After the reaction mixture was stirred at RT for 1 h, 0.1% aq. TFA (20 mL) was added. 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 0-70% EtOAc in hexanes to afford 0.45 g of compound **24** as a colorless oil. MS: (+) m/z 449.2 (M+23).

[00182] *Compound 25*. TFA (3 mL, 1.437 mmol) was added to a solution of compound **24** (0.45 g, 1.055 mmol) in DCM (0.5 mL) at RT. The reaction mixture was stirred at RT overnight. The solvent was evaporated. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-30% MeOH in DCM to afford 0.39 g of compound **25** as a white solid. MS: (+) m/z 371.1 (M+1).

[00183] *Compound 26*. *N,N'*-methanediylidenedicyclohexanamine (DCC, 0.261 g, 1.267 mmol) was added to a solution of compound **25** and 1-hydroxypyrrolidine-2,5-dione (0.146 g, 1.267 mmol) in DCM (6 mL) at RT. After the reaction mixture was stirred at RT overnight, the solid was filtered off. The filtrate was then concentrated. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-100% EtOAc in hexanes to afford 0.43 g of compound **26** as a colorless oil.

[00184] *Compound 27*. DIEA was added to a solution of compound **21** (50 mg, 0.086 mmol) and compound **26** (60.6 mg, 0.130 mmol) in DMF at RT, adjusting the pH to 8-9. After the reaction mixture was stirred at RT for 4 h, the reaction was quenched by addition of 10 mL of 1:1 mixture of 0.1% aq. TFA and acetonitrile. Preparative HPLC purification afforded 55 mg of compound **27** as a white solid, MS: (+) m/z 931.4 (M+1).

[00185] *Compound 28*. TFA (1 mL, 0.059 mmol) was added to a solution of compound **27** (55 mg, 0.059 mmol) in DCM (2 mL) at RT. The reaction mixture was stirred at RT for 10 min. The solvent was evaporated.

[00186] DIEA was added to a solution of compound **9** (32.7 mg, 0.059 mmol) and HATU (22.47 mg, 0.059 mmol) in DMF (1 mL). The pH of the reaction mixture was adjusted to 8-9. After the reaction mixture was stirred at RT for 10 min, DMF (2 mL) and DIEA were added.

The pH of the reaction solution was adjusted to 8-9. After the reaction mixture was stirred at RT for 10 min, LCMS showed the reaction was complete. The reaction was quenched by addition of 20 mL 1:1 (v/v) mixture of water containing 0.1% TFA and acetonitrile. Preparative HPLC purification afforded 70 mg of compound **28** as a white solid. MS: (+) m/z 684.1 (M/2+1).

[00187] *Compound (III-2)*. Piperidine was added to a solution of compound **28** (70 mg, 0.051 mmol) in DMF (4 mL). After the reaction mixture was stirred at RT for 20 min, a 1:1 mixture of acetonitrile and 0.1% aq. TFA (40 mL) was added. Preparative HPLC purification afforded 46 mg of compound **(III-2)** as a white solid. MS: (+) m/z 1144.6 (M+1).

[00188] Compounds such as **(III-2)**, having a hydroxylamine group, can be used to form conjugates with an antibody or other ligand having an aldehyde or ketone functionality, for example by incorporation of the unnatural amino acid 4-acetylphenylalanine.

Example 3 – Compounds (I-2) and (I-3)

[00189] The synthesis of compounds (I-2) and (I-3) is shown schematically in **Fig. 5**.

[00190] *Compound (I-3)*. DIEA was added to a solution of compound **9** (10 mg, 0.018 mmol) and HATU (6.87 mg, 0.018 mmol) in DMF (0.3 mL), adjusting the pH to 8-9. After the reaction mixture was stirred at RT for 10 min, compound **29** (prepared according to Cheng *et al.* 2011, Example 17; 4.56 mg, 0.018 mmol) in DMF (0.5 mL) and DIEA were added, adjusting pH to 8-9. After the reaction mixture was stirred at RT for 20 min, the reaction was quenched by addition of 4 mL of 1:1 mixture of acetonitrile and 0.1% aq. TFA. Preparative HPLC purification afforded 12 mg of compound **(I-3)** (I-2) as a white solid. MS: (+) m/z 788.4 (M+1).

[00191] *Compound (I-2)*. A mixture of compound **(I-3)** (12 mg, 0.015 mmol) and Pd/C, 10 wt % (4 mg, 0.015 mmol) in methanol (0.5 mL) was stirred under an H_2 atmosphere overnight. The catalyst was filtered off, and the filtrate concentrated. Preparative HPLC purification afforded 8.1 mg of compound **(I-2)** as a white solid. MS: (+) m/z 758.4 (M+1).

[00192] Compound **(I-1)** can be analogously prepared by replacing compound **29** with the compound having mixed stereochemistry at the alpha-methyl position (Cheng *et al.* 2011).

Example 4 – Compound (III-4)

[00193] **Figs. 6a** through **6c** in combination show schematically the synthesis of compound (III-4).

[00194] *Compound 32.* Compound **30** (Aldrich, 3.5g, 13.9 mmol) was dissolved in 50 mL DCM. To this solution was added Dess-Martin periodinane (11.8 g, 27.9 mmol) at 5 °C. After 10 min the mixture was warmed to RT. After another hour the reaction was quenched with saturated aq. $NaHCO_3$ and saturated aq. NaS_2O_3. After extraction with ether, the ether extract was washed with aq. $NaHCO_3$ and then brine and dried and evaporated down to a sticky oil. The oil was dissolved in 50 mL of DCM, to which was added the commercially available compound **31** (5.05 g, 13.93 mmol). After 10 min the reaction mixture was taken up in EtOAc, washed with aq. $NaHCO_3$ and then brine and dried, filtered and the solvent evaporated. After column chromatography (EtOAc: hexane, 0-20% gradient) compound **32** (2.3 g, 6.90 mmol, 49.5 % yield) was obtained as a white solid. It had an NMR spectrum consistent with literature (Wipf *et al.* 2004a).

[00195] *Compound 33.* Hydrochloric acid (7.80 mL, 31.2 mmol, 4M in dioxane) was added at 5 °C to a DCM solution of compound **32** (5.2 g, 15.60 mmol). After the deprotective reaction was complete, the reaction mixture was evaporated and compound **33** (4.21 g, 15.60 mmol, 100 % yield, hydrochloride) was obtained as a white solid, which is used for next step without further purification.

[00196] *Compound 35.* To a solution of compound **34** (prepared per Sani *et al.* 2007, 4.00 g, 11.6 mmol) in 20 mL DMF at 5 °C were added HATU (4.61 g, 12.12 mmol) and DIPEA (6 ml, 34.4 mmol). After 10 min compound **33** (2.71 g, 11.60 mmol) was added. After another half hour the mixture was taken up in EtOAc, which was washed with 10% aq. citric acid, saturated aq.$NaHCO_3$ and brine. After drying and filtration, the organic phase was evaporated down to give compound **35** (6.49 g, 11.60 mmol, 100 % yield, $[M+Na]^+$, calculated 582.3, found 582.3) as an oil, which was used for next step without further purification.

[00197] *Compound 36.* $NaBH_4$ (4.66 g, 123 mmol) was added, in portions, to a 100 mL methanol solution of compound **35** (6.49 g, 11.6 mmol) and $NiSO_4(H_2O)_6$ (6.48 g, 24.66 mmol) at 5 °C. (Caution: Hydrogen was generated.) After 30 min, saturated aq. $NaHCO_3$ was added, followed by EtOAc. After filtration through CELITE™, the organic phase was separated from the aqueous phase, washed with brine, dried, filtered and evaporated down to give compound **36** (5.6 g, 9.97 mmol, 81 % yield, $[M+1]^+$, calculated 562.3, found 562.4), which was used for next step without further purification.

[00198] *Compound 37.* Compound **36** (5.6 g, 9.97 mmol) was dissolved in 30 mL pyridine at 5 °C. Acetic anhydride (4 g, 39.2 mmol) was added to this solution. After 10 min

the mixture was warmed to RT. After about an hour the reaction mixture was concentrated down. The resulting residue was taken up in EtOAC and the organic phase was washed with 10% aq. citric acid, saturated aq. $NaHCO_3$, and brine, sequentially. The organic phase was dried, filtered and concentrated to give compound **37** (5.8 g, 9.61 mmol, 100 % yield, $[M+1]^+$, calculated 604.3, found 604.4), which was used for next step without further purification.

[00199] *Compounds **38a** and **38b**.* Compound **37** (0.8 g, 1.3 mmol) was dissolved in 5 mL methanol at -78 °C. To this solution was added NaOMe (331 uL, 1.33 mmol, 4M in MeOH). The mixture was allowed to warm up to RT over1 hr. The mixture was taken up in EtOAc, washed with 10% aq. citric acid, saturated aq. $NaHCO_3$ solution, and brine. The separated organic phase was dried, filtered and evaporated to give a mixture of ethyl and methyl esters (compounds **38a** and **38b**, respectively). The mixture of esters was not separated during the next few steps, until both were hydrolyzed to the carboxylic acid at a later step.

[00200] *Compounds **40a** and **40b**.* The mixture of compounds **38a** and **38b** from the above reaction was dissolved in 20 mL DCM. To this solution was added 4-nitrophenyl carbonochloridate **39** (524 mg, 2.6 mmol) and pyridine (210 µl, 2.6 mmol) at 5 °C. The temperature was allowed to rise to room temperature after 1 h and methylamine (1.950 mL, 3.9 mmol, 2M in THF) was added. After 10 min the solvent was evaporated and the residue was passed through a chromatography column to give a mixture of compounds **40a** and **40b** (ethyl and methyl esters, respectively; 420 mg, **40a/40b** ratio 3:1 from HPLC, about 53% yield for two steps, $[M+1]^+$: calculated 603.3, found 603.4 for **40a**; calculated 589.3, found 589.4 for **40b**).

[00201] *Compounds **41a** and **41b**.* The mixture of compounds **40a** and **49b** (420 mg, about 0.68 mmol) was dissolved in 3 mL DCM, to which was added HCl (4.8 mmol, 1.2 mL, 4N in dioxane). After 1 h at 5 °C the solvent was evaporated and the mixture of compounds **41a** (ethyl ester) and **41b** (methyl ester), ratio about 3:1, was used for next step without further purification.

[00202] *Compounds **43a** and **43b**.* A mixture of compounds **41a** and **41b** (400 mg, ≈0.72 mmol), compound **42** (commercially available from Anichem, 170 mg, 0.721 mmol) and acetic acid (0.041 mL, 0.721 mmol) were mixed in DCM at 5 °C. Sodium triacetoxyborohydride (306 mg, 1.44 mmol) was added. The mixture was taken up in EtOAc after 30 min. After washed with 7% aq. K_2CO_3 and brine, the organic phase was dried, filtered and evaporated down to give a residue. After column chromatography purification

(McOH: DCM, 0-7% gradient), compounds **43a** (ethyl ester) and **43b** (methyl ester) were obtained (310 mg, approximately 0.42 mmol, approximately 58.3 % yield, **43a**:**43b** ratio about 3:1, $[M+1]^+$, calculated 738.4, found 738 for **43a**; calculated 724.4, found 724 for **43b**).

[00203] *Compounds **45a** and **45b**.* A mixture of compounds **43a** and **43b** (310 mg, approximately 0.42 mmol) was dissolved in 5 mL DCM at RT. To this solution were added 2,6-di-*tert*-butylpyridine (161 mg, 0.840 mmol) and a 2 mL DCM solution of compound **44** (prepared per Peltier *et al.* 2006, 73.8 mg, 0.420 mmol). After half an hour Et_3N (58.6 µl, 0.420 mmol) was added. The mixture was then taken up in EtOAc, which was washed with 10% aq. citric acid, saturated aq. $NaHCO_3$ solution and brine. The organic phase was dried, filtered and evaporated down to a residue. After column chromatography purification, compounds **45a** and **45b** were obtained (294 mg, approximately 0.334 mmol, 80 % yield, **45a**:**45b** ratio 3:1, $[M+1]^+$, calculated 877.5, found 877 for **45a;** calculated 863.5 found 863 for **45b**) as a sticky oil.

[00204] *Compounds **46a** and **46b**.* A mixture of compounds **45a** and **45b** (100 mg, approximately 0.114 mmol) was added to a suspension of Pd/C (65 mg, 10%) in 20 mL MeOH. HCl (28.5 µL, 0.114 mmol, 4M in dioxane) was added. The flask was evacuated and refilled with H_2, this process being repeated three times. After 2 h the suspension was filtered and the solvent was evaporated to give a residue. A suspension of compound **5a** (19.59 mg, 0.137 mmol) in 500 uL DMF, HATU (43.4 mg, 0.114 mmol) and DIPEA (49.8 µl, 0.285 mmol) were added at 5 °C. After the suspension became homogeneous the above residue was added as a DMF (1 mL) solution. More DIPEA was added to adjust the pH to about 12. After 10 min the mixture was taken up in EtOAc, which was washed with 10% aq. citric acid, saturated aq. $NaHCO_3$ and brine. The separated organic phase was dried, filtered and evaporated. The resulting residue was passed through a chromatographic column to give a mixture of compounds **46a** and **46b** (ethyl and methyl esters, respectively, 80 mg, about 0.082 mmol, 71.9 % yield, **46a**:**46b** ratio 3:1, $[M+1]^+$, calculated 976.5, found 976.5 for **46a**; calculated 962.5, found 962.5 for **46b**).

[00205] *Compounds **47a** and **47b**.* HCl (256 µl, 1.024 mmol, 4M in dioxane) was added to a 2 mL MeOH solution of compounds **46a** and **46b** (200 mg, 0.205 mmol) at 5 °C. After 1 h the solution was evaporated down and dried on high vacuum overnight to give a sticky oil. This sticky oil, Fmoc-protected citrulline **18a** (81 mg, 0.205 mmol), and DIPEA (179 µl, 1.024 mmol) were dissolved in 2 mL DMF at RT. Propylphosphonic acid anhydride (T3P,

178 μL, 0.410 mmol, 2.3 M in EtOAc) was added. After 1h the reaction mixture was taken up in EtOAc, which was washed with saturated aq. $NaHCO_3$ solution and brine. After separation, drying and evaporation, the resulting residue was passed through a chromatography column (MeOH: DCM, 0-10% gradient) to give a mixture of compounds **47a** and **47b** (ethyl and methyl esters, respectively, 150 mg, approximately 0.119 mmol, about 58.3 % yield, **47a**:**47b** ratio 3:1, $[M+1]^+$, calculated 1255.6, found 1255.6 for **47a**; calculated 1241.6, found 1241.6 for **47b**).

[00206] *Compound 49*. A mixture of compounds **47a** and **47b** (200 mg, about 0.165 mmol) was dissolved in 5 mL of DMF (with 5% piperidine) at room temperature. The solution was evaporated to dryness after 30 min. The resulting residue was mixed with Boc-protected valine N-hydroxysuccinimide ester **48** (61.9 mg, 0.198 mmol), 5 mL DMF and DIPEA (87 μL, 0.496 mmol). After allowing reaction to proceed overnight, the reaction mixture was evaporated to dryness. The resulting mixture was dissolved in a 5 mL mixture of MeOH, THF and water (1:1:1). NaOH was added and the pH of the final solution was 14. After allowing reaction to proceed overnight at RT, the mixture was acidified with HCl to a pH of 3 and evaporated under high vacuum. The obtained solid was treated with TFA and the mixture was evaporated after 10 min, affording compound **49** (80 mg, 0.072 mmol, 43.8 % yield, $[M+1]^+$, calculated 1104.6, found 1104.6) after preparative HPLC purification.

[00207] *Compound (III-4)*. Compound **49** (80 mg, 0.072 mmol), commercially available compound **21a** (Aldrich, 26.6 mg, 0.087 mmol) and DIPEA (38.0 μl, 0.217 mmol) were dissolved in 2 mL of DMF. After reaction was allowed to proceed overnight, the mixture was evaporated down and the residue was purified by preparative HPLC to give a major isomer (15 mg, 16% yield, $1/2[M+2]^{2-}$, calculated 649.3, found 649.5) and a minor isomer (3.7 mg, 4% yield, $1/2[M+2]^{2+}$, calculated 649.3, found 649.5)). The major isomer was tentatively assigned as **(III-4)**, having the natural tubulysin stereochemistry at the alpha-methyl of the Tup subunit and the minor isomer was assigned as compound **(III-5)**, having the inverted stereochemistry there.

Example 5 – Compounds (I-5), (I-6), and (I-7)

[00208] A small portion of the sticky oil described above from the treatment of cmopounds **46a** and **46b** with HClwas not coupled to compound **18a** but was instead dissolved in a mixture of THF, MeOH and water (1:1:1). The pH of the reaction mixture was adjusted to 14. After allowing the reaction to proceed overnight, half of the reaction mixture

was evaporated and purified by preparative chromatography to give compounds **(I-5)** (1 m;, M+1, 876.6), **(I-6)** (1 mg; M+1, 862.5) and **(I-7)** (1 mg; M+1, 848.5).

Example 6 – Compound (I-1)

[00209] A scheme for the synthesis of compound **(I-1)** is shown in **Fig. 7**.

[00210] *Compound **51***. HCl (6 N, 0.2 mL) was added to a solution of compound **50** (Cheng *et al.* 2011; 50 mg, 0.198 mmol) and 2,2-dimethoxypropane (0.244 mL, 1.982 mmol) in MeOH (1 mL). After the reaction mixture was stirred at RT overnight, the solvent was evaporated to afford 52.8 mg of compound **51**. MS: (+) m/z 267.2 (M+1).

[00211] *Compound **52***. A mixture of compound **51** (52.8 mg, 0.198 mmol) and palladium on carbon (10 wt%, 8 mg) in MeOH (1 mL) was stirred under an H_2 atmosphere overnight. The catalyst was then filtered off and the solvent evaporated to afford 46.9 mg of compound **52**. MS: (+) m/z 237.3 (M+1).

[00212] *Compound **(I-1)***. A mixture of pentafluorophenol (2.493 mg, 0.014 mmol), 1,3-dicyclohexylcarbodiimide (2.049 mg, 9.93 μmol), and compound **9** (5 mg, 9.03 μmol) in DCM (0.5 mL) was stirred at RT overnight. The solvent was then evaporated.

[00213] To a solution of the resulting residue (6.50 mg, 9.03 μmol) and compound **52** (4.27 mg, 18.06 μmol) in DMF (0.2 mL) was added DIEA (1 drop). After the reaction mixture was stirred at RT for 10 min, the reaction was quenched by addition of a 1:1 mixture of acetonitrile and water containing 0.1% TFA (4 mL). Preparative HPLC purification afforded 2.5 mg of compound **(I-1)** as a white solid. MS: (+) m/z 772.5 (M+1).

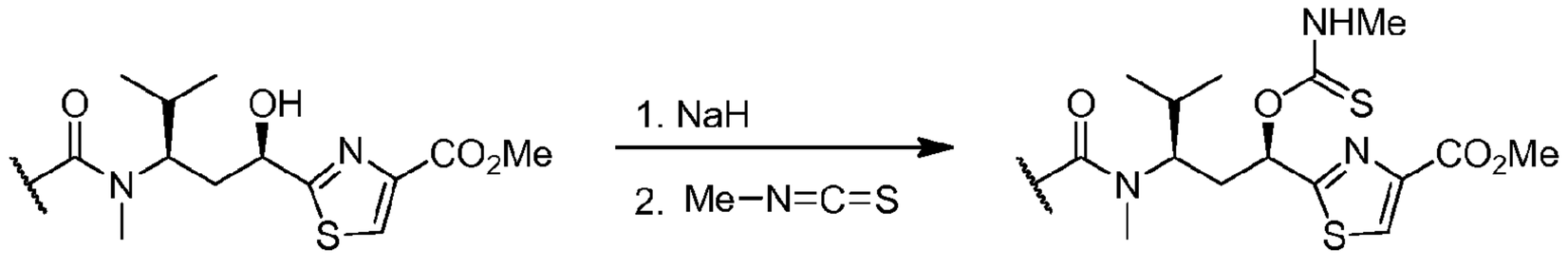
Example 6 – Compound (I-4)

[00214] **Fig. 8** shows a scheme for the synthesis of compound **(I-4)**.

[00215] DIEA was added to a solution of compound **9** (10.69 mg, 0.019 mmol) and HATU (7.34 mg, 0.019 mmol) in DMF (0.3 mL), adjusting pH to 8-9. After the reaction mixture was stirred at rt for 10 min, compound **53** (4 mg, 0.019 mmol; prepared according to Sani *et al.* 2007) in DMF (0.5 mL) and DIEA were added, adjusting the pH to 8-9. After the reaction mixture was stirred at RT for 20 min, the reaction was quenched by addition of a 1:1 mixture of acetonitrile and water containing 0.1% TFA (4 mL). Preparative HPLC purification afforded 12.5 mg of compound **(I-4)** as a white solid. MS: (+) m/z 743.4 (M+1).

Example 7 – Thiocarbamates

[00216] Compounds according to formula (I) in which W is S (that is, thiocarbamates) can be made by treating a suitable precursor such as compound **6** (**Fig. 1**) with sodium hydride and then a thioisocyanate, as illustrated below:



6 (partial structure)

Example 8 – Compound (III-6)

[00217] A scheme for the synthesis of compound **56** is shown in **Figs. 9a** and **9b**.

[00218] *Compound 56.* 1,3-Dicyclohexylcarbodiimide (DCC, 0.160 g, 0.778 mmol) was added to a solution of tert-butyl 1-amino-3,6,9,12-tetraoxapentadecan-15-oate **54** (Quanta Biosciences, 0.25 g, 0.778 mmol) and 2-(((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-oxy)acetic acid **55** (Chem-Impex, 0.244 g, 0.778 mmol) in DCM (5 mL) at RT. After the reaction mixture was stirred at RT overnight, 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 0-10% methanol in DCM to afford 0.30 g of compound **56** as a colorless oil. MS: (+) m/z 617.4 (M+1).

[00219] *Compound 57.* A solution of compound **56** (0.303 g, 0.491 mmol) in TFA (2 mL, 0.662 mmol) was stirred at RT for 2 h. After the solution was concentrated, the residue was washed with hexanes to afford 0.28 g of compound **57**. MS: (+) m/z 561.3 (M+1).

[00220] *Compound 58.* DCC (0.199 g, 0.963 mmol) was added to a solution of compound **57** (0.27 g, 0.482 mmol) and 1-hydroxypyrrolidine-2,5-dione (also known as N-hydroxysuccinimide or NHS, 0.111 g, 0.963 mmol) in DCM (5 mL) at RT. After the reaction mixture was stirred at RT overnight, the solid was filtered off. The filtrate was then concentrated. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-100% ethyl acetate in hexanes to afford 0.12 g of compound **58** as a colorless oil. MS: (+) m/z 658.3 (M+1).

[00221] *Compound 59.* DIEA (2 drops) was added to a solution of compound **58** (0.12 g, 0.182 mmol) and compound **21** (0.106 g, 0.182 mmol) in DMF (2 mL) at RT. After the reaction mixture was stirred at RT for 1h, the reaction was quenched by addition of a mixture

of acetonitrile and water containing 0.1% TFA. The crude product was purified by prep HPLC to afford 0.12 g of compound **59** as a white solid. MS: (+) m/z 1121.6 (M+1).

[00222] *Compound **60***. TFA (0.5 mL) was added to a solution of compound **59** (20 mg, 0.018 mmol) in DCM (1 mL) at RT. After the reaction mixture was stirred at RT for 20 min, the solution was concentrated to afford 18.2 mg of compound **60**. MS: (+) m/z 1021.6 (M+1).

[00223] *Compound **61***. DIEA was added to a solution of compound **9** (9.87 mg, 0.018 mmol) and HATU (6.78 mg, 0.018 mmol) in DMF (0.4 mL). The pH of the reaction mixture was adjusted to 8-9. After the reaction mixture was stirred at RT for 10 min, compound **60** (18.2 mg, 0.018 mmol) in DMF (1 mL) and DIEA were added. The pH of the reaction mixture was adjusted to 8-9. After the reaction mixture was stirred at RT for 10 min, the reaction was quenched by addition of 10 mL 1:1 (v/v) mixture of water containing 0.1% TFA and acetonitrile. The crude product was purified by prep HPLC to afford 25 mg of compound **61** as a white solid. MS: (+) m/z 779.0 (M/2+1).

[00224] *Compound **(III-6)***. One drop of piperidine was added to a solution of compound **61** (25 mg, 0.016 mmol) in DMF (1 mL) at RT. After the reaction mixture was stirred at RT for 1h, the reaction was quenched by addition of a mixture of acetonitrile and water containing 0.1% TFA. The crude product was purified by prep HPLC to afford 20 mg of compound **(III-6)** as a white solid. MS: (+) m/z 668.0 (M/2+1). Compound (III-6) has a hydroxylamine group, which can be use for conjugation via oxime formation, for instance with the ketone group of a p-acetylphenyalanine residue that has been introduced into a protein, as discussed above.

[00225] A conjugate of compound **(III-6)** and an anti-mesothelin antibody modified to contain a p-acetylphenylalanine residue exhibited an EC_{50} of 0.14 against N87 gastric cancer cells, using a ^{3}H thymidine incorporation assay.

[00226] In one embodiment, this invention provides a conjugate of compound **(III-6)** and an anti-mesothelin antibody modified to contain a ketone group. Preferably, the modification is by the incorporation of a p-acetylphenylalanine residue into the antibody polypeptide chain. Also preferably, the antibody so modified is 6A4.

Example 9 – Intermediate Compound 69

[00227] **Fig. 10** shows a scheme for the synthesis of compound **69**, which can be used as an intermediate for the synthesis of compounds of this invention.

[00228] *Compound 63*. Compound **62** (Chem-Impex, 5 g, 23.8 mmol) was added into a solution of $SOCl_2$ (3.47 mL, 47.6 mmol) in 20 mL MeOH at 5 °C. After allowing reaction to procede overnight, the reaction mixture was heated for half an hour at 45 °C. The volatile materials were evaporated and the residue was dissolved in 20 mL DCM. Boc_2O (7.8 g, 35.7 mmol) was added. Et_3N was used to adjust the pH of the solution to 9 (tested with moistened pH paper). After a few hours the reaction mixture was taken up in EtOAc. The EtOAc solution was washed with 10% citric acid solution, sat. aq. $NaHCO_3$ solution and brine. The organic phase was dried, separated and evaporated down. The final residue from the evaporation of the dried organic phase was passed through a column to give compund **63** (5 g, 65% yield, $[M+1\text{-Boc}]^-$, calculated 225.1, found 225.2).

[00229] *Compound 65*. To a 20 mL DCM solution of comound **63** (2 g, 6.2 mmol) was added DIBAL-H (12.4 mL, 12.4 mmol, 1M in DCM) at -78 °C. After half an hour, MeOH was used to quench the reaction. HCl was used to adjust the pH of the solution to around 2. The mixture was taken up in EtOAc, which was washed with 10% citric acid, brine, dried, separated and evaporated. The residue was dissolved in 30 mL of DCM. Compound **64** (2.4g, 6.2 mmol, US 4,894,386) was added at 0 °C. The mixture was evaporated after 1h at RT, the residue was passed through a column to give compound **65** (2 g, 79% yield, $[M+1\text{-Boc}]^+$, calculated 307.2, found 307.1).

[00230] *Compound 66*. Compound **65** (600 mg, 1.48 mmol) was dissolved in 75 mL EtOAc at RT. The solution was transferred a flask filled with N_2 and Pd/C (600 mg, 10%). The flask was evacuated and refilled with H_2; the cycle was repeated three times. After 1 h the solution was filtered and evaporated to give compound **66** (560 mg, 100% yield, $[M+1]^+$, calculated 379.3, found 379.3) as a mixture of epimers. This mixture was not separated until later.

[00231] *Compound 67*. Compound **66** (1.30 g, 3.43 mmol), Fmoc-protected citrulline **18a** (1.6 g, 4.12 mmol) and 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ, 1.06 g, 4.3 mmol) were dissolved in a mixture of DCM and MeOH (22 mL, 10:1). After allowing reaction to proceed overnight, the mixture was evaporated and the residue was passed through a column (MeOH:DCM, 0-5% gradient) to give compound **67** ($[M+1]^+$, 7calculated 758.4, found 758.4) as a mixture of epimers (≈4:1 from HPLC analysis). It was used for next step without further purification.

[00232] *Compound 68a.* The mixture of compound **67** from the above reaction was dissolved in 10 mL of DMF (with 5% piperidine) at RT. After 30 min, the mixture was evaporated and dried with a high vacuum pump overnight. The residue was mixed with compound **48** (1.5 g, 3.45 mmol) in 5 mL DMF. Et_3N was used to adjust the pH of the solution to 12. After 1h the reaction mixture was taken up in EtOAc, which was washed with 10% citric acid, sat. aq. $NaHCO_3$ solution and brine. The organic phase was dried, filtered and evaporated to dryness. The residue was deprotected with 5% piperidine in DMF in the same way as the deprotection of compound **67**. The amine obtained in this step and compound **21a** (1 g, 3.27 mmol) were mixed in 10 mL of DMF. Et_3N was used to adjust the pH of the solution to 12 at RT. After allowing the reaction to proceed overnight, the mixture was taken up in EtOAc, which was washed with 10% citric acid, sat. aq. $NaHCO_3$ and brine. The organic phase was dried, filtered and evaporated to give a residue. The residue was passed through a regular silica column to give a mixture of compounds **68a** and **68b** (1g, 35% yield from compound **66**, $[M+1]^+$, calculated 828.5, found 828.5). After separation on a preparative HPLC column, 600 mg of the major epimer was obtained (structure tentatively assigned as compound **68a**, with compound **68b** assigned as the minor one).

[00233] *Compound 69.* Compound **68a** (600 mg, 0.72 mmol) was dissolved in a 5 mL mixture of DCM and TFA (1:1). After 2 h, the reaction mixture was evaporated to give compound **69** (quantitative yield, [M+1], calculated 672.4, found 672.4).

Example 10 – Intermediate Compound 75

[00234] **Fig. 11** shows a scheme for the synthesis of intermediate compound **75**, which can be used for the synthesis of compounds of this invention.

[00235] *Compound 71.* Compound **70** (Cheng *et al.* 2011, 25 mg, 0.044 mmol), N,N'-diisopropylcarbodiimide (DIC, 0.014 mL, 0.088 mmol), 4-(dimethylamino)pyrdine (DMAP, 10.78 mg, 0.088 mmol) and MeOH (0.036 mL, 0.882 mmol) were mixed at RT. After an hour, the reaction mixture was evaporated and passed through a column to give compound **71** (10 mg, 39% yield, M+1, 581.4).

[00236] *Compound 72.* Compound **71** (122 mg, 0.210 mmol, from another synthetic batch) was dissolved in MeOH (2 mL) at 5 °C. NaOMe (0.441 mL, 0.221 mmol) was added. After 0.5 h the mixture was neutralized with HCl (4M in dioxane) and evaporated to give compound **72** (m+1, 539.4), which was used for next step without further purification.

[00237] *Compound 73.* Compound **72** (60 mg, 0.111 mmol) was dissolved in DCM (1.5 mL) at 5 °C. Pyridine (0.045 mL, 0.557 mmol). 4-Nitrophenylcarbonochloridate **72a** (67.3 mg, 0.334 mmol, Aldrich) in 0.5 mL DCM was added slowly. After allowing reaction to proceed overnight, the mixture was evaporated and purified by column chromatography to give compound **73** (42 mg, 0.060 mmol, 53.6 % yield) (m+1, 704.4).

[00238] *Compound 74.* Compound **73** (42 mg, 0.060 mmol) was dissolved in DCM (1 mL) at 5 °C. Methylamine (1.853 mg, 0.060 mmol) was added. After 0.5 h the mixture was evaporated and the residue was passed through a chromatographic column (MeOH:DCM, 0-15% gradient, product eluting out at 7-10%) to give compund **74** (35 mg, 0.059 mmol, 98 % yield) (m+1, 596.4).

[00239] *Compound 75.* Compound **74** (93 mg, 0.156 mmol) was dissolved in THF (1 mL) at 5 °C. LiOH (7.47 mg, 0.94 mmol) in 0.34 mL water was added. After the reaction finished, the reaction mixture was neutralized with HCl (4M in dioxane) and dried on high vacuum to give compound **75** (m+1, 582.3), which was used for next step without further purification.

Example 11 – Compounds (III-7) and (III-8)

[00240] **Fig. 12** shows schemes for the synthesis of of compounds (III-7) and (III-8).

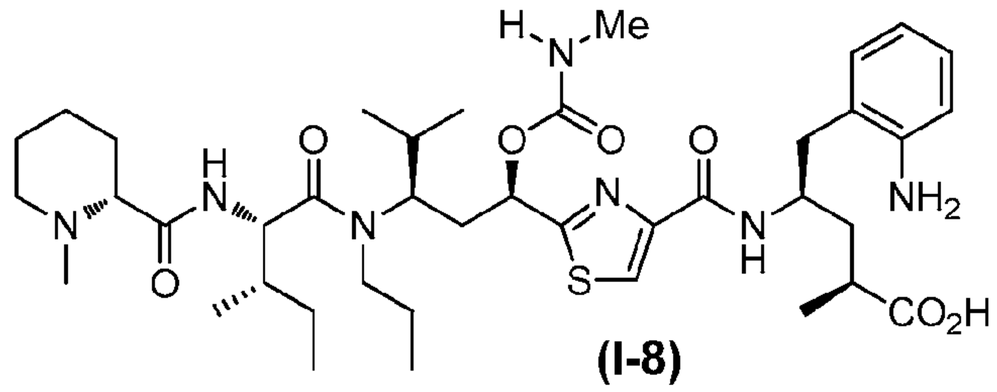
[00241] *Compound **(III-7)**.* Compound **75** (11 mg, 0.019 mmol) was activated in DMF (0.5 mL) with HATU (6.83 mg, 0.018 mmol) and DIEA (14.86 µl, 0.085 mmol). Compound **69** (15.24 mg, 0.023 mmol) was then added. After 10 min the reaction mixture was taken up in DMSO and purified by preparative chromatography to give compund **(III-7)** (12 mg, 9.71 µmol, 51.4 % yield) (m+1, 1235.7).

[00242] *Compound **(III-8)**.* Compound **75** (10 mg, 0.017 mmol) was activated in DMF (0.5 mL) with HATU (6.21 mg, 0.016 mmol) and DIEA (0.014 mL, 0.077 mmol). Compound **23** (13.86 mg, 0.021 mmol) was then added. After 10 min the reaction mixture was taken up in DMSO and purified by preparative chromatography to give compound **(III-8)** (13 mg, 10.52 µmol, 61.2 % yield) (m+1, 1235.7).

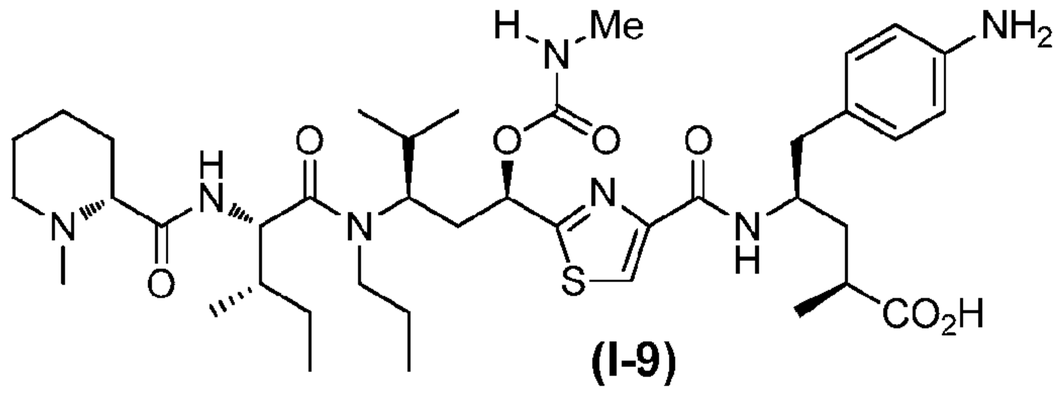
Example 12 – Compounds (I-8) and (I-9)

[00243] In principle, compounds **(I-8)** and **(I-9)** can be obtained by treatment of compounds **(III-7)** and **(III-8)**, respectively, with the protease cathepsin B, for which the dipeptide Val-Cit is a substrate motif. Compound **(III-7)**, with its *ortho*-amino group, may be subject to more steric hindrance against cleavage.

Compound (III-7) → Cathepsin B → **(I-8)**



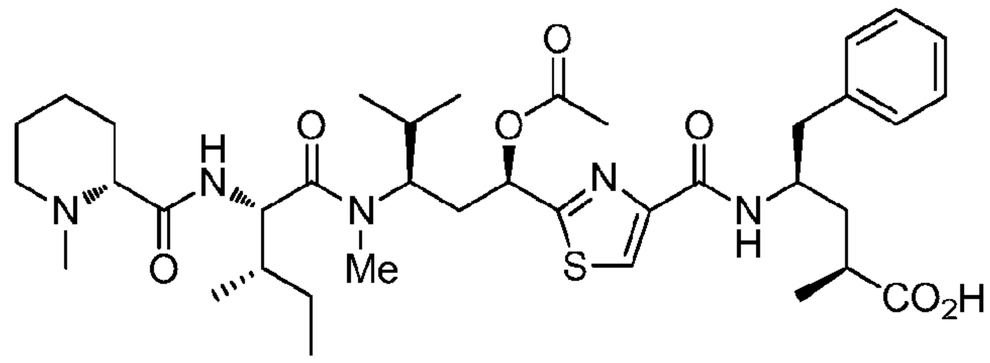
Compound (III-8) → Cathepsin B → **(I-9)**



Example 13 – Biological Activity of Compounds

[00244] **Figs. 13a** and **13b** show the biological activity of compounds **(I-4)** and **(I-2)** of this invention against H226 lung cancer and 786-O renal cancer cells, respectively, using an ATP luminescence assay. As controls, doxorubicin and the tubulysin analog Compound A, which contains an acetate group instead of a carbamate group at the Tuv subunit, were used. Compound A can be prepared according to the teachings of Cheng *et al.* 2011.

Compound A



[00245] Against H226 cells, the EC_{50} values were: doxorubicin, 115.4 nM; Compound A, 2.4 nM; and compound (I-4), 12.1 nM. Against 786-O cells, the EC_{50} values were: doxorubicin, 68.9 nM; Compound A, 1.2 nM, and compound (I-4), 7.1 nM.

[00246] **Figs. 13c** and **13d** present the similar type of data for compounds **(I-5)**, **(I-6)**, and **(I-7)**. The comparison compounds were doxorubicin and tubulysin D. The EC_{50} values against H226 cells were: doxorubicin, 115.4 nM; tubulysin D, 0.05 nM; compound (I-5), 19.5 nM; compound (I-6), 9.9 nM; and compound (I-7), 15.4 nM. Against 786-O cells, the EC_{50} values were: doxorubicin, 68.9 nM; tubulysin D, 0.02 nM; compound (I-5), 22.9 nM; compound (I-6), 22.8 nM; and compound (I-7), 12.5 nM.

[00247] Tumor cell lines were obtained from the American Type Culture Collection (ATCC), P.O. Box 1549, Manassas, VA 20108, USA, and cultured according to ATCC instructions. Cells were seeded at 1.0 x 10^3 cells/well in 96-well plates for 3 h for the ATP assays. 1:3 serial dilutions of the compounds were added to the wells. Plates were allowed to incubate for 24 to 72 h. 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 – In Vitro Activity of a Conjugate

[00248] **Fig. 14** shows the in vitro activity of conjugate (II-1), against N87 gastric cancer cells (American Type Culture Collection (ATCC), P.O. Box 1549, Manassas, VA 20108, USA).

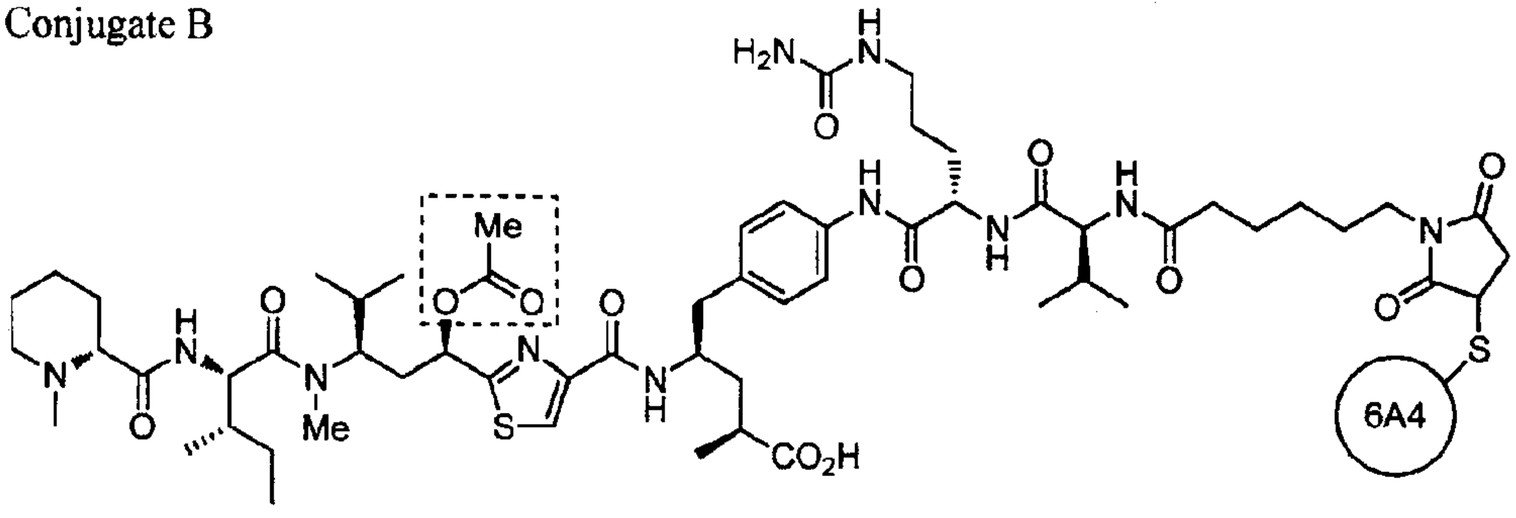
[00249] Cells were seeded at 1.0 x 10^4 cells/well in 96-well plates for 3 h for ^{3}H thymidine assays, respectively. Serial dilutions (1:3) of conjugate (II-1) were added to the wells. Plates were allowed to incubate for 120 h. The plates were pulsed with 1.0 μCi of ^{3}H-thymidine per well for the last 24 h of the total incubation period, harvested, and read on a Top Count Scintillation Counter (Packard Instruments, Meriden, CT). The EC_{50} value – the concentration at which an agent inhibits or reduces cell proliferation by 50% of the maximum inhibition – was determined using PRISM™ software, version 4.0 (GraphPad Software, La Jolla, CA, USA) to be 0.2 nM

[00250] A comparison of the EC_{50} values from **Figs. 13a-13d** and **Fig. 14** illustrates two points. First is the potency enhancement associated with the targeted delivery of a cytotoxin via a conjugate and then the active internalization mechanism triggered by binding of the antibody component of the conjugate to its antigen (Schrama *et al.* 2006). Second, the unconjugated toxins are relatively polar compounds and, in the absence of an active internalization mechanism, have difficulty diffusing across a cell membrane, thus resulting in higher (lower potency) measured EC_{50} values.

Example 15 – In Vivo Activity of a Conjugate

[00251] In this example, the in vivo activity of conjugate (II-1) was compared against that of Conjugate B (Cheng *et al.* 2011), which is structurally identical to conjugate (II-1), except that it has an acetate in the Tuv subunit instead of a carbamate:

Conjugate B



[00252] Five million OVCAR3 ovarian cancer cells, resuspended in 0.1 mL phosphate buffered saline ("PBS") plus 0.1 mL matrigel, were implanted subcutaneously at the flank region of SCID mice. Tumor measurements started 28 days later, and mice were randomized into groups of 7 mice each with average tumor sizes of 60 mm^3 estimated by LWH/2 of tumors. At 29 days post tumor implantation, mice were dosed intraperitoneally singly with testing compounds. **Fig. 15** shows that, against OVCAR3 xenografts, conjugate (II-1) suppressed tumor growth more effectively than conjugate B. The differential is especially notable after 20 days.

[00253] **Figs. 16a, 16b, 17a, 17b, 18a, 18b, 19a, 19b,** and **20** present additional in vivo efficacy data for conjugates of this invention, generated per the protocol described above, *mutatis mutandis*.

[00254] **Fig. 16a** shows the data for OVCAR3 (ovarian cancer) tumor volume versus time for a series of conjugates of compound (III-1) with anti-CD70 antibody 1F4 or anti-mesothelin antibody 6A4. The legend provides, in order, the DAR (*e.g.*, "2.7") and dosage in μmol/kg (*e.g.*, "0.1"). In each instance the mode of administration was intraperitoneal, in a single dose (SD), except for last data set (♦), for which the conjugate was administered Q7Dx3. **Fig. 16b** shows the body weight change for the same experiment.

[00255] The preparation and characterization of human monoclonal antibody 6A4 is described in Terrett *et al.* 2012.

The V_H CDR1, CDR2, and CDR3 and V_K CDR1, CDR2, and CDR3 sequences for antibody 6A4 are given in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6, respectively. The variable region V_H and V_K sequences of antibody 6A4 are provided in SEQ ID NO:7 and SEQ ID NO:8, respectively.

[00256] The preparation and characterization of human monoclonal antibody 1F4 is cescribed in Coccia *et al.* 2010.

The V_H CDR1, CDR2, and CDR3 and V_K CDR1, CDR2, and CDR3 sequences for antibody 1F4 are given in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, and SEQ ID NO:14, respectively. The variable region V_H and V_K sequences of antiobdy 1F4 are provided in SEQ ID NO:15 and SEQ ID NO:16, respectively.

[00257] **Figs. 17a** and **17b** present the results for a similar experiment, but with conjugates of compound (III-8) and anti-mesothelin antibody 6A4. In each instance, a single dose was administered intraperitoneally.

[00258] The efficacy of a conjugate of compound (III-1) and anti-mesothelin antibody 6A4 against H226 (lung cancer) tumors is presented in **Figs. 18a** and **18b**, with DAR and dosage information found again in the legends. In each instance the conjugate was administered intraperitoneally in a single dose.

[00259] **Figs. 19a** and **19b** present H226 data for a conjugate of compound (III-8) and anti-mesothelin antibody 6A4. In the first data set (●) the administration was a single dose, while for the last two data sets (▼and ♦) the administration regimen was Q7Dx3.

[00260] **Fig. 20** shows the efficacy data for conjugates of compound (III-1) with anti-mesothelin antibody 6A4 or anti-CD70 antibody 1F4 against N87 gastric cancer tumors.

[00261] Antibodies, especially when used in conjugates, may have either a natural constant region or an engineered (modified) one, designed to reduce or eliminate effector functions such as ADCC. Examples of such modified antibody constant regions are provided by the polypeptides of SEQ ID NO:25 and SEQ ID NO:26. SEQ ID NO:25 is a constant region of the IgG4 isotype modified with certain amino acid substitutions. SEQ ID NO:26 is a hybrid IgG1/IgG4 constant region. In both SEQ ID NO:25 and SEQ ID NO:26 the presence of the C-terminal lysine is optional.

Example 16 – Stability Studies

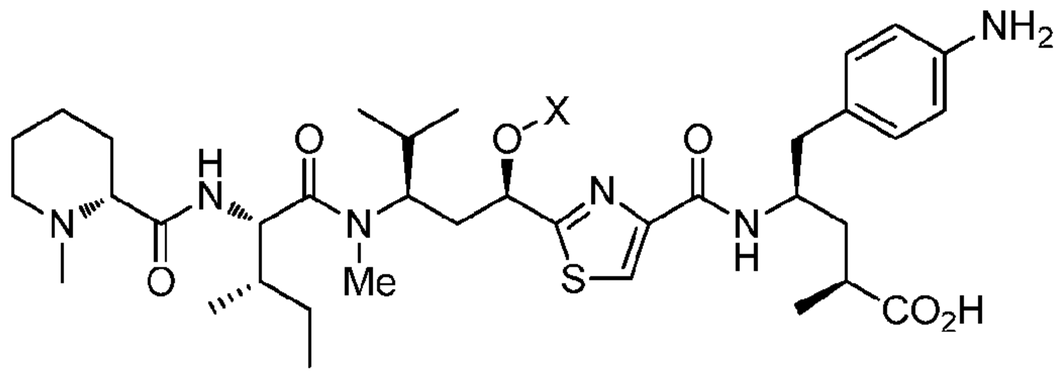
[00262] In this example, the mouse serum stability of Conjugate (II-1) and Conjugate B, with their respective carbamate and acetate groups, were compared.

[00263] The conjugate was injected into mice at a dose of 0.1 μmol/kg. In the case of conjugate (II-1) the administration concentration was 3.4 mg/mL and the conjugate had a substitution ratio (SR) of 4.2. In the case of conjugate B the administration concentration was 1.2 mg/mL and the SR was 2.3. Approximately 100 uL of serum from each of 3 animals at each time point was taken for analysis.

[00264] Serum samples from the different animals were pooled, giving 200-300 uL at each time point. The pooled volume was centrifuged to remove solids and the supernatant was used for analysis. The conjugate was isolated from serum by immuno-afinity capture using an anti-idiotypic monoclonal antibody coupled to SEPHAROSE™ beads. After capture, the conjugate was eluted by exposure to low pH followed by neutralization with Tris base. Cytotoxin present on the conjugate was released by addition of activated cathepsin B to cleave the Cit-Val peptide linker. Cathepsin B digestion was done at 37 °C for 3 h, followed by addition of 1 volume of cold methanol. The solvent-extracted cytotoxin was analyzed by LC-MS using an ESI-TOF MS in-line to a UPLC with reverse phase chromatography (Acquity HSS T3 2.1 X 50mm).

[00265] For conjugate (II-1), the presence of the hydroxyl compound from hydrolysis of the carbamate group was not detected at any time, through time points up to 240 h. Only the carbamate compound was detected.

[00266] Conversely, for conjugate B, the hydrolysis product was detectable after 6 hours and about 50 per cent hydrolysis had occurred by 72 h. Table 2 below shows the relative amounts of the acetate and hydroxyl compounds, based on mass spectrum intensities for the respective doubly charged ions ($M[H^{2+}]$ 372.2 Da and 351.2 Da):



Carbamate: X = C(=O)NHMe
Acetate: X = C(=O)Me
Hydroxyl: X = H

Table 2			
Time (h)	Relative Peak Intensity		Hydroxyl Ion (% of total)
	Acetate Ion	Hydroxyl Ion	
0.25	360,587	—	0
2	649,982	—	0
6	165,680	11,779	7
24	77,110	14,619	16
48	26,760	16,312	38
72	18,243	17,124	48
168	7,978	18,330	70
240	4,268	15,654	79

[00267] These results show that while replacement of the acetate group in the Tuv subunit leads to a much more stable compound, which however still retains substantial biological activity.

Example 17 – Compounds 72a, 72b, and 72c

[00268] This example describes the preparation and properties of compounds of this invention having structural variations at the carbamate group. Reference is made to **Fig. 21** for the synthetic scheme.

[00269] *Compound **70a***. Ammonia in MeOH (2M, 0.089 mL, 0.178 mmol) was added to compound **7** (0.1 g, 0.148 mmol) in MeOH (2 mL). After the reaction mixture was stirred at RT for 20 min, LCMS showed the reaction went to completion. The solvent was evaporated. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-15% MeOH in DCM to afford 55 mg of compound **70a** as a white solid. MS: (+) m/z 554.3 (M+1).

[00270] *Compound **71a*** LiOH (4.41 mg, 0.184 mmol) in water (0.5 mL) was added to a solution of compound **70a** (51 mg, 0.092 mmol) in THF (1 mL) at RT. After the reaction mixture was stirred at RT for 2 h, LCMS showed the reaction had gone to completion. The solvent was evaporated. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-30% MeOH in DCM to afford 47 mg of compound **71a** as a white solid. MS: (+) m/z 540.3 (M+1).

[00271] *Compouind **72a***. DIEA (6.45 μL, 0.037 mmol) was added to a solution of compound **71a** (20 mg, 0.037 mmol) and HATU (14.09 mg, 0.037 mmol) in DMF (0.5 mL). The pH of the reaction mixture was adjusted to 8-9. After the reaction mixture was stirred at

RT for 20 min, compound **23** (24.9 mg, 0.037 mmol) in DMF (1 mL) and DIEA (6.45 μL, 0.037 mmol) were added. The pH of the reaction solution was adjusted to 8-9. After the reaction mixture was stirred at RT for another 20 min, LCMS showed the reaction went to completion. The reaction was quenched by the addition of 10 mL 1:1 (v/v) mixture of water containing 0.1% TFA and acetonitrile containing 0.1% TFA. The crude product was purified by preparative HPLC to afford 35.2 mg of compound **72a** as a white solid. MS: (+) m/z 1193.6 (M+1). Compound **72a** is also referred to herein above as compound (III-9).

[00272] A conjugate of compound **72a** and anti-CD70 antibody 1F4 had an EC_{50} of 0.19 nM against 786-O renal cancer cells. A conjugate of compound **72a** and anti-mesothelin antibody 6A4 had an EC_{50} of 0.16 nM against N87 gastric cancer cells. In both instances, an ^{3}H-thymidine incorporation assay was used.

[00273] *Compound 70b*. A solution of compound **7** (0.1 g, 0.148 mmol) and aniline (0.041 mL, 0.444 mmol) in DMF (1.8 mL) was heated at 50 °C overnight. The solvent was evaporated. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-15% MeOH in DCM to afford 58 mg of compound **5** as a white solid. MS: (+) m/z 630.4 (M+1).

[00274] *Compound 71b*. LiOH (4.03 mg, 0.168 mmol) in water (0.5 mL) was added to a solution of compound **70b** (53 mg, 0.084 mmol) in THF (1 mL) at RT. After the reaction mixture was stirred at RT for 2 h, LCMS showed the reaction went to completion. The solvent was evaporated. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-30% MeOH in DCM to afford 43 mg of compound **71b** as a white solid. MS: (+) m/z 616.3 (M+1).

[00275] *Compound 72b*. DIEA(5.66 μL, 0.032 mmol) was added to a solution of compound **71b** (20 mg, 0.032 mmol) and HATU (12.35 mg, 0.032 mmol) in DMF (0.5 mL). The pH of the reaction mixture was adjusted to 8-9. After the reaction mixture was stirred at RT for 20 min, compound **23** (21.82 mg, 0.032 mmol) in DMF (1 mL) and DIEA (5.66 μL, 0.032 mmol) were added. The pH of the reaction solution was adjusted to 8-9. After the reaction mixture was stirred at RT for another 20 min, LCMS showed the reaction went to completion. The reaction was quenched by the addition of 10 mL 1:1 (v/v) mixture of water containing 0.1% TFA and acetonitrile containing 0.1% TFA. The crude product was purified by preparative HPLC to afford 35 mg of compound **72b** as a white solid. MS: (+) m/z 1269.7 (M+1). Compound **72b** is also referred to hereinabove as compound (III-10).

[00276] A conjugate of compound **72b** and anti-CD70 antibody 1F4 had an EC_{50} of 0.58 nM against 786-O renal cancer cells. A conjugate of compound **72b** and anti-mesothelin antibody 6A4 had an EC_{50} of 0.47 against N87 gastric cancer cells. In both instances a ^{3}H thymidine incorporation assay was used.

[00277] *Compound 70c*. Dimethylamine in DMF (1 mL, 0.148 mmol) was added dropwise to a solution of compound **7** (0.1 g, 0.148 mmol) in DMF (1 mL) at RT, until the reaction went to completion. The solvent was evaporated. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-15% MeOH in DCM to afford 56 mg of compound **70c** as a white solid. MS: (+) m/z 582.3 (M+1).

[00278] *Compound 71c*. LiOH (8.23 mg, 0.344 mmol) in water (0.5 mL) was added to a solution of compound **70c** (0.1 g, 0.172 mmol) in THF (1 mL) at RT. After the reaction mixture was stirred at RT for 2 h, LCMS showed the reaction went to completion. The solvent was evaporated. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-30% MeOH in DCM to afford 50.8 mg of compound **71c** as a white solid. MS: (+) m/z 568.3 (M+1).

[00279] *Compound 72c*. DIEA (6.14 μL, 0.035 mmol) was added to a solution of compound 71c (20 mg, 0.035 mmol) and HATU (13.39 mg, 0.035 mmol) in DMF (0.5 mL). The pH of the reaction mixture was adjusted to 8-9. After the reaction mixture was stirred at RT for 20 min, compound **23** (23.67 mg, 0.035 mmol) in DMF (1 mL) and DIEA (6.14 μL, 0.035 mmol) were added. The pH of the reaction solution was adjusted to 8-9. After the reaction mixture was stirred at RT for another 20 min, LCMS showed the reaction went to completion. The reaction was quenched by the addition of 10 mL 1:1 (v/v) mixture of water containing 0.1% TFA and acetonitrile containing 0.1% TFA. The crude product was purified by preparative HPLC to afford 23.8 mg of compound **72c** as a white solid. MS: (+) m/z 1221.6 (M+1). Compound **72c** is also referred to hereinabove as compound (III-11).

[00280] A conjugate of compound **72c** and anti-CD70 antibody 1F4 had an EC_{50} of 0.32 nM against 786-O renal cancer cells. A conjugate of compound **72c** and anti-mesothelin antibody 6A4 had an EC_{50} of 0.34 nM against N87 gastric cancer cells. In both instances a ^{3}H thymidine incorporation assay was used.

Example 18 – Compounds 75a, 75b, and 75c

[00281] This example describes the preparation of the tubulysin analogs of the previous example, but without the linker moieties attached. Reference is made to **Fig. 22** for the synthetic scheme.

[00282] *Compound 73.* LiOH (0.071 g, 2.97 mmol) in water (5 mL) was added to a solution of compound **18** (0.25 g, 0.743 mmol) in THF (5 mL) at RT. After the reaction mixture was stirred at RT for 2 h, the solvent was evaporated. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-20% MeOH in DCM to afford 0.22 g of compound **73** as a white solid. MS: (+) m/z 223.3 (M+1-Boc).

[00283] *Compound 74.* A mixture of compound **73** (0.2 g, 0.620 mmol) and 4N HCl in 1,4-dioxane (4 mL, 0.620 mmol) was stirred at RT for 1 h. The solvent was evaporated. The white solid compound **74** was washed with hexanes twice. MS: (+) m/z 223.3 (M+1).

[00284] *Compound 75a.* DIEA (3.23 μL, 0.019 mmol) was added to a solution of compound **71a** (10 mg, 0.019 mmol) and HATU (7.05 mg, 0.019 mmol) in DMF (0.5 mL), adjusting pH to 8-9. After the reaction mixture was stirred at RT for 20 min, DIEA (3.23 μL, 0.019 mmol) and compound **74** (5.35 mg, 0.024 mmol) in DMF (0.5 mL) were added, adjusting the pH to 8-9. After the reaction mixture was stirred at RT for 1 h, LCMS showed the reaction went to completion. The reaction was quenched by addition of 10 mL of 1:1 (v/v) mixture of acetonitrile and water containing 0.1 % TFA. The crude product was purified by preparative HPLC to afford 6.0 mg of compound **75a** as a white solid. MS: (+) m/z 744.4 (M+1). Compound **75a** is also referred to hereinabove as compound (I-10).

[00285] Compound **75a** had an EC_{50} of 75.8 nM against N87 gastric cancer cells, using an ATP luminescence assay.

[00286] *Compound 75b.* DIEA (2.83 μL, 0.016 mmol) was added to a solution of compound **71b** (10 mg, 0.016 mmol) and HATU (6.17 mg, 0.016 mmol) in DMF (0.5 mL), adjusting pH to 8-9. After the reaction mixture was stirred at RT for 20 min, DIEA (2.83 μL, 0.016 mmol) and compound **74** (4.69 mg, 0.021 mmol) in DMF (0.5 mL) were added, adjusting the pH to 8-9. After the reaction mixture was stirred at RT for 1 h, LCMS showed the reaction went to completion. The reaction was quenched by addition of 10 mL of 1:1 (v/v) mixture of acetonitrile and water containing 0.1 % TFA. The crude product was purified by preparative HPLC to afford 7.6 mg of compound **75b** as a white solid. MS: (+) m/z 820.4 (M+1). Compound **75b** is also referred to hereinabove as compound (I-11).

[00287] Compound **75b** had an EC_{50} of 0.39 nM against N87 gastric cancer cells, using an ATP luminescence assay.

[00288] *Compound 75c.* DIEA (3.07 μL, 0.018 mmol) was added to a solution of compound **71c** (10 mg, 0.018 mmol) and HATU (6.70 mg, 0.018 mmol) in DMF (0.5 mL), adjusting the pH to 8-9. After the reaction mixture was stirred at RT for 20 min, DIEA (3.07 μL, 0.018 mmol) and compound **74** (5.09 mg, 0.023 mmol) in DMF (0.5 mL) were added, adjusting the pH to 8-9. After the reaction mixture was stirred at RT for 1 h, LCMS showed the reaction went to completion. The reaction was quenched by addition of 10 mL of 1:1 (v/v) mixture of acetonitrile and water containing 0.1 % TFA.The crude product was purified by preparative HPLC to afford 7.6 mg of compound **75c** as a white solid. MS: (+) m/z 772.5 (M+1). Compound **75c** is also referred to hereinabove as compound (I-12).

[00289] Compound **75c** had an EC_{50} of 5.4 nM against N87 gastric cancer cells, using an ATP luminescence assay.

Example 19 – Compound 80

[00290] This example describes the synthesis of a compound **80** suitable for conjugation via "click" chemistry, having a cyclooctyne group that can react with an azide group in a partner molecule. The corresponding synthetic scheme is shown in **Fig. 23**.

[00291] *Compound 77.* DCC (22.40 mg, 0.109 mmol) was added to a solution of DBCO-PEG4-Acid **76** (50 mg, 0.090 mmol, purchased from Click Chemistry Tools, Scottsdale, AZ) and *N*-hydroxysuccinimide (NHS, 20.83 mg, 0.181 mmol) in DCM (1 mL) at RT. The reaction mixture was stirred at RT overnight. The solid was filtered off, and the filtrate concentrated to afford compound **77**. MS: (+) m/z 650.3 (M+1).

[00292] *Compound 78.* DIEA was added to a solution of compound **77** (58.8 mg, 0.091 mmol) and compound **21** (57.6 mg, 0.100 mmol) in DMF (1 mL) at RT, adjusting the pH to 8-9. After the reaction mixture was stirred at RT for 1 h, LCMS showed the reaction went to completion. The solvent was evaporated. The crude product was purified by preparative HPLC to afford 20 mg of compound **78** as a white solid. MS: (+) m/z 1113.6 (M+1).

[00293] *Compound 79.* TFA (0.5 mL, 6.53 mmol) was added to a mixture of compound **78** (17.2 mg, 0.015 mmol) in DCM (1 mL) at RT. After the reaction mixture was stirred at RT for 1 h, LCMS showed the reaction went to completion. The solvent was evaporated to afford compound **79**. MS: (+) m/z 1013.5 (M+1).

[00294] *Compound 80*. DIEA (2.96 µL, 0.017 mmol) was added to a solution of compound **9** (8.55 mg, 0.015 mmol) and HATU (5.87 mg, 0.015 mmol) in DMF (0.4 mL). The pH of the reaction mixture was adjusted to 8-9. After the reaction mixture was stirred at RT for 20 min, compound **79** (15.65 mg, 0.015 mmol) in DMF (1 mL) and DIEA (2.96 µL, 0.017 mmol) were added. The pH of the reaction mixture was adjusted to 8-9. After the reaction mixture was stirred at RT for another 20 min, LCMS showed the reaction went to completion. The solvent was evaporated. The crude product was redissolved in 1 mL of DMSO, and purified by preparative HPLC to afford compound **80** as a white solid. MS: (+) m/z 775.0 (M/2+1). Compound **80** is also referred to hereinabove as compound (III-12).

[00295] A conjugate of compound **80** and seven variants of anti-glypican 3 antibody 4A6 modified to have an azide group at various locations exhibited EC_{50}'s of 1.4, 0.17, 0.13, 0.22, 0.14, 0.064, and 0.25 nM against N87 gastric cancer cells, using a ^3H thymidine incorporation assay.

[00296] The preparation and characterization of antibody 4A6 is described in Terrette *et al.* 2010. The V_H CDR1, CDR2, and CDR3 and V_K CDR1, CDR2, and CDR3 sequences for antibody 4A6 are given in SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, and SEQ ID NO:22, respectively. The variable region V_H and V_K sequences are provided in SEQ ID NO:23 and SEQ ID NO:24, respectively.

[00297] In one embodiment, this invention provides a conjugate of compound **80** and a polypeptide, preferably an antibody, modified to include an azide group.

Example 20 – Compound 88

[00298] This example describes the synthesis of compound **88**, which has an alkyl primary amine functionality that can be used for conjugation. Reference is made to the synthetic scheme of **Fig. 24**.

[00299] *Compound 82*. A mixture of *tert*-butyl 1-amino-3,6,9,12-tetraoxapentadecan-15-oate **54** (0.285 g, 0.888 mmol, purchased from VWR; see also Example 8) and 2,5-dioxo-pyrrolidin-1-yl 6-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)hexanoate **81** (0.4 g, 0.888 mmol, purchased from Chem-Impex) in DMF (3 mL) was stirred at RT for 2 h. The solvent was evaporated. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-10% MeOH in DCM to afford 0.4 g of compound **82** as a white solid. MS: (+) m/z 657.4 (M+1).

[00300] *Compound 83*. A mixture of compound **82** (0.258 g, 0.393 mmol) in TFA (2 mL, 0.393 mmol) was stirred at RT for 1 h. The solvent was evaporated. Compound **83** (white solid) was washed twice with hexanes and used for the next step reaction without further purification.

[00301] *Compound 84*. DCC (0.162 g, 0.786 mmol) was added to a mixture of compound **83** (0.236 g, 0.393 mmol) and NHS (0.090 g, 0.786 mmol) in DCM (5 mL) at RT. After the reaction mixture was stirred at RT overnight, the solid was filtered off, and the filtrate concentrated. The crude product was purified by flash chromatography eluting from silica gel with a gradient of 0-100% EtOAc in hexanes to afford 0.195 g of compound **84** as a colorless oil. MS: (+) m/z 698.3 (M+1).

[00302] *Compound 85*. DIEA was added to a solution of compound **21** (0.162 g, 0.279 mmol) and compound **84** (0.195 g, 0.279 mmol) in DMF (1.5 mL) at RT. After the reaction mixture was stirred at RT for 1 h, the reaction was quenched by addition of 6 mL of 1:1 mixture of acetonitrile and water containing 0.1% TFA. The crude product was purified by preparative HPLC to afford 0.292 g of compound **85** as a white solid. MS: (+) m/z 1161.6 (M+1).

[00303] *Compound 86*. TFA (0.5 mL) was added to a mixture of compound **85** (22.1 mg, 0.019 mmol) in DCM (1 mL) at RT. After the reaction mixture was stirred at RT for 20 min, the solvent was evaporated to afford compound **86**. MS: (+) m/z 1061.6 (M+1).

[00304] *Compound 87*. DIEA was added to a solution of compound **9** (10.53 mg, 0.019 mmol) and HATU (7.23 mg, 0.019 mmol) in DMF (0.4 mL). The pH of the reaction mixture was adjusted to 8-9. After the reaction mixture was stirred at RT for 10 min, compound **86** (20.19 mg, 0.019 mmol) in DMF (1 mL) and DIEA were added. The pH of the reaction solution was adjusted to 8-9. After the reaction mixture was stirred at RT for 10 min, LCMS showed the reaction went to completion. The reaction was quenched by addition of 10 mL of a 1:1 (v/v) mixture of water (0.1% TFA) and acetonitrile. The crude product was purified by preparative HPLC to afford 27.3 mg of compound **87** as a white solid. MS: (+) m/z 799.1 (M/2+1).

[00305] *Compound 88*. Piperidine was added to a solution of compound **87** (27.3mg, 0.017 mmol) in DMF (2 mL) at RT, adjusting the pH to 9-10. After the reaction mixture was stirred at RT for 1 h, the reaction was quenched by addition of 6 mL of a 1:1 mixture of acetonitrile and water containing 0.1 % TFA. The crude product was purified by preparative

HPLC to afford 22.5 mg of compound **88** as a white solid. MS: (+) m/z 688.1 (M/2+1). Compound **88** is also referred to hereinabove as compound (III-13).

[00306] In one embodiment, there is provided a conjugate in which compound **88** is conjugated to a polypeptide, which preferably is an antibody, via amide formation between the alkyl primary amine of compound **88** and a carboxyl group on a side chain residue of an amino acid – preferably glutamic acid – in the polypeptide. This invention also provides a method of making such a conjugate, comprising combining compound **88** and the polypeptide in the presence of the enzyme transglutaminase.

[00307] 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.

[00308] 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.

REFERENCES

[00309] Full citations for the references cited in abbreviated fashion by first author (or inventor) and date earlier in this specification are provided below.

The citation of a reference hereinbelow or elsewhere in this specification is not an admission that such reference is material prior art.

[00310] Abe *et al.*, WO 97/21712 (1997).

[00311] Boyd *et al.*, US 2008/0279868 A1 (2008).

[00312] Boyd *et al.*, US 7,691,962 B2 (2010).

[00313] Balasubramanian *et al.*, *Bioorg. Med. Chem. Lett.* **2008**, 18, 2996-2999.

[00314] Balasubramanian *et al.*, *J. Med. Chem.* **2009**, 52 (2), 238-240.

[00315] Chai *et al.*, *Chem. & Biol.* **2010**, 17(3), 296-309.

[00316] Chai *et al.*, US 2011/0245295 A1 (2011).

[00317] Cheng *et al.*, US 2011/0027274 A1 (2011).

[00318] Coccia *et al.*, US 2010/0150950 (2010)

[00319] Davis *et al.*, US 2008/0176958 A1 (2008).

[00320] Domling, DE 10 2004 030 227 A1 (2006).

[00321] Domling *et al.*, US 2005/0239713 A1 (2005) [2005a].

[00322] Domling *et al.*, US 2005/0249740 A1 (2005) [2005b].

[00323] Domling *et al.*, *Mol. Diversity* **2005**, 9, 141-147 [2005c].

[00324] Domling *et al.*, *Ang. Chem. Int. Ed.* **2006**, 45, 7235-7239.

[00325] Ellman *et al.*, US 8,476,451 B2 (2013).

[00326] Hamel *et al.*, *Curr. Med. Chem. – Anti-Cancer Agents* **2002**, 2, 19-53.

[00327] Hoefle *et al.*, DE 100 08 089 A1 (2001).

[00328] Hoefle *et al.*, *Pure Appl. Chem.* **2003**, 75 (2-3), 167-178.

[00329] Hoefle *et al.*, US 2006/0128754 A1 (2006) [2006a].

[00330] Hoefle *et al.*, US 2006/0217360 A1 (2006) [2006b].

[00331] Jackson *et al.*, US 2013/0224228 A1 (2013).

[00332] Kaur *et al.*, *Biochem. J.* **2006**, 396, 235-242.

[00333] Khalil *et al.*, C*hemBioChem* **2006**, 7, 678-683.

[00334] Leamon *et al.*, Cancer Res. **2008**, 68 (23), 9839-9844.

[00335] Leamon *et al.*, US 2010/0323973 A1 (2010).

[00336] Leung *et al.*, US 2002/0169125 A1 (2002).

[00337] Low *et al.*, US 2010/0324008 A1 (2010).

[00338] Lundquist *et al.*, *Org. Lett.* **2001**, 3, 781-783.

[00339] Neri *et al.*, *ChemMedChem* **2006**, 1, 175-180.

[00340] Pando *et al., J. Am. Chem. Soc.*, **2011**, 133, 7692-7696.

[00341] Patterson *et al.*, *Chem. Eur. J.* **2007**, 13, 9534-9541.

[00342] Patterson *et al.*, *J. Org. Chem.* **2008**, 73, 4362-4369.

[00343] Peltier *et al.*, *J. Am. Chem. Soc.* **2006**, 128, 16018-16019.

[00344] Reddy *et al.*, *Mol. Pharmaceutics* **2009**, 6 (5), 1518-1525.

[00345] Reichenbach *et al.* WO 98/13375 A1 (1998).

[00346] Richter, US 2012/0129779 A1 (2012) [2012a]

[00347] Richter, US 2012/0252738 A1 (2012) [2012b].

[00348] Richter, US 2012/0252739 A1 (2012) [2012c].

[00349] Sani *et al.*, *Angew. Chem. Int. Ed.* **2007**, 46, 3526-3529.

[00350] Sasse *et al.*, *J. Antibiotics* **2000**, 53 (9), 879-885.

[00351] Sasse *et al.*, *Nature Chem. Biol.* **2007**, 3 (2), 87-89.

[00352] Schluep *et al.*, Clin. Cancer Res. **2009**, 15 (1), 181-189.

[00353] Schrama *et al.*, *Nature Rev. Drug Disc.* **2006**, 5, 147-159.

[00354] Shankar *et al.*, *SYNLETT* **2009**, 8, 1341-1345.

[00355] Shankar *et al.*, *Org. Biomol. Chem.*, **2013**, 11(14), 2273-2287.

[00356] Shibue *et al.*, *Tetrahedron Lett.* **2009**, 50, 3845-3848.

[00357] Shibue *et al.*, *Chemistry Eur. J.*, **2010**, 16(38), 11678-11688.

[00358] Shibue *et al.*, *Bioorg. Med. Chem.*, **2011**, 21, 431-434.

[00359] Sreejith *et al.*, *SYNLETT* **2011**, No. 12, 1673-1676x.

[00360] Steinmetz *et al.*, *Angew. Chem. Int. Ed.* **2004**, 43, 4888-4892.

[00361] Terrett *et al.*, US 8,268,970 B2 (2012).

[00362] Terrette *et al.*, US 2010/0209432 A1 (2010).

[00363] Ullrich *et al.*, *Angew. Chemie Int. Ed.* **2009**, 48, 4422-4425.

[00364] Vlahov *et al.*, *Bioorg. Med. Chem. Lett.* **2008**, 18 (16), 4558-4561 [2008a].

[00365] Vlahov *et al.*, US 2008/0248052 A1 (2008) [2008b].

[00366] Vlahov *et al.*, US 2010/0240701 A1 (2010) [2010a].

[00367] Vlahov *et al.*, US 2010/0048490 A1 (2010) [2010b].

[00368] Wang *et al.*, *Chem. Biol. Drug. Des* **2007**, 70, 75-86.

[00369] Wipf *et al.*, *Org. Lett.* **2004**, 6 (22), 4057-4060.

[00370] Wipf *et al.*, *Org. Lett.* **2007**, 9 (8), 1605-1607.

[00371] Wipf *et al.*, US 2010/0047841 A1 (2010).

[00372] Zanda *et al.*, US 8,580,820 B2 (2013).

SEQUENCE LISTING

[00373] Incorporated herein by reference in its entirety is a Sequence Listing named "SEQT_12026WOPCT.txt," comprising SEQ ID NO:1 through SEQ ID NO:26, which include nucleic acid and/or amino acid sequences disclosed herein. The Sequence Listing has been submitted herewith in ASCII text format via EFS-Web, and thus constitutes both the paper and computer readable form thereof. The Sequence Listing was first created using PatentIn 3.5 on January 4, 2014, and is approximately 15 KB in size.

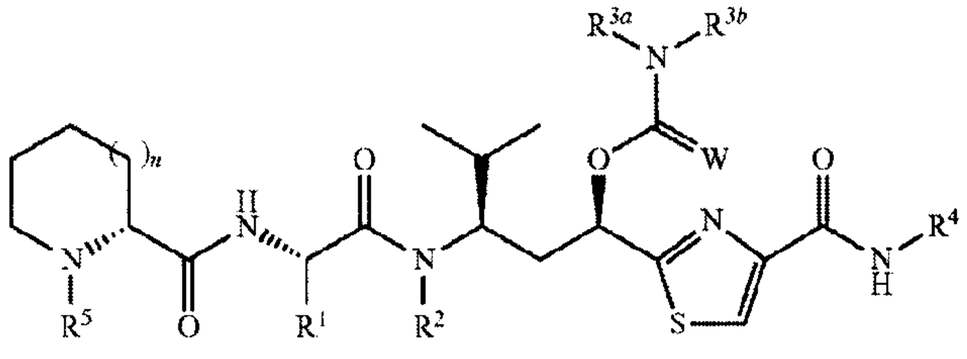
[00374] The following Table 3 summarizes the descriptions of the sequences disclosed in this application.

Table 3 – Sequence Summary	
SEQ ID NO:	SEQUENCE DESCRIPTION
1	6A4 V_H CDR1 amino acid
2	6A4 V_H CDR2 amino acid
3	6A4 V_H CDR3 amino acid
4	6A4 V_K CDR1 amino acid
5	6A4 V_K CDR2 amino acid
6	6A4 V_K CDR3 amino acid
7	6A4 V_H amino acid
8	6A4 V_K amino acid
9	1F4 V_H CDR1 amino acid
10	1F4 V_H CDR2 amino acid
11	1F4 V_H CDR3 amino acid
12	1F4 V_K CDR1 amino acid
13	1F4 V_K CDR2 amino acid
14	1F4 V_K CDR3 amino acid
15	1F4 V_{II} amino acid
16	1F4 V_K amino acid
17	4A6 V_H CDR1 amino acid
18	4A6 V_H CDR2 amino acid
19	4A6 V_H CDR3 amino acid
20	4A6 V_K CDR1 amino acid
21	4A6 V_K CDR2 amino acid
22	4A6 V_K CDR3 amino acid
23	4A6 V_H amino acid
24	4A6 V_K amino acid
25	Modified antibody constant region
26	Modified antibody constant region

What is claimed is:

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

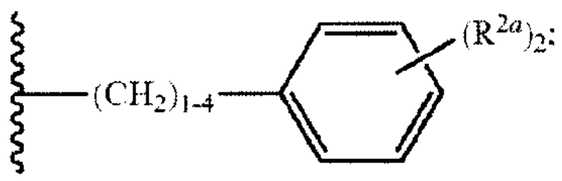
(I)



wherein

R^1 is 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;

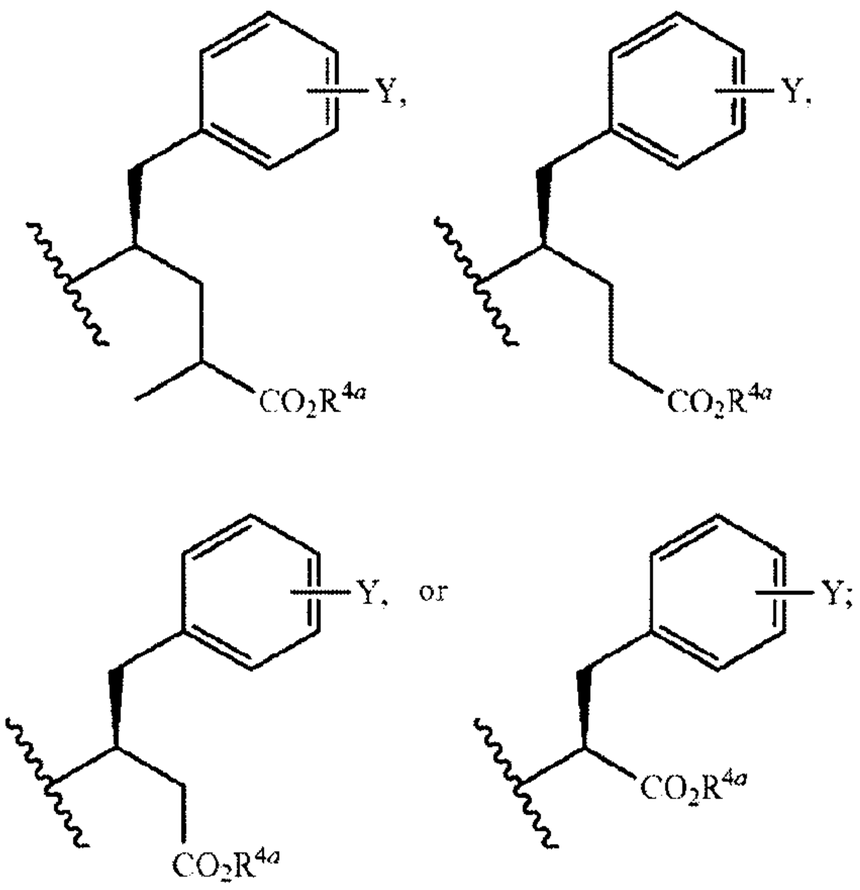
R^2 is H, unsubstituted or substituted C_1-C_{10} alkyl, unsubstituted or substituted C_2-C_{10} alkenyl, unsubstituted or substituted C_2-C_{10} alkynyl, unsubstituted or substituted aryl, unsubstituted or substituted heteroaryl, unsubstituted or substituted $(CH_2)_{1-2}O(C_1$-C_{10} alkyl), unsubstituted 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, unsubstituted or substituted alkylaryl, or



wherein each R^{2a} is independently H, NH_2, NHMe, Cl, F, Me, Et, or CN;

R^{3a} and R^{3b} are independently H, C_1-C_5 alkyl, CH_2(C_5-C_6 cycloalkyl), $CH_2C_6H_5$, C_6H_5, or CH_2CH_2OH;

R^4 is



wherein R^{4a} is H or C_1-C_3 alkyl; and Y is H, OH, Cl, F, CN, Me, Et, NO_2, or NH_2;

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);

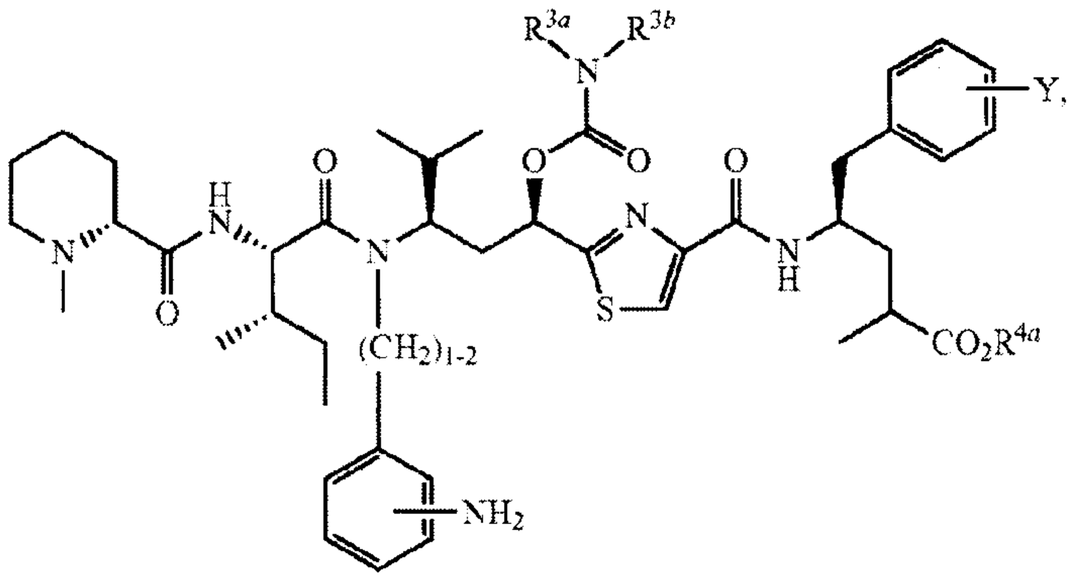
W is O or S; and

n is 0, 1, or 2;

or a pharmaceutically acceptable salt thereof.

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

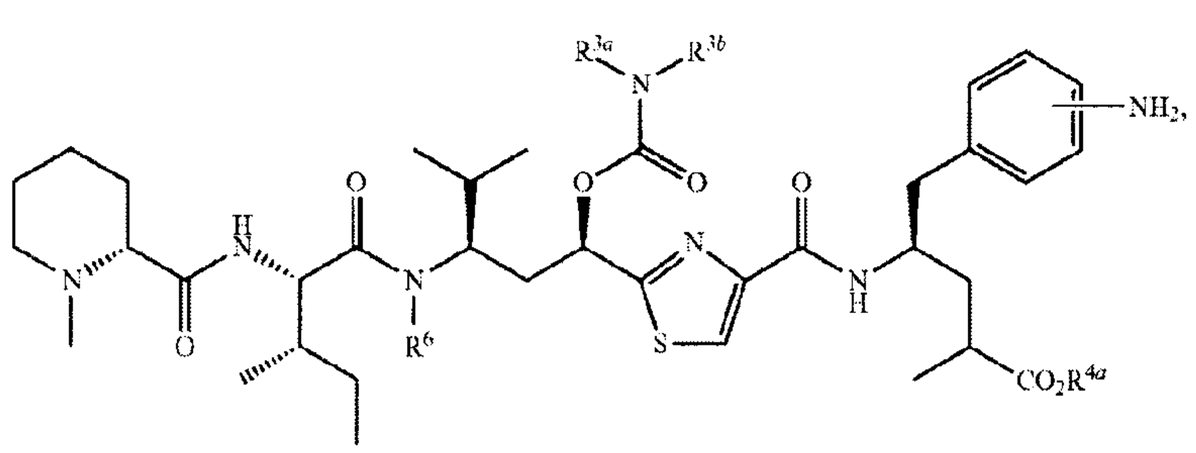
(Ia)



wherein Y is H or NO_2; R^{4a} is H, Me, or Et; and R^{3a} and R^{3b} are independently H, Me, or Et.

3. A compound according to claim 1, having a structure represented by formula (Ib):

(Ib)

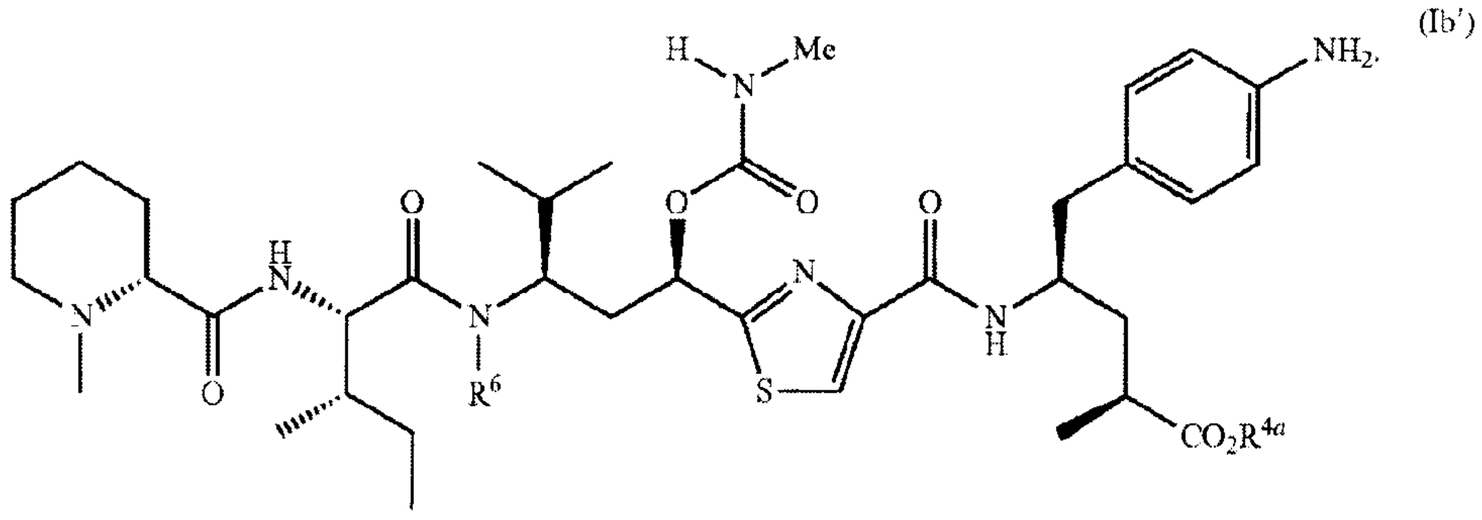


where R^{4a} is H, Me, or Et; R^{3a} and R^{3b} are independently H, Me, or Et; and R^6 is C_1-C_5 alkyl, $CH_2OC(=O)C_1$-C_5 alkyl, or $(CH_2)_{1\text{-}2}C_6H_5$.

4. A compound according to claim 3, wherein one of R^{3a} and R^{3b} is H and the other is Me.

5. A compound according to claim 3, having a structure represented by formula (Ib'):

(Ib')

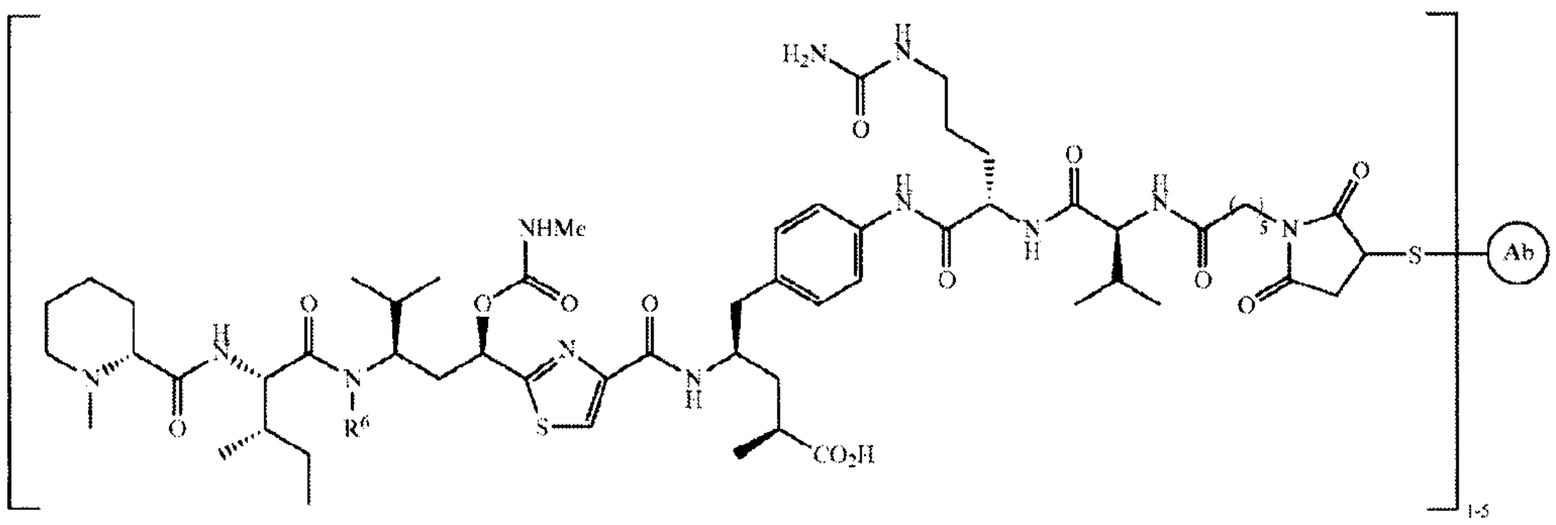


where R^{4a} is H, Me, or Et and R^6 is Me or n-Pr.

6. A conjugate comprising a compound according to claim 1 covalently linked to a targeting moiety that specifically or preferentially binds to a tumor associated antigen.

7. A conjugate according to claim 6, having a structure represented by formula (II-1'):

(II-1')



where R^6 is Me or n-Pr and Ab is an antibody.

8. A conjugate according to claim 7, wherein the antibody is an anti-CD70, anti-mesothelin, or anti-glypican 3 antibody.

9. A conjugate according to claim 6, having a structure represented by formula (II)

$[D(X^D)_aC(X^Z)_b]_mZ$ (II)

wherein

Z is a targeting moiety;

X^D is a first spacer moiety;

X^Z is a second spacer moiety;

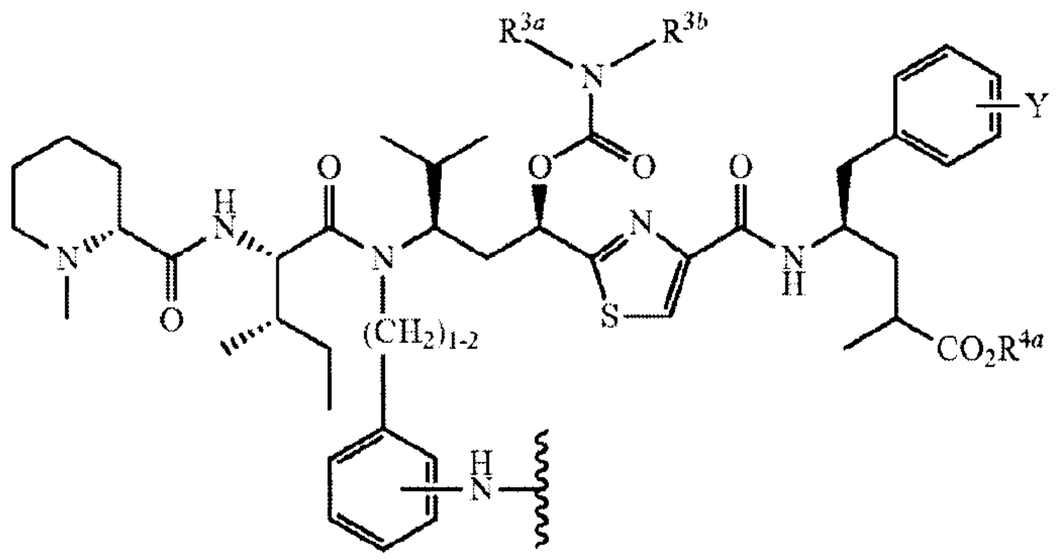
C is a cleavable group;

subscripts a and b are independently 0 or 1;

subscript m is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10; and

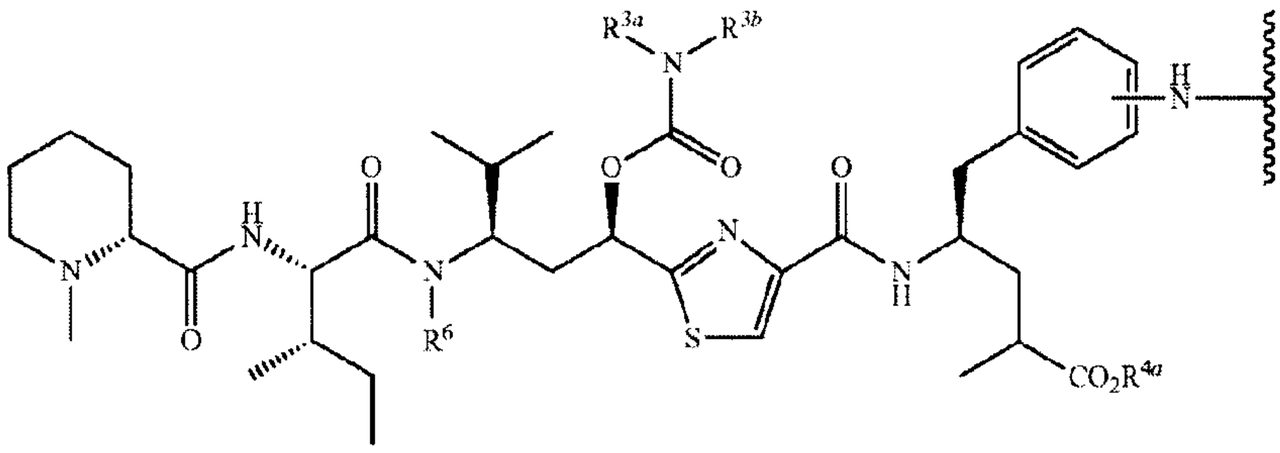
D is according to formula (D-a)

(D-a)



or formula (D-b)

(D-b)



wherein Y is H or NO_2; R^{4a} is H, Me, or Et; R^{3a} and R^{3b} are independently H, Me, or Et; and R^6 is C_1-C_5 alkyl, $CH_2OC(=O)C_1$-C_5 alkyl, or $(CH_2)_{1-2}C_6H_5$;

or a pharmaceutically acceptable salt thereof.

10. A conjugate according to claim 9, wherein Z is an antibody.

11. A drug-linker compound having a structure according to formula (III)

$D\text{-}(X^D)_aC(X^Z)_b—R^{31}$ (III)

wherein

R^{31} is a reactive functional group;

X^D is a first spacer moiety;

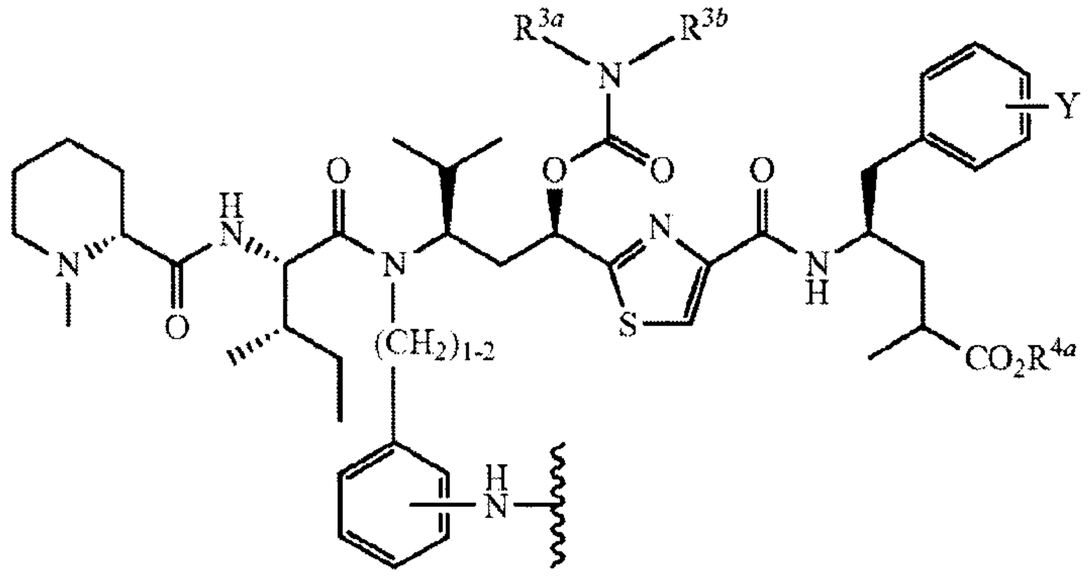
X^Z is a second spacer moiety;

C is a cleavable group;

subscripts a and b are independently 0 or 1; and

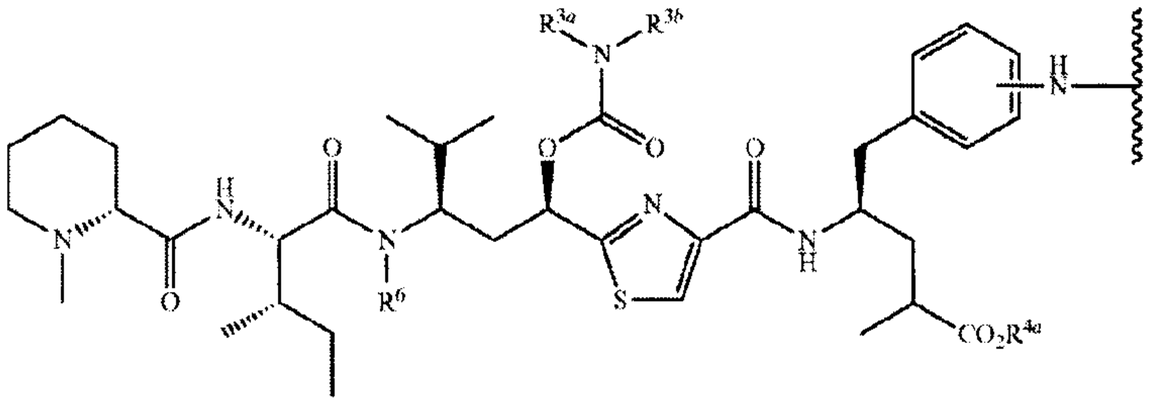
D is according to formula (D-a)

(D-a)



or formula (D-b)

(D-b)



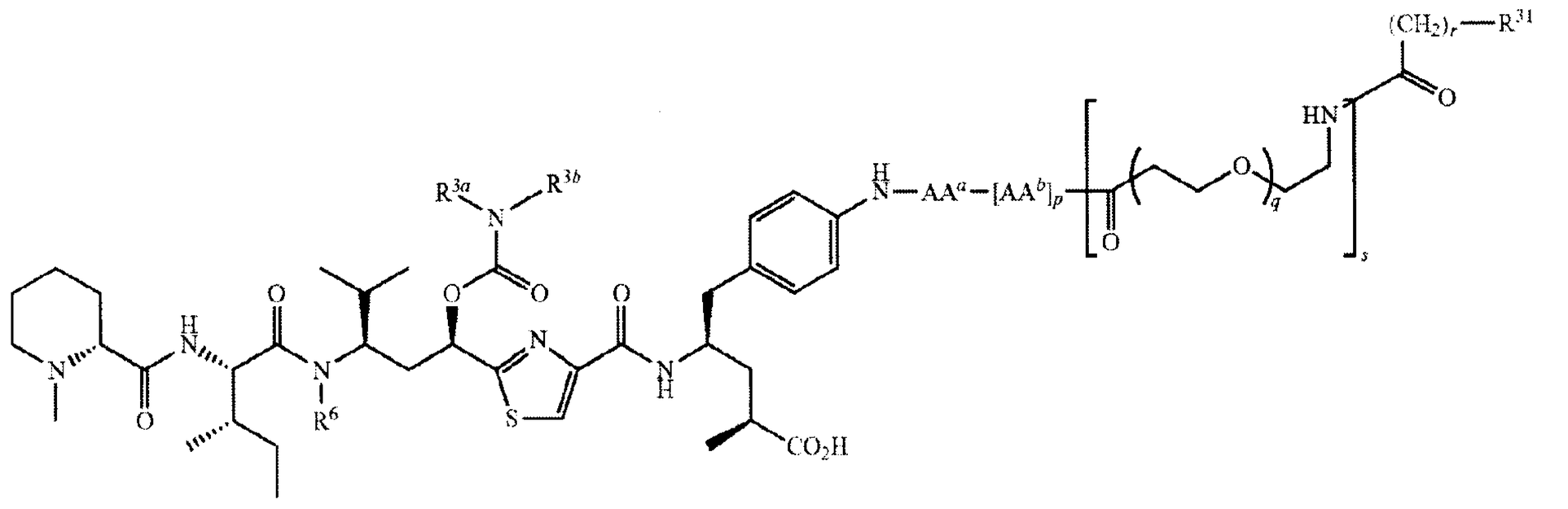
wherein Y is H or NO_2; R^{4a} is H, Me, or Et; and R^{3a} and R^{3b} are independently H, Me, or Et; and R^6 is C_1-C_5 alkyl, $CH_2OC(=O)C_1$-C_5 alkyl, or $(CH_2)_{1-2}C_6H_5$;

or a pharmaceutically acceptable salt thereof.

12. A drug-linker compound according to claim 11, wherein R^{31} is —NH_2, —OH, —CO_2H, —SH, maleimido, cyclooctyne, azido, hydroxylamino, or N-hydroxysuccinimido.

13. A drug-linker compound according to claim 11, having a structure represented by formula (III-a):

(III-a)



where

R^{3a} and R^{3b} are independently H, Me, or Et;

R^6 is Me, Et, or n-Pr;

AA^a and each AA^b are independently alanine, β-alanine, γ-aminobutyric acid, arginine, asparagine, aspartic acid, γ-carboxyglutamic acid, citrulline, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, norleucine, norvaline, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, or valine;

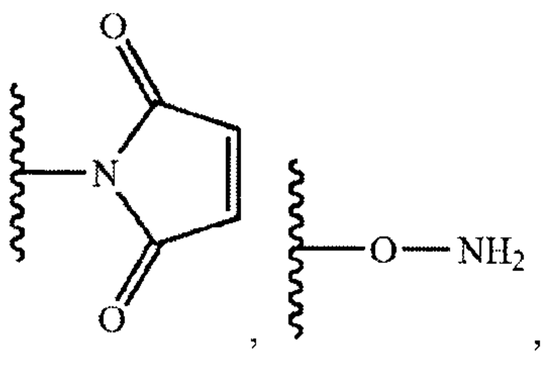
p is 1, 2, 3, or 4;

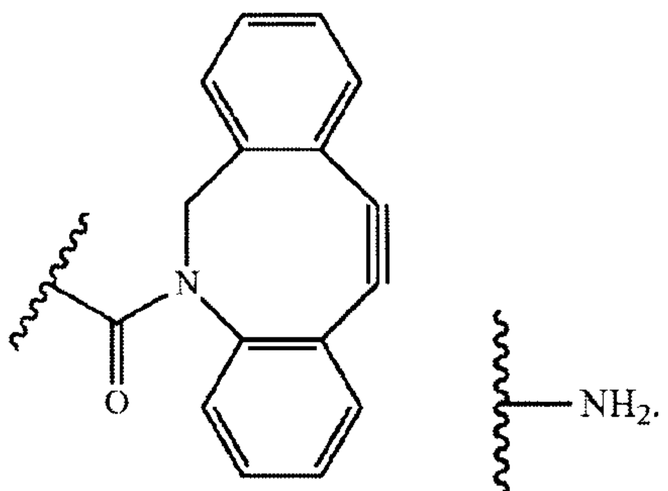
q is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10;

r is 1, 2, 3, 4, or 5;

s is 0 or 1; and

R^{31} is

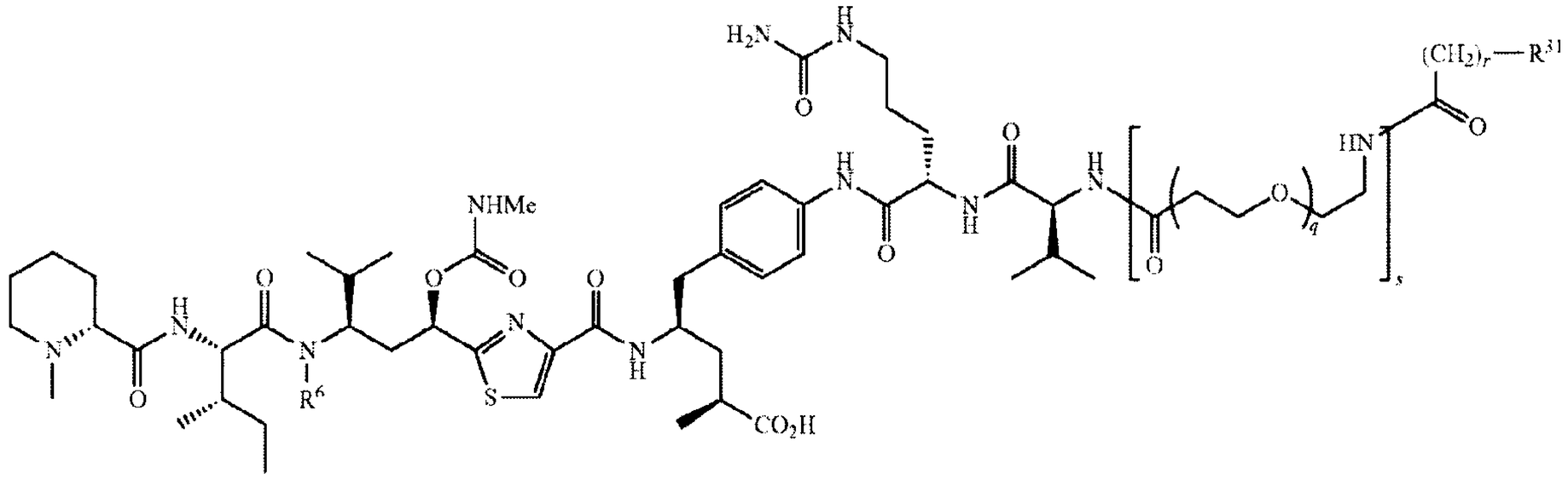




, or .

14. A drug-linker compound according to claim 11, having a structure represented by formula (III-b):

(III-b)



where

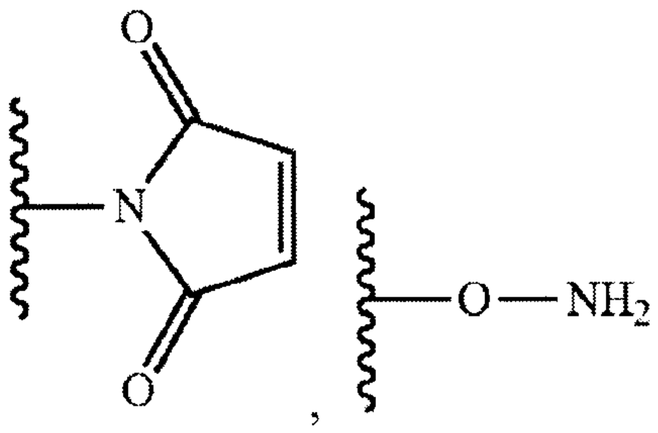
R^6 is Me or n-Pr;

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

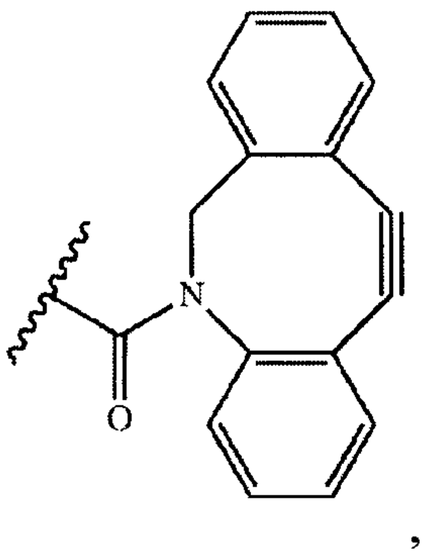
r is 1, 2, 3, 4, or 5;

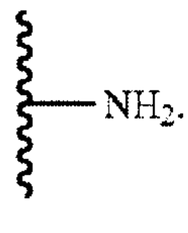
s is 0 or 1; and

R^{31} is


,

,



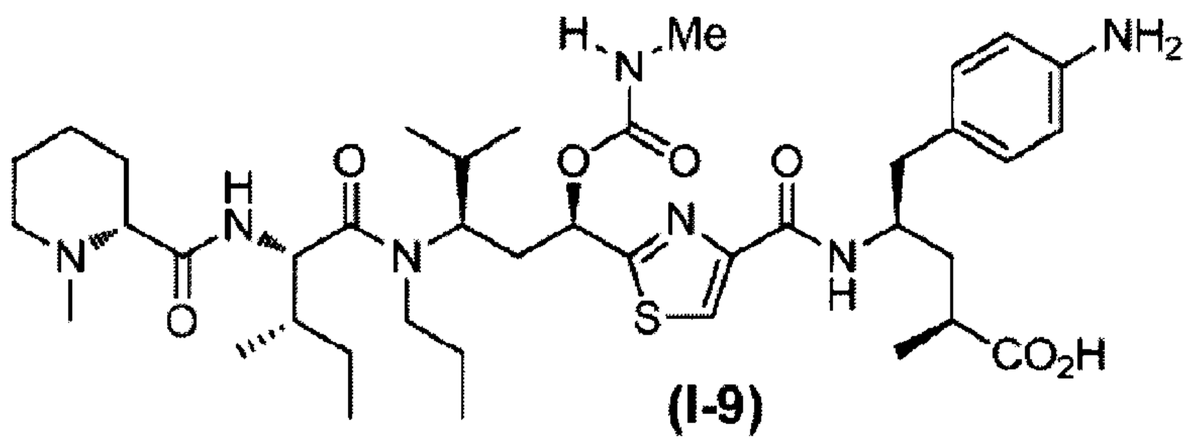

.

, or

15. A pharmaceutical composition comprising a compound according to claim 1, or a conjugate thereof with a targeting moiety, and a pharmaceutically acceptable carrier.

16. A pharmaceutical composition according to claim 15, wherein the compound according to claim 1 is conjugated to a targeting moiety that is an antibody.

17. A compound according to claim 1, having a structure represented by formula (I-9):

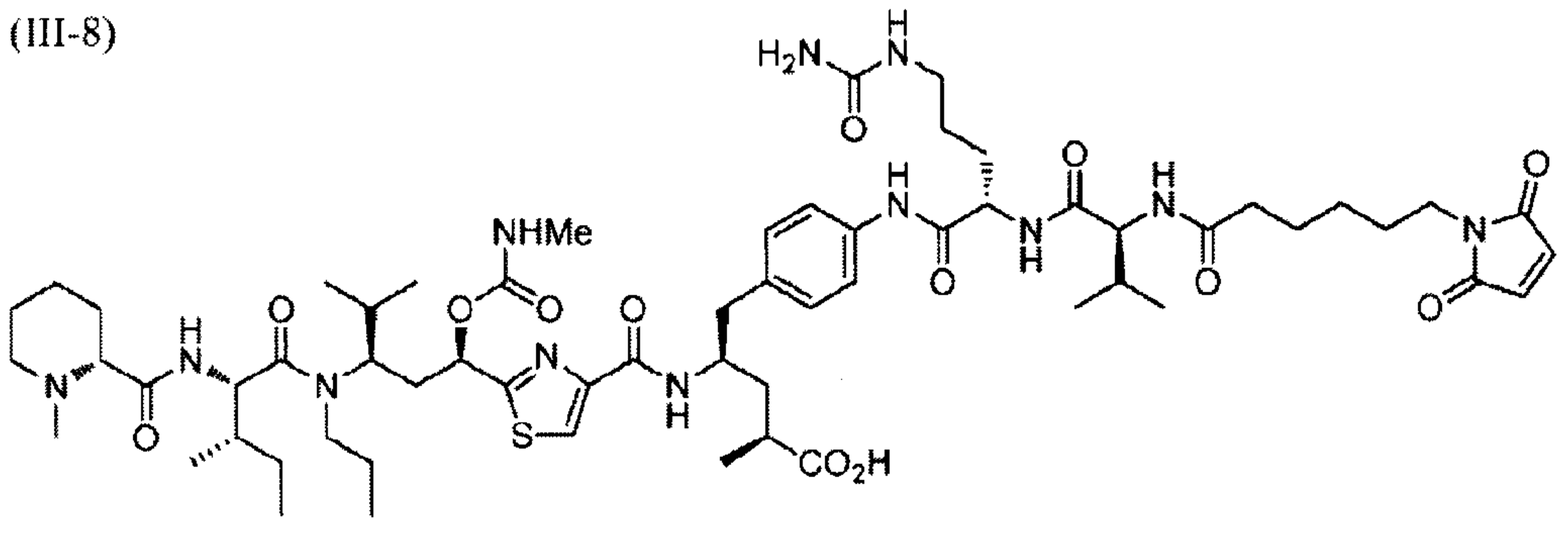


(I-9)

18. A conjugate according to claim 7, wherein the antibody is an anti-mesothelin antibody and R^6 is n-Pr.

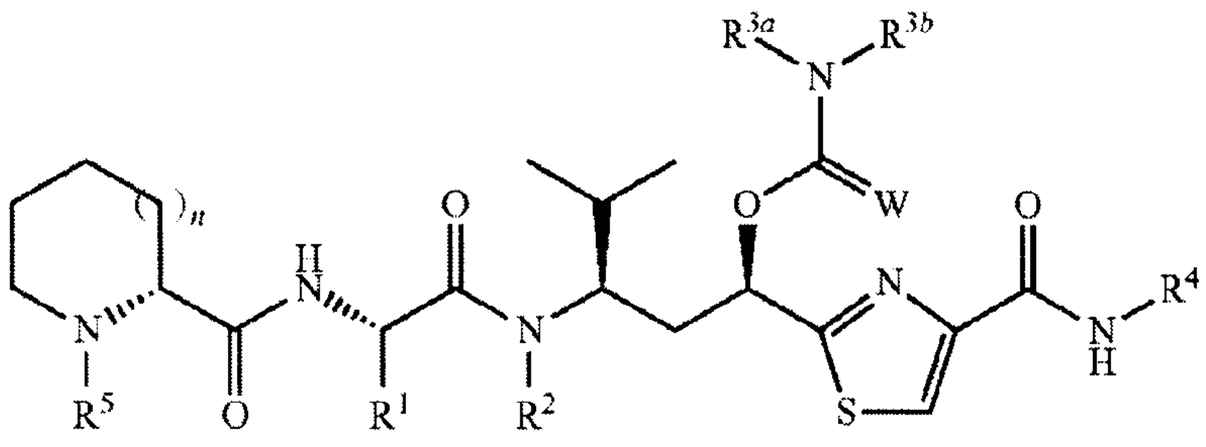
19. A drug-linker compound according to claim 11, having a structure represented by formula (III-8):

(III-8)



20. Use of a compound according to formula (I) or a conjugate thereof with a targeting moiety, for treating a cancer in a subject:

(I)

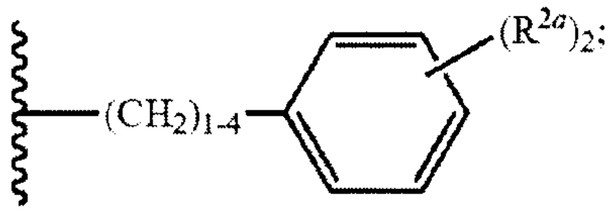


wherein

R^1 is 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^2 is 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}

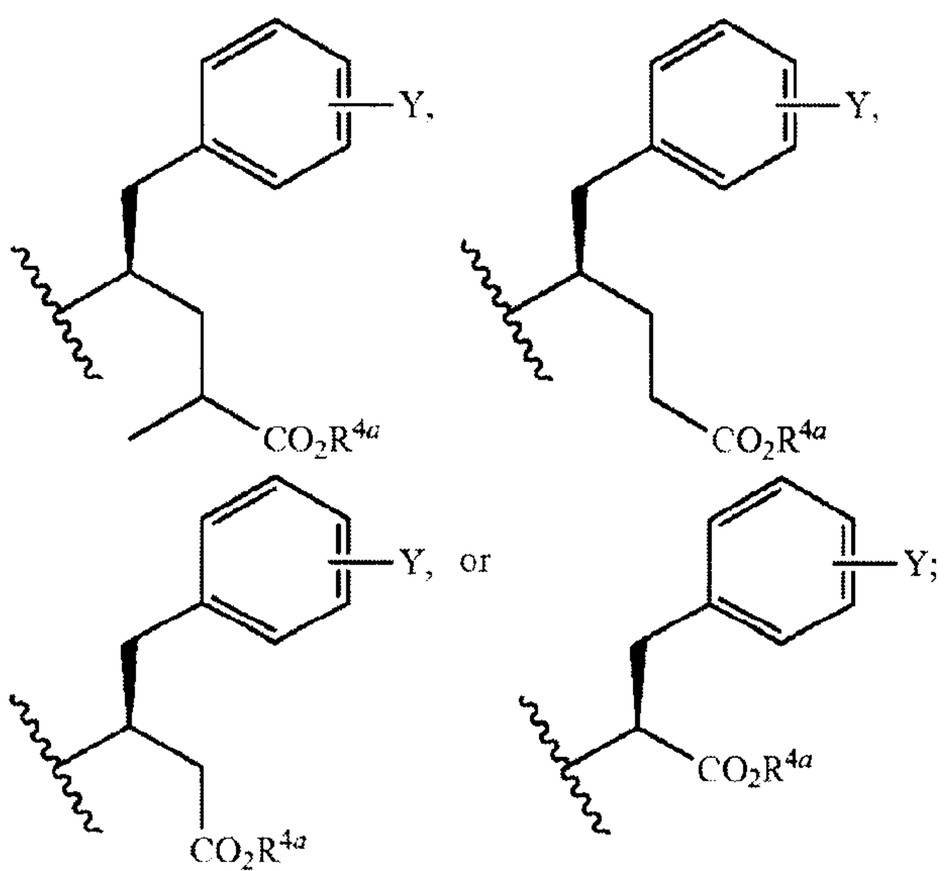
alkyl), unsubstituted or substituted $(CH_2)_{1-2}OC(=O)(C_2-C_{10}$ alkenyl), unsubstituted or substituted $(CH_2)_{1-2}OC(=O)(C_2-C_{10}$ alkynyl), unsubstituted or substituted $C(=O)(C_1-C_{10}$ alkyl), unsubstituted or substituted $C(=O)(C_2-C_{10}$ alkenyl), unsubstituted or substituted $C(=O)(C_2-C_{10}$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



wherein each R^{2a} is independently H, NH_2, NHMe, Cl, F, Me, Et, or CN;

R^{3a} and R^{3b} are independently H, C_1-C_5 alkyl, $CH_2(C_5-C_6$ cycloalkyl), $CH_2C_6H_5$, C_6H_5, or CH_2CH_2OH;

R^4 is



wherein R^{4a} is H or C_1-C_3 alkyl; and Y is H, OH, Cl, F, CN, Me, Et, NO_2, or NH_2;

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);

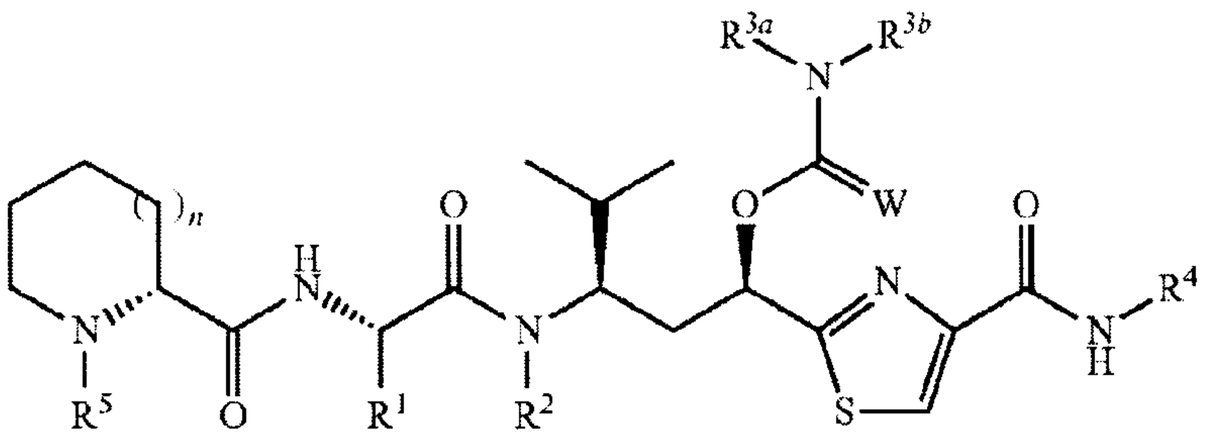
W is O or S; and

n is 0, 1, or 2;

or a pharmaceutically acceptable salt thereof.

21. Use of a compound according to formula (I) or a conjugate thereof with a targeting moiety, in the manufacture of a medicament for treating a cancer in a subject:

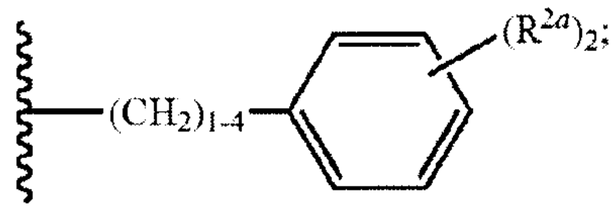
(I)



wherein

R^1 is 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;

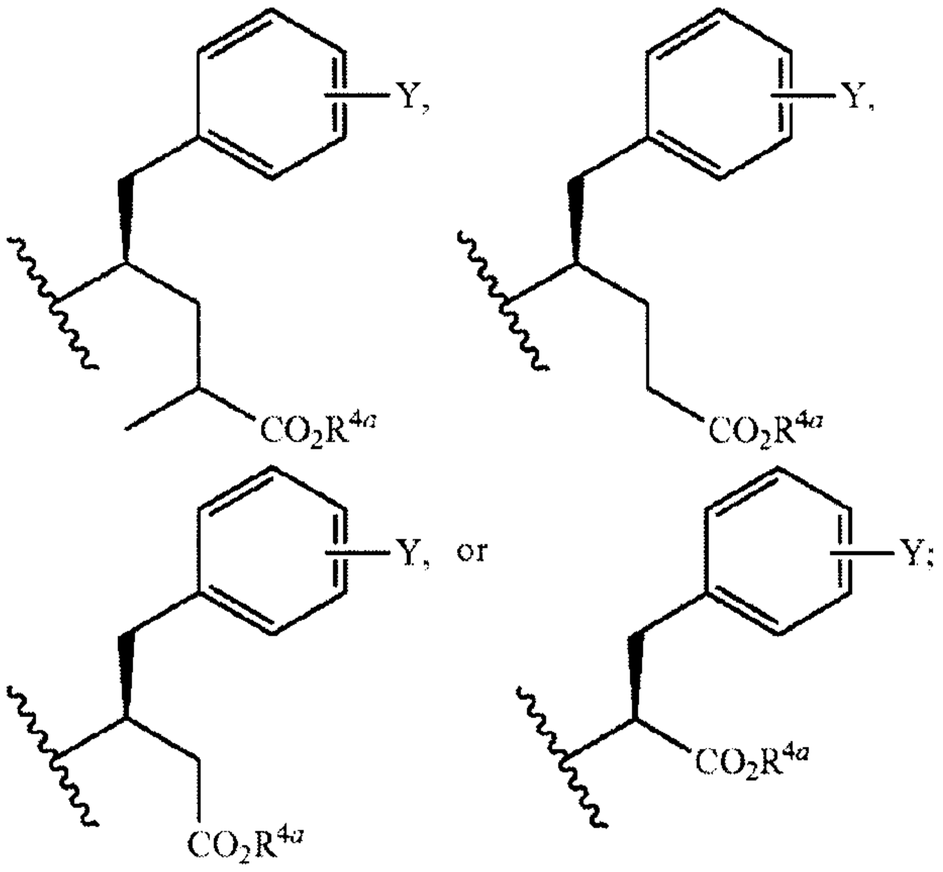
R^2 is H, unsubstituted or substituted C_1-C_{10} alkyl, unsubstituted or substituted C_2-C_{10} alkenyl, unsubstituted or substituted C_2-C_{10} alkynyl, unsubstituted or substituted aryl, unsubstituted or substituted heteroaryl, unsubstituted or substituted $(CH_2)_{1-2}O(C_1$-C_{10} alkyl), unsubstituted 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, unsubstituted or substituted alkylaryl, or



wherein each R^{2a} is independently H, NH_2, NHMe, Cl, F, Me, Et, or CN;

R^{3a} and R^{3b} are independently H, C_1-C_5 alkyl, CH_2(C_5-C_6 cycloalkyl), $CH_2C_6H_5$, C_6H_5, or CH_2CH_2OH;

R^4 is



wherein R^{4a} is H or C_1-C_3 alkyl; and Y is H, OH, Cl, F, CN, Me, Et, NO_2, or NH_2;

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);

W is O or S; and

n is 0, 1, or 2;

or a pharmaceutically acceptable salt thereof.

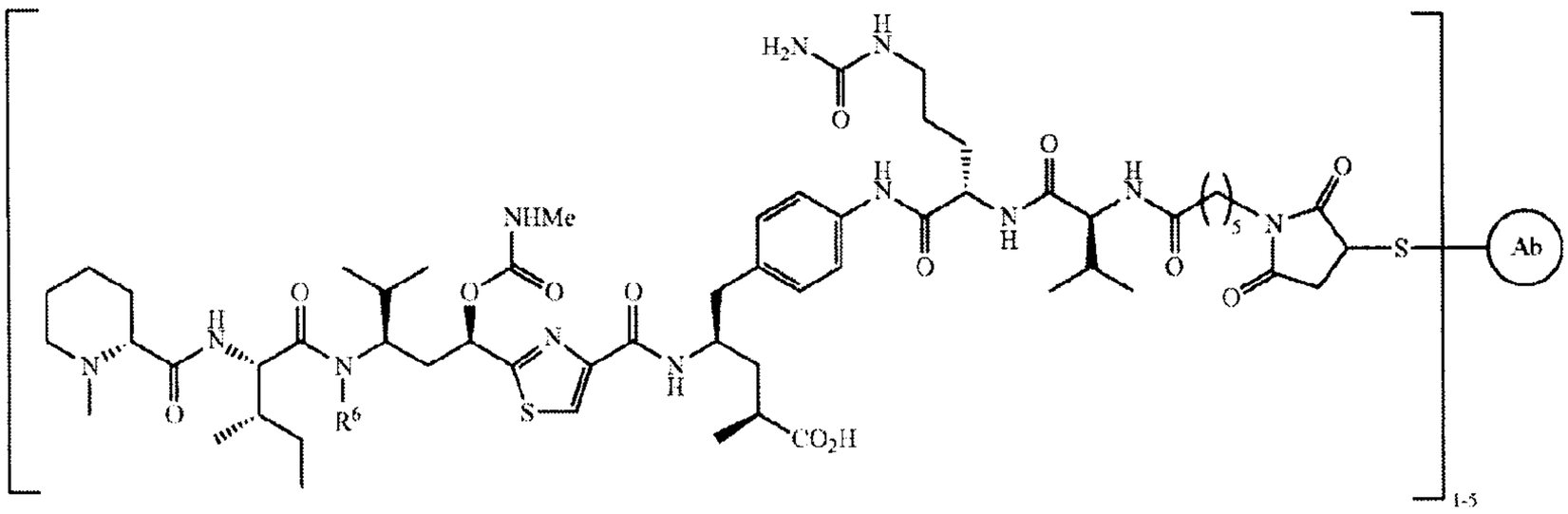
22. The use according to claim 20 or 21, wherein the compound is conjugated to a targeting moiety that is an antibody.

23. The use according to claim 22, wherein the antibody binds to an antigen that is overexpressed or uniquely expressed by the cancer.

24. The use according to claim 23, wherein the cancer is renal, lung, gastric, or ovarian cancer.

25. The use according to claim 20 or 21, wherein the conjugate is of formula (II-1'):

(II-1')



where R^6 is Me or n-Pr and Ab is an antibody.

26. The use according to claim 25, wherein the antibody is an anti-CD70, anti-mesothelin or an anti-glypican-3 antibody.

27. The use according to claim 26, wherein the antibody is anti-mesothelin antibody 6A4 having a heavy chain CDR1 comprising the amino acid sequence of SEQ ID NO:1, a heavy chain CDR2 comprising the amino acid sequence of SEQ ID NO:2, a heavy chain CDR3 comprising the amino acid sequence of SEQ ID NO:3, a light chain CDR1 comprising the amino acid sequence of SEQ ID NO:4, a light chain CDR2 comprising the amino acid sequence of SEQ ID NO:5, and a light chain CDR3 comprising the amino acid sequence of SEQ ID NO:6.

Fig. 1

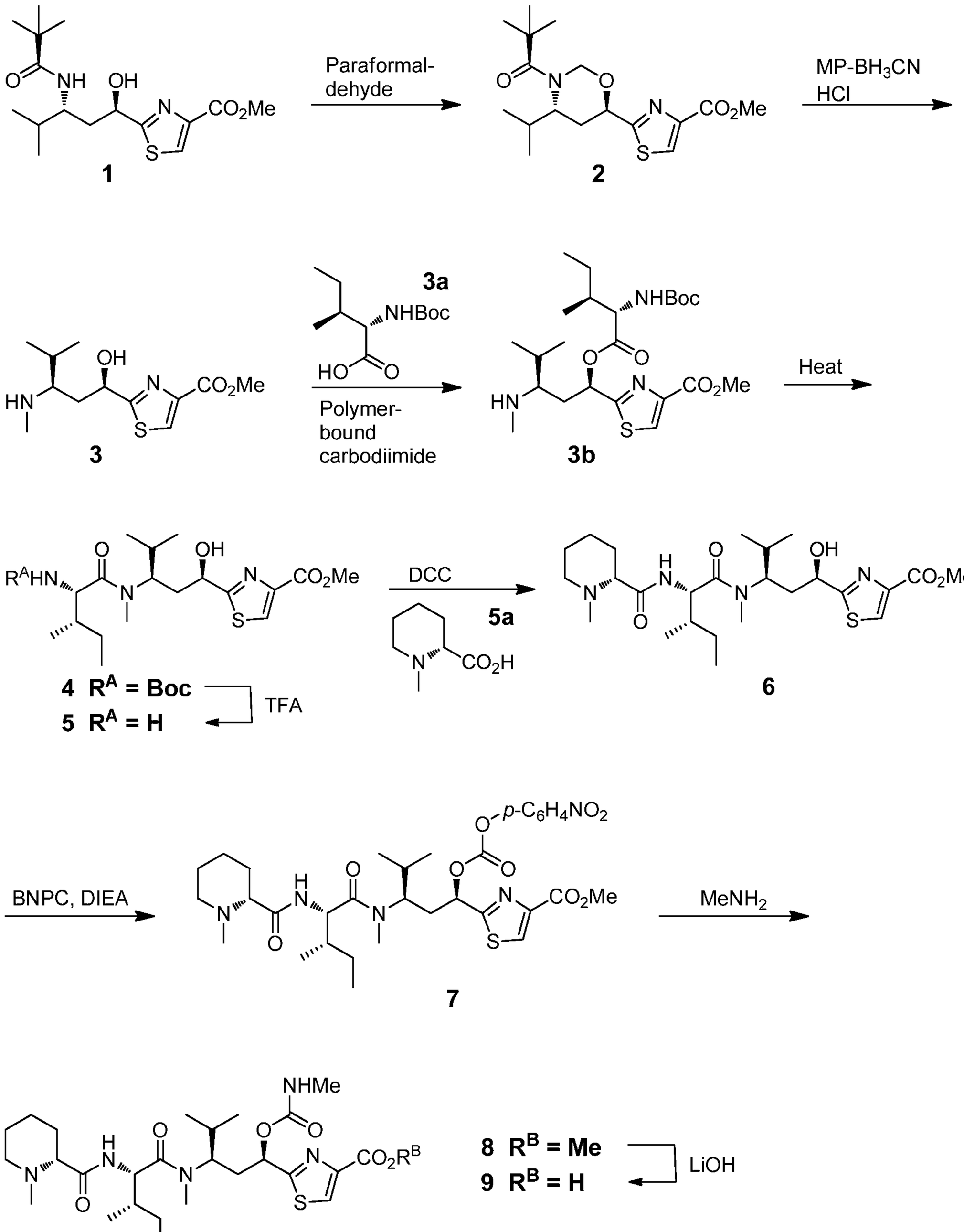

Paraformal-
dehyde
MP-BH3CN
HCl
1
2
3a
NHBoc
HO
O
Polymer-
bound
carbodiimide
3
3b
Heat
RAHN
4 RA = Boc
5 RA = H
TFA
DCC
5a
6
BNPC, DIEA
p-C6H4NO2
7
MeNH2
NHMe
8 RB = Me
9 RB = H
LiOH

Fig. 2a

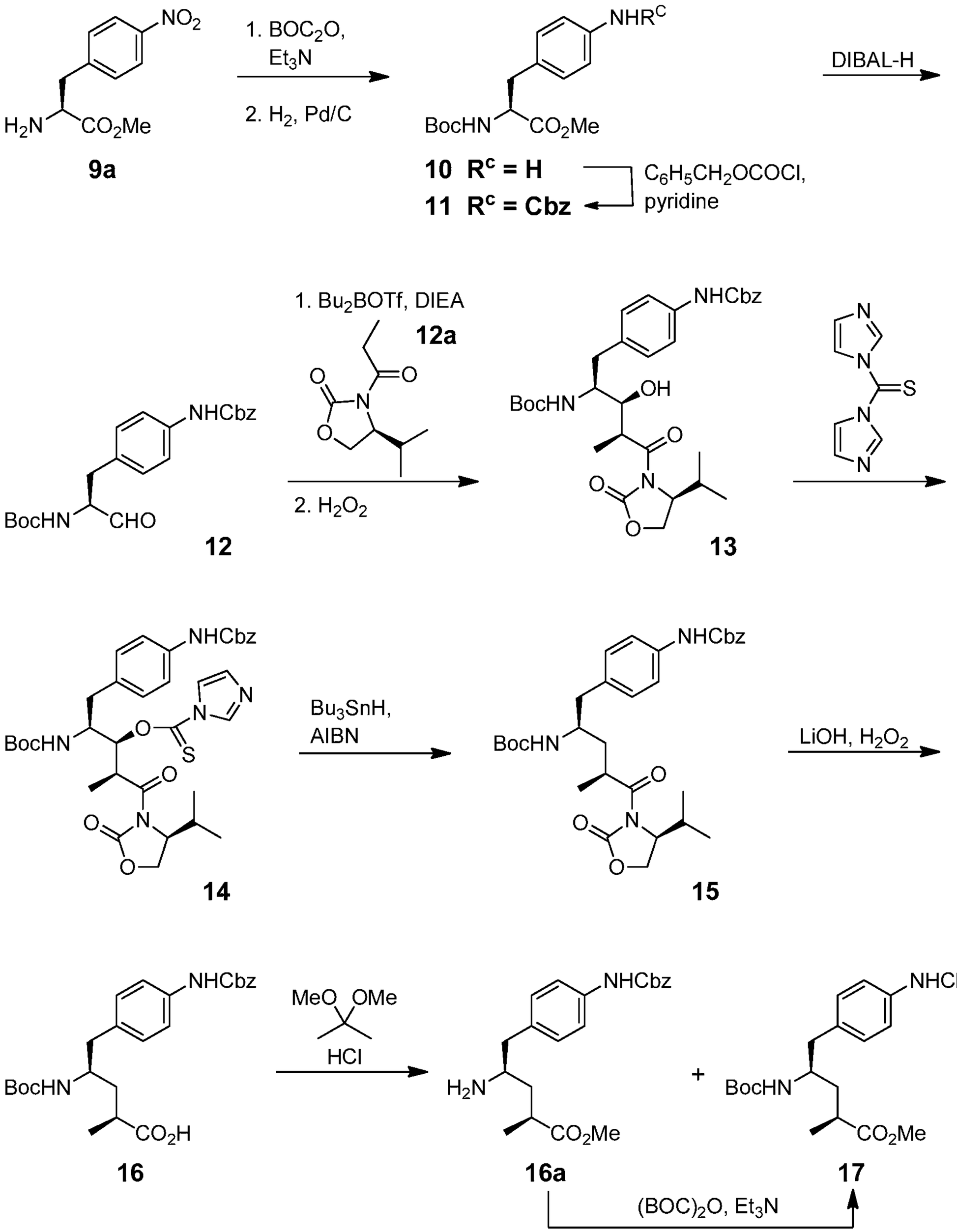

NO2
1. BOC2O, Et3N
2. H2, Pd/C
NHRC
DIBAL-H
H2N
CO2Me
9a
10 Rc = H
11 Rc = Cbz
C6H5CH2OCOCl, pyridine
1. Bu2BOTf, DIEA
12a
2. H2O2
NHCbz
BocHN
CHO
12
OH
13
N
S
Bu3SnH, AIBN
14
15
LiOH, H2O2
MeO OMe
HCl
16
16a
17
(BOC)2O, Et3N

Fig. 2b

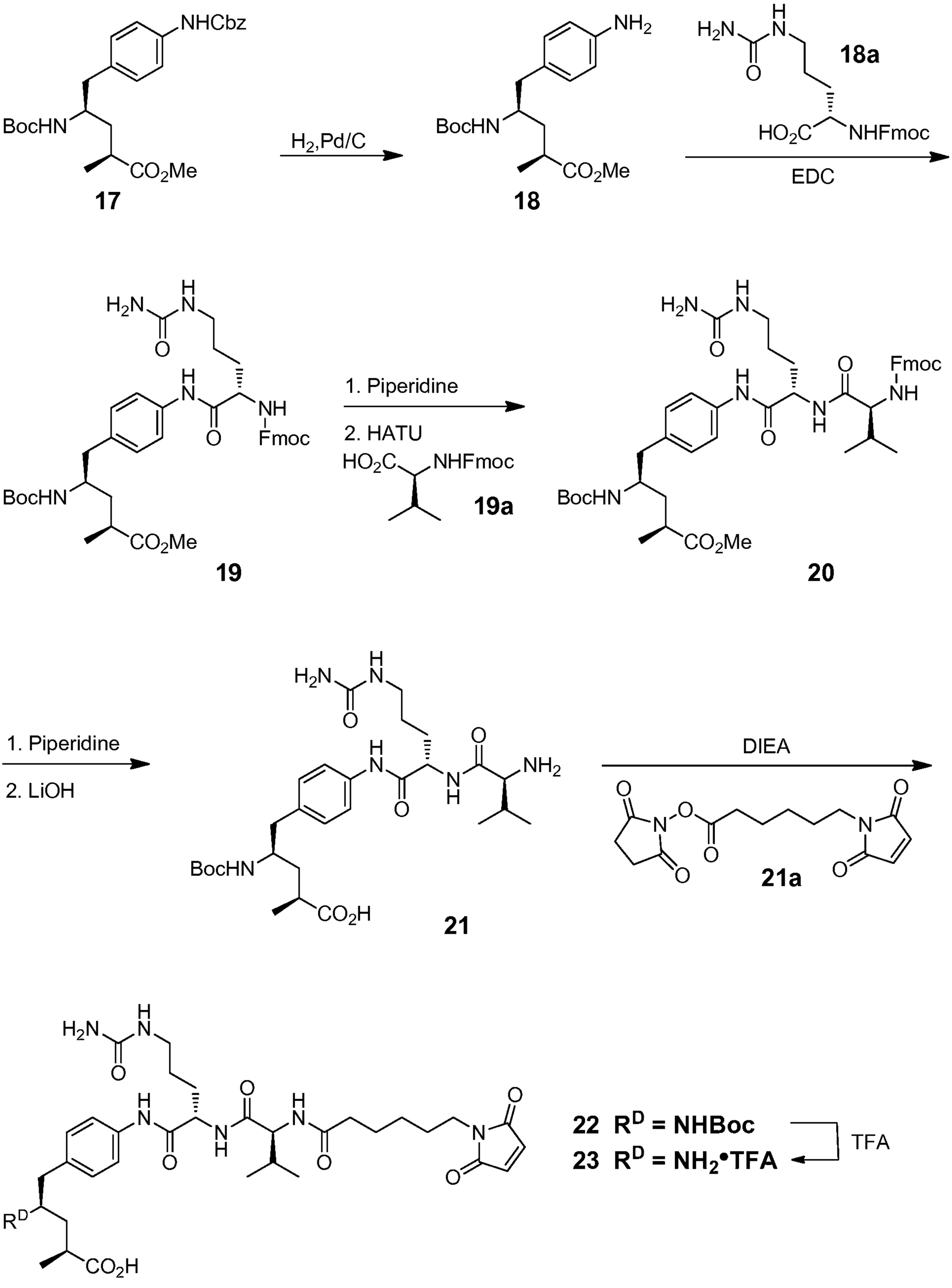

NHCbz
BocHN
CO2Me
17
H2,Pd/C
NH2
BocHN
CO2Me
18
H2N
H
N
O
18a
HO2C
NHFmoc
EDC
H2N
H
N
O
N
H
NH
Fmoc
O
BocHN
CO2Me
19
1. Piperidine
2. HATU
HO2C
NHFmoc
19a
H2N
H
N
O
N
H
N
H
O
Fmoc
NH
BocHN
CO2Me
20
1. Piperidine
2. LiOH
H2N
H
N
O
N
H
N
H
O
NH2
BocHN
CO2H
21
DIEA
O
N
O
O
O
N
O
O
21a
H2N
H
N
O
N
H
N
H
O
O
N
O
O
RD
CO2H
22 RD = NHBoc
23 RD = NH2•TFA
TFA

Fig. 3

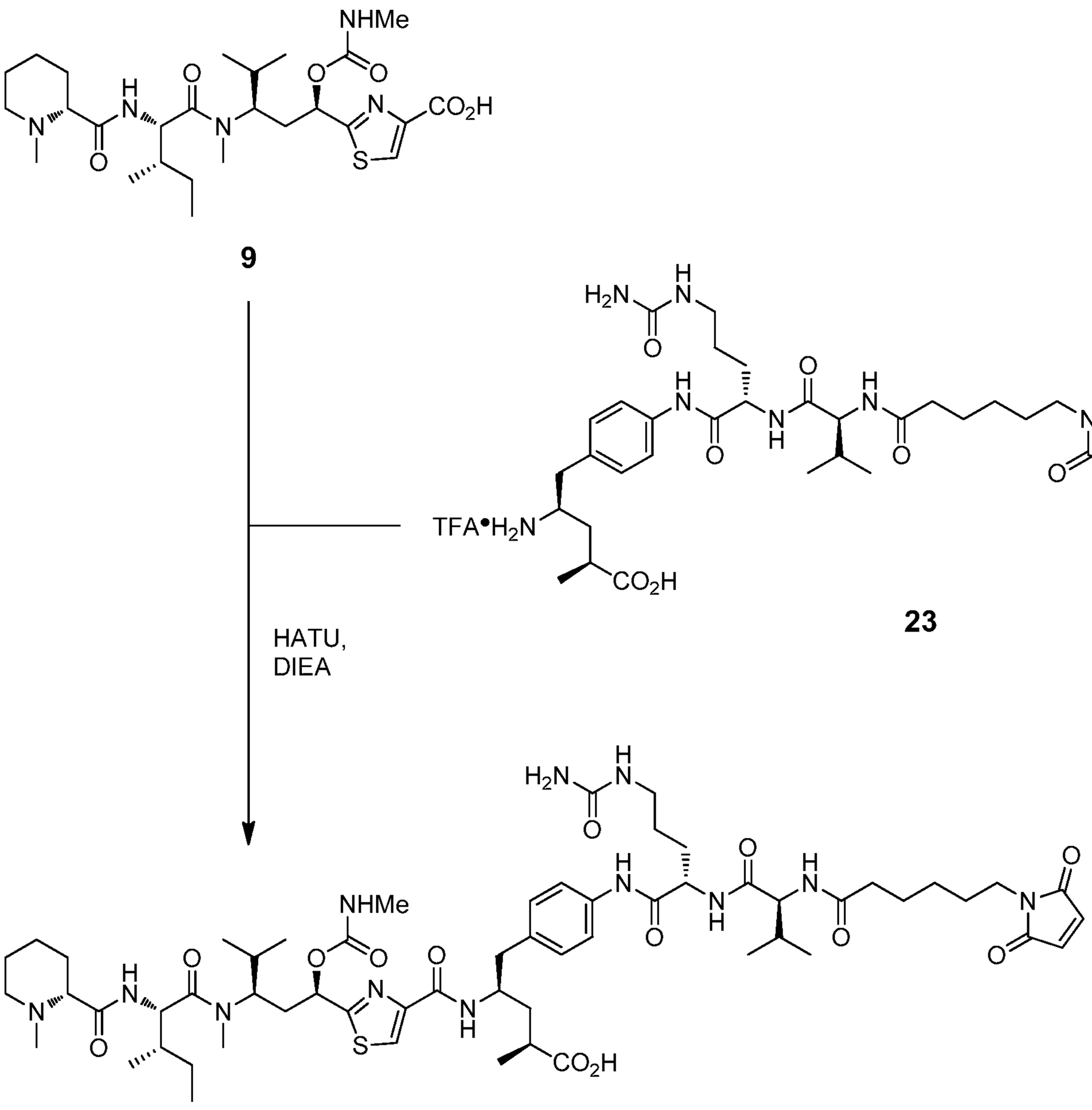


Fig. 4

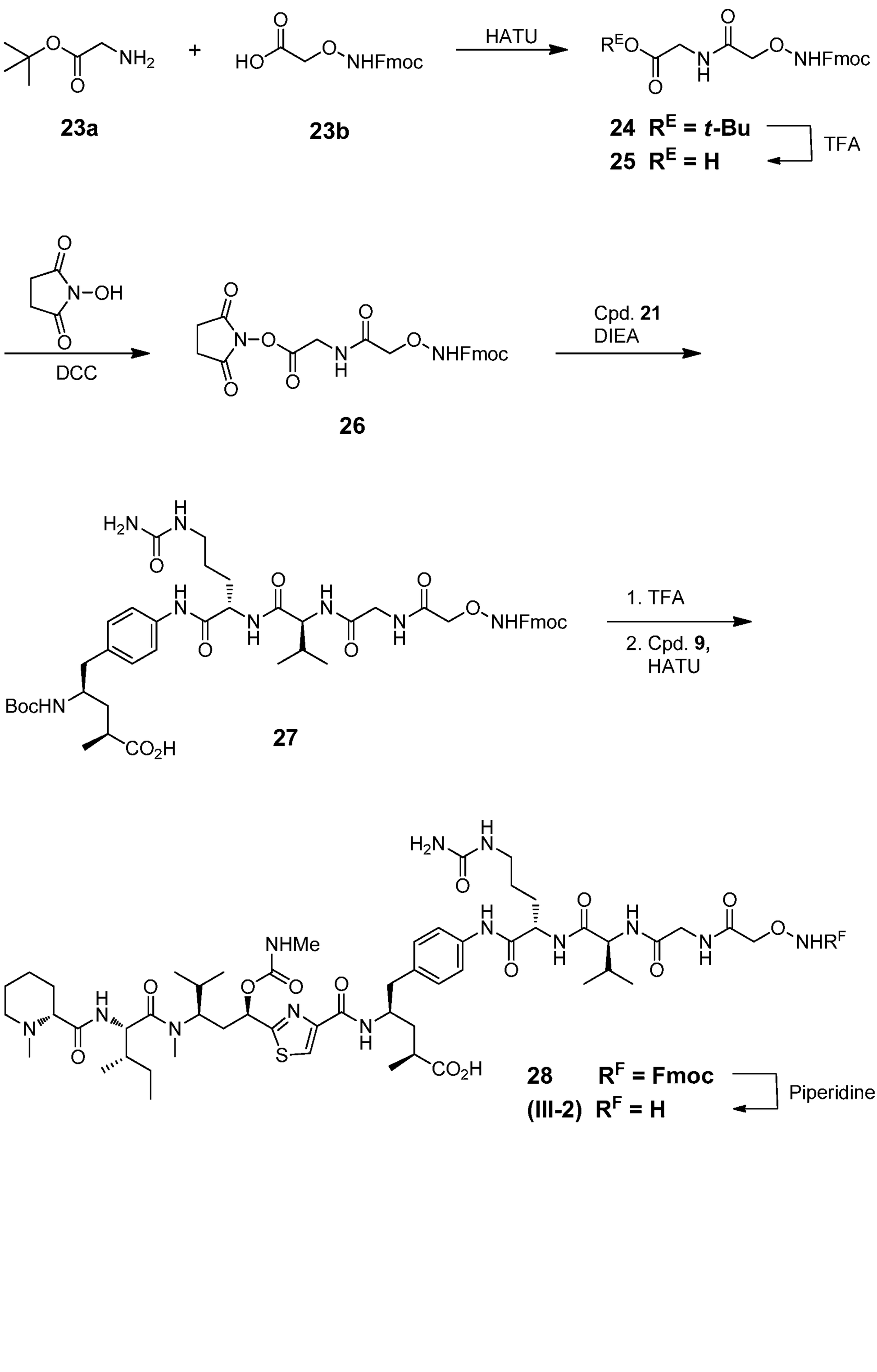
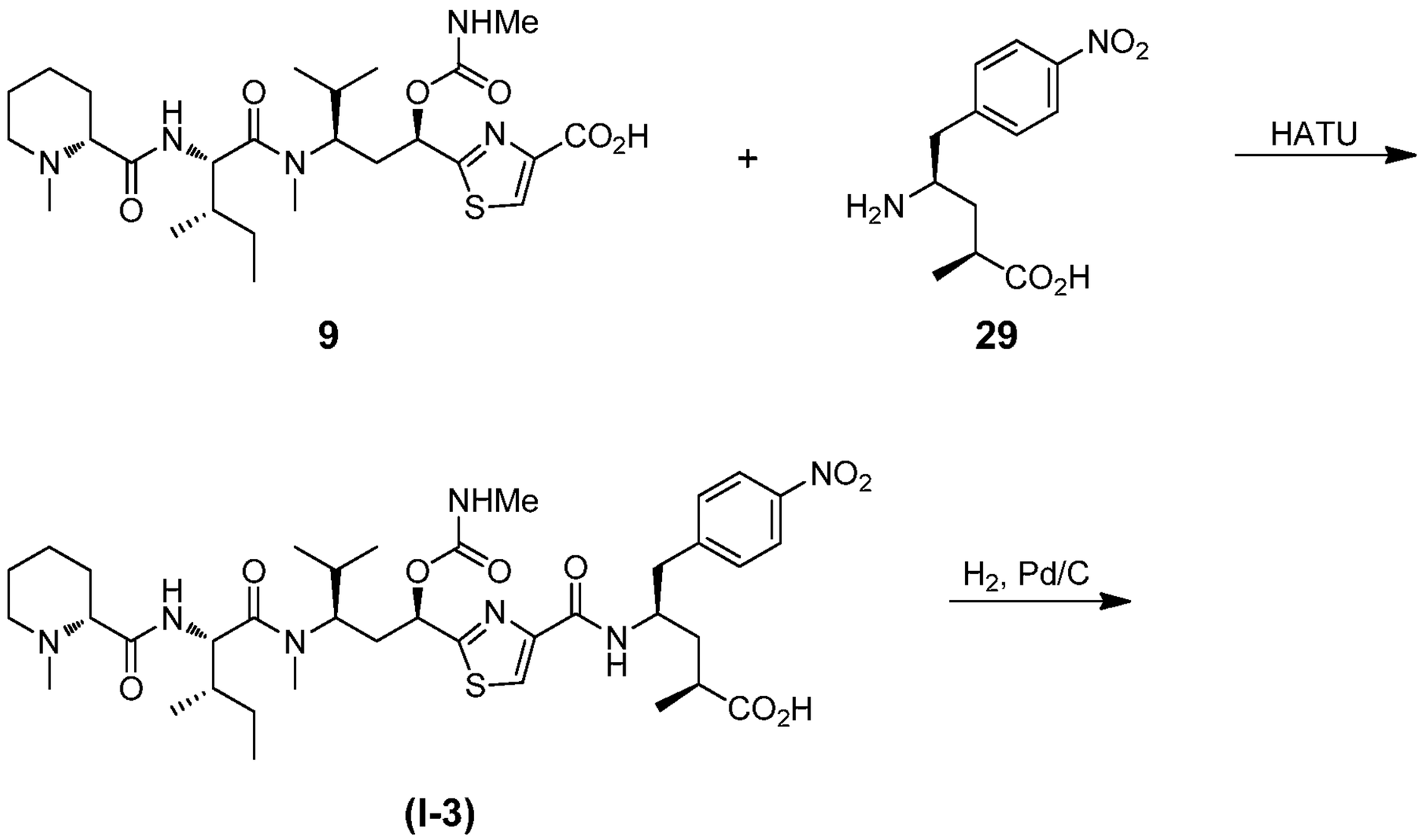

NH2
HO
NHFmoc
HATU
REO
N
H
O
NHFmoc
23a
23b
24 RE = t-Bu
25 RE = H
TFA
N–OH
DCC
26
Cpd. 21
DIEA
H2N
BocHN
CO2H
27
1. TFA
2. Cpd. 9,
HATU
NHMe
S
NHRF
28 RF = Fmoc
(III-2) RF = H
Piperidine

Fig. 5



9

29

(I-3)

(I-2)

Fig. 6a

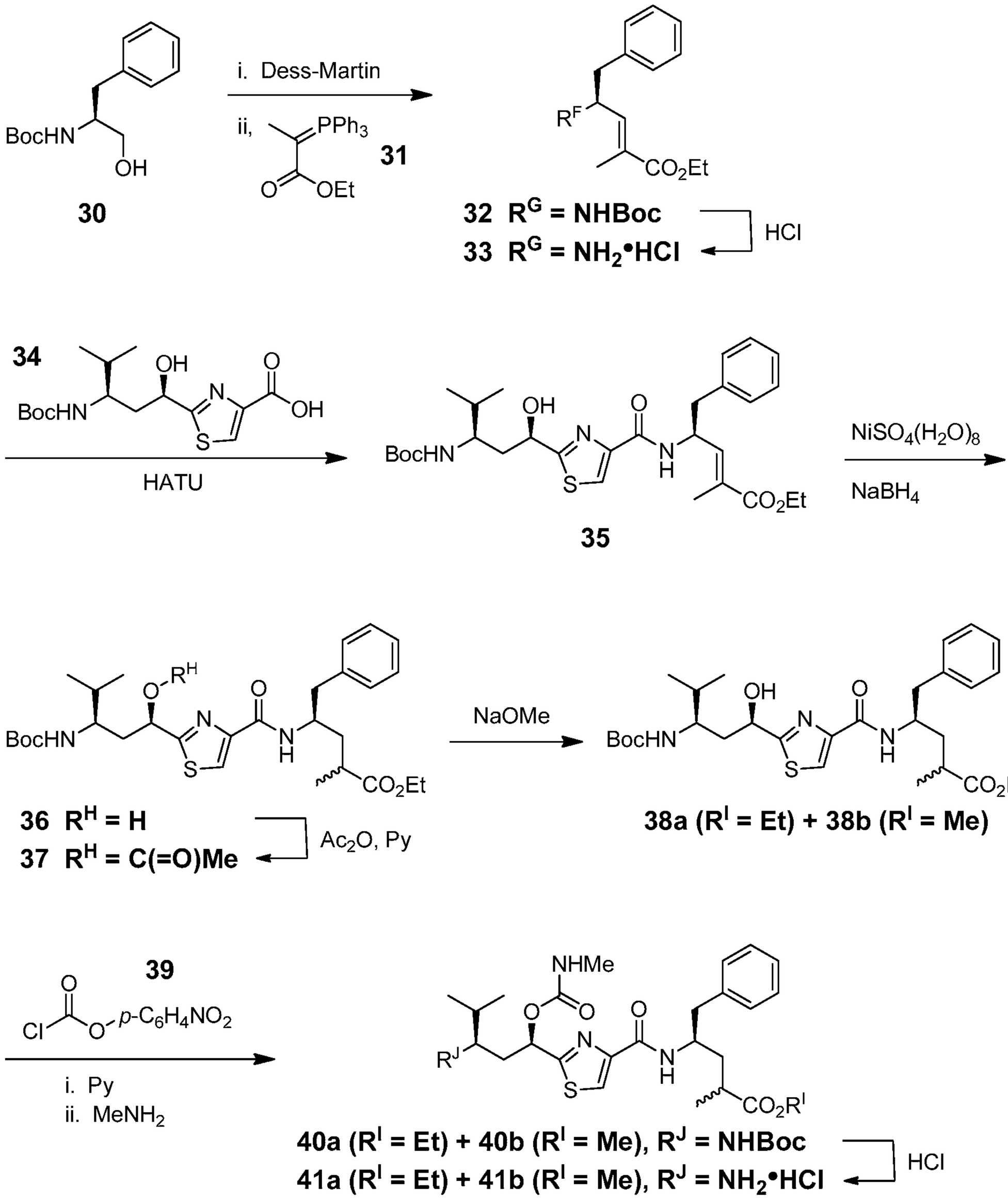

i. Dess-Martin
ii,
PPh3
31
BocHN
OH
30
R^F
CO2Et
32 R^G = NHBoc
33 R^G = NH2•HCl
HCl
34
BocHN
OH
N
S
O
OH
HATU
35
NiSO4(H2O)8
NaBH4
R^H
36 R^H = H
37 R^H = C(=O)Me
Ac2O, Py
NaOMe
38a (R^I = Et) + 38b (R^I = Me)
39
Cl
p-C6H4NO2
i. Py
ii. MeNH2
NHMe
R^J
CO2R^I
40a (R^I = Et) + 40b (R^I = Me), R^J = NHBoc
41a (R^I = Et) + 41b (R^I = Me), R^J = NH2•HCl
HCl

Fig. 6b

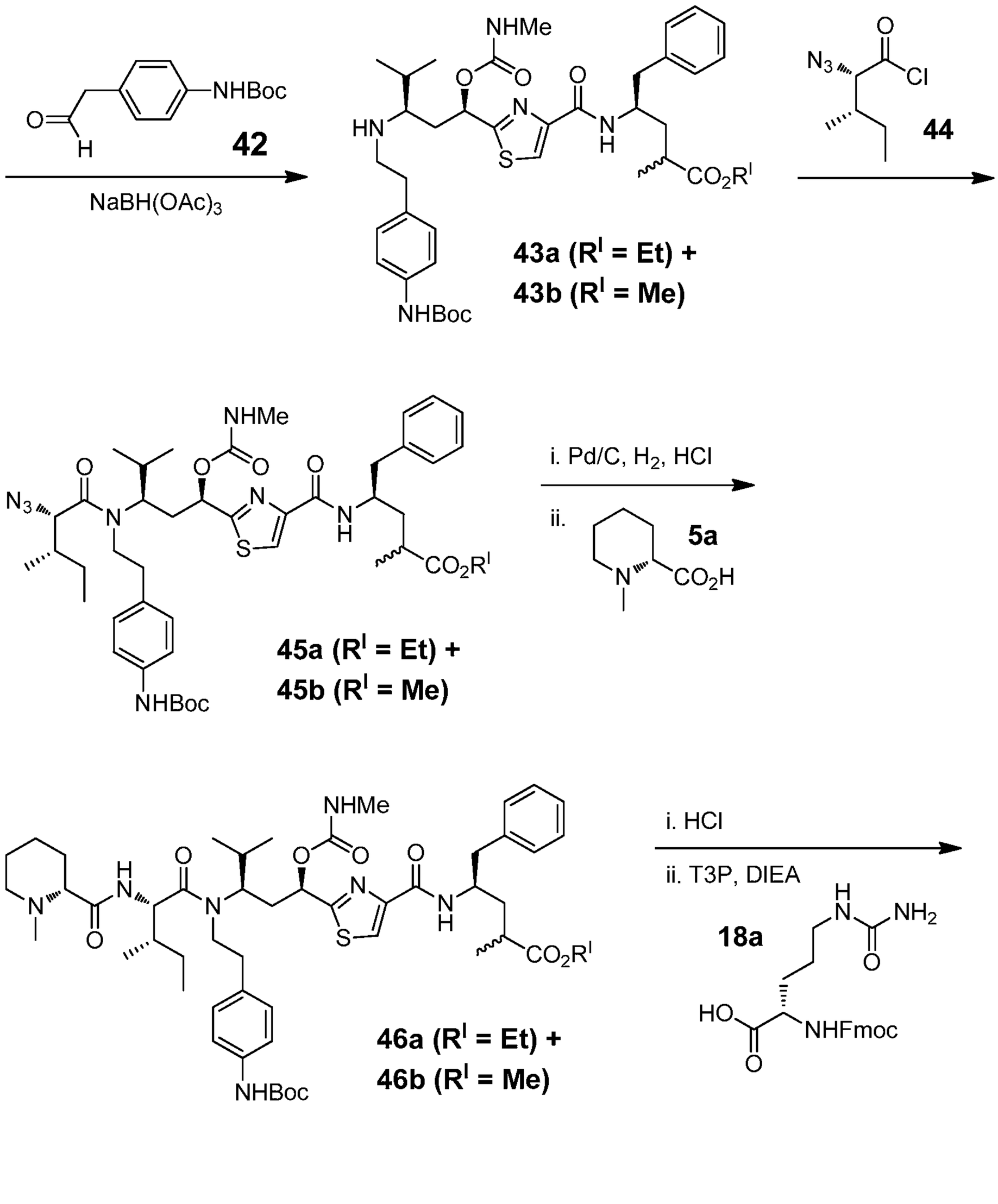

NHBoc
42
NaBH(OAc)3
NHMe
O
N
HN
S
NH
CO2RI
NHBoc
43a (RI = Et) +
43b (RI = Me)
N3
Cl
44
NHMe
NHBoc
45a (RI = Et) +
45b (RI = Me)
i. Pd/C, H2, HCl
ii.
5a
CO2H
46a (RI = Et) +
46b (RI = Me)
i. HCl
ii. T3P, DIEA
18a
NH2
HO
NHFmoc

Fig. 6c

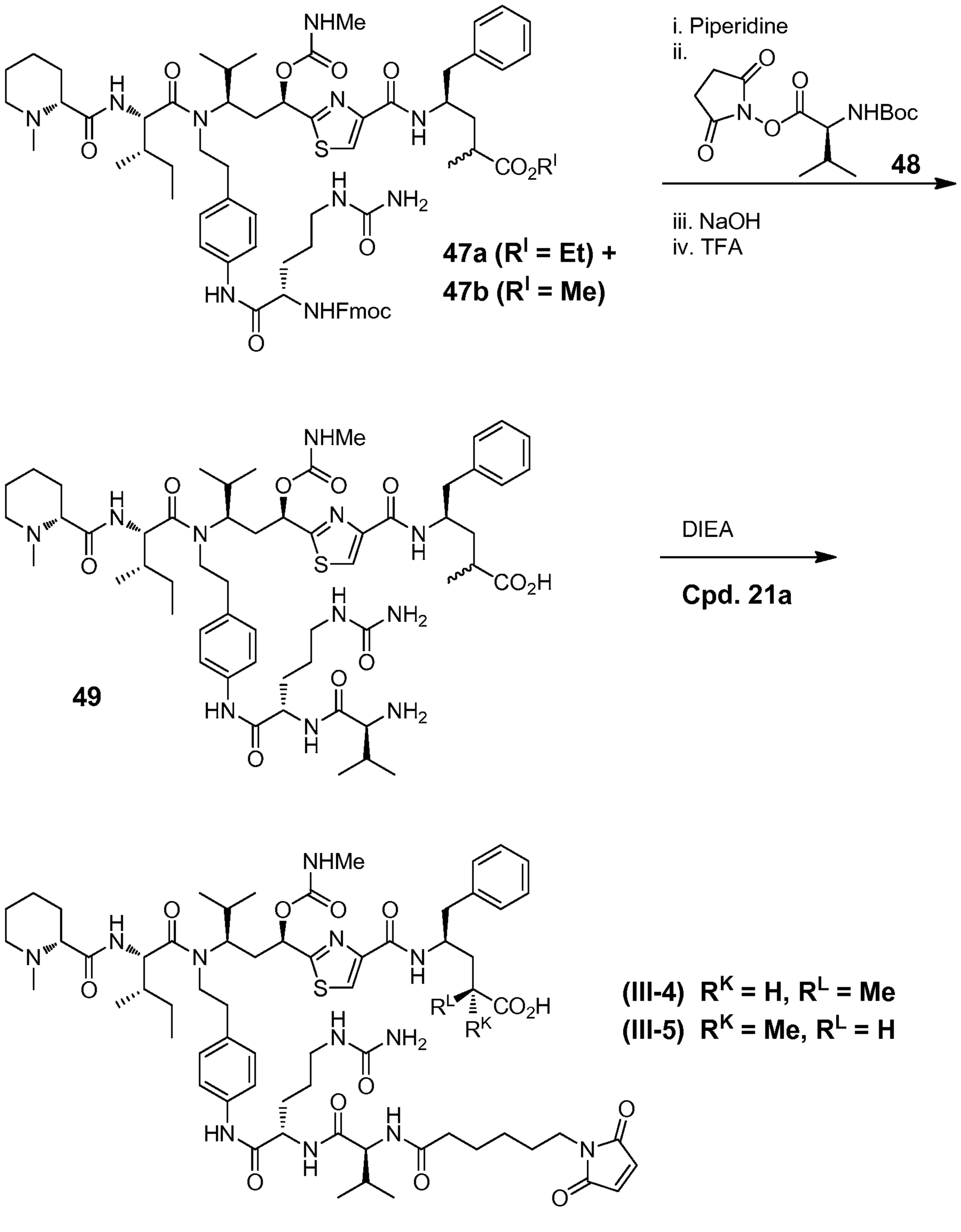
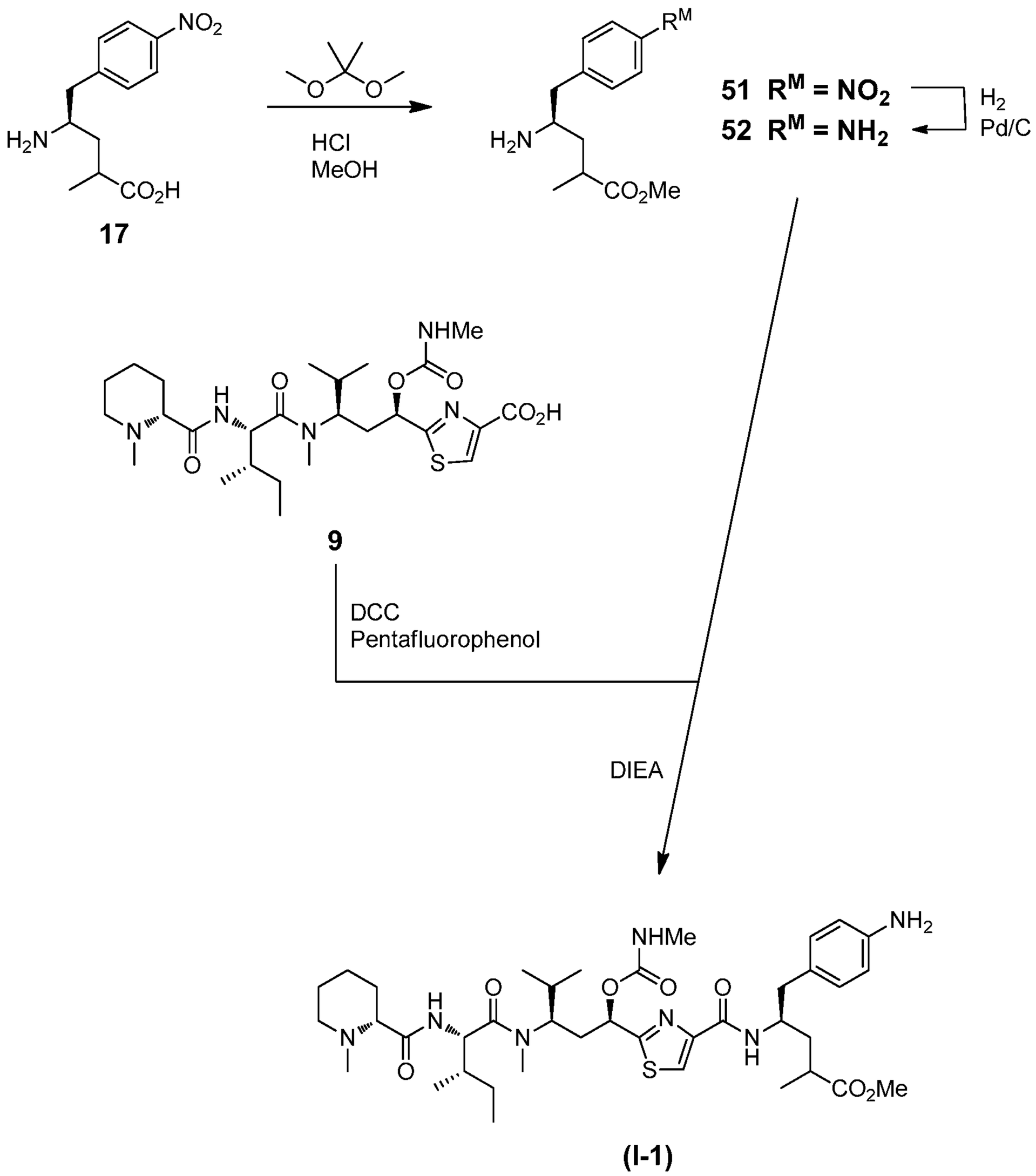
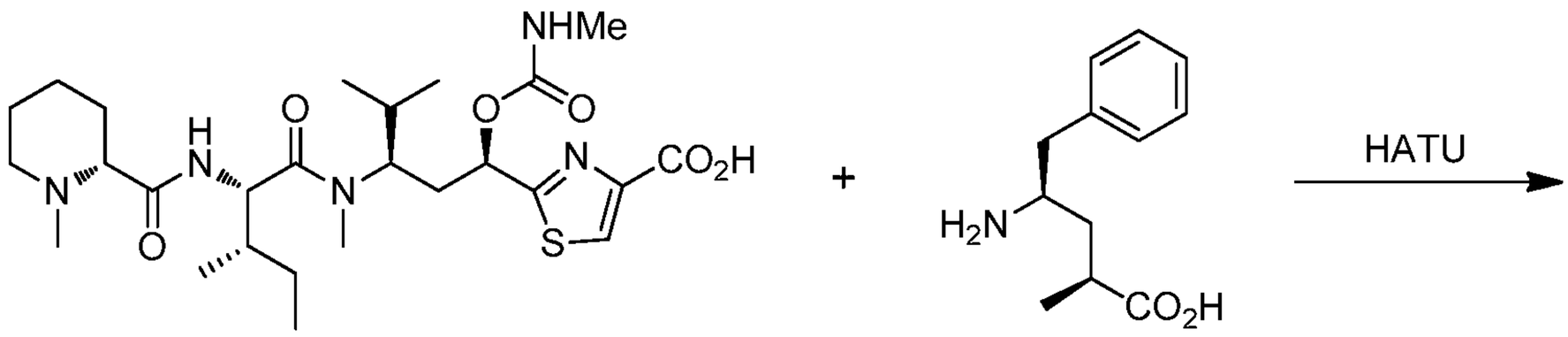

NHMe
O
O
O
N
H
N
O
N
S
CO2RI
H
N
NH2
O
HN
NHFmoc
O
i. Piperidine
ii.
O
N
O
O
NHBoc
48
iii. NaOH
iv. TFA
47a (RI = Et) +
47b (RI = Me)
NHMe
CO2H
DIEA
Cpd. 21a
49
NH2
HN
N
H
RL
RK
(III-4) RK = H, RL = Me
(III-5) RK = Me, RL = H

Fig. 7

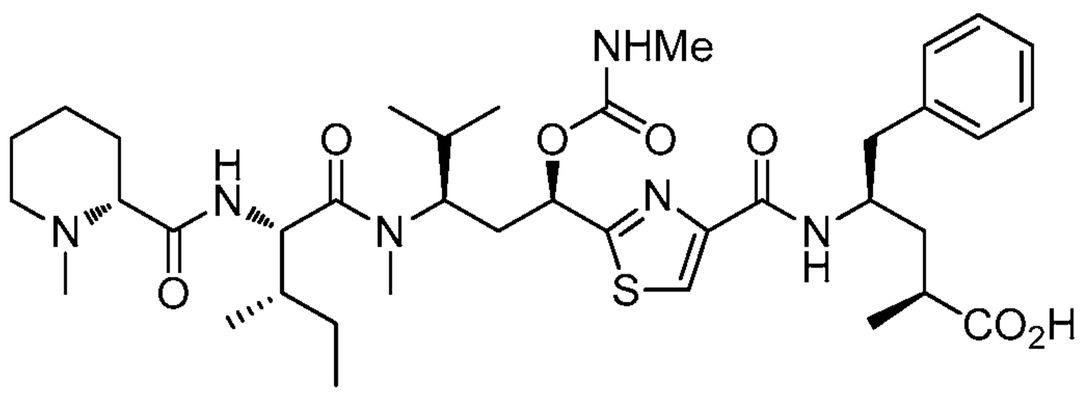

NO2
H2N
CO2H
17
HCl
MeOH
RM
H2N
CO2Me
51 RM = NO2
52 RM = NH2
H2
Pd/C
NHMe
O
O
H
N
O
N
N
CO2H
S
9
DCC
Pentafluorophenol
DIEA
NH2
NHMe
O
O
H
N
O
N
N
O
O
N
S
H
N
CO2Me

(I-1)

Fig. 8



9 53



(I-4)

Fig. 9a

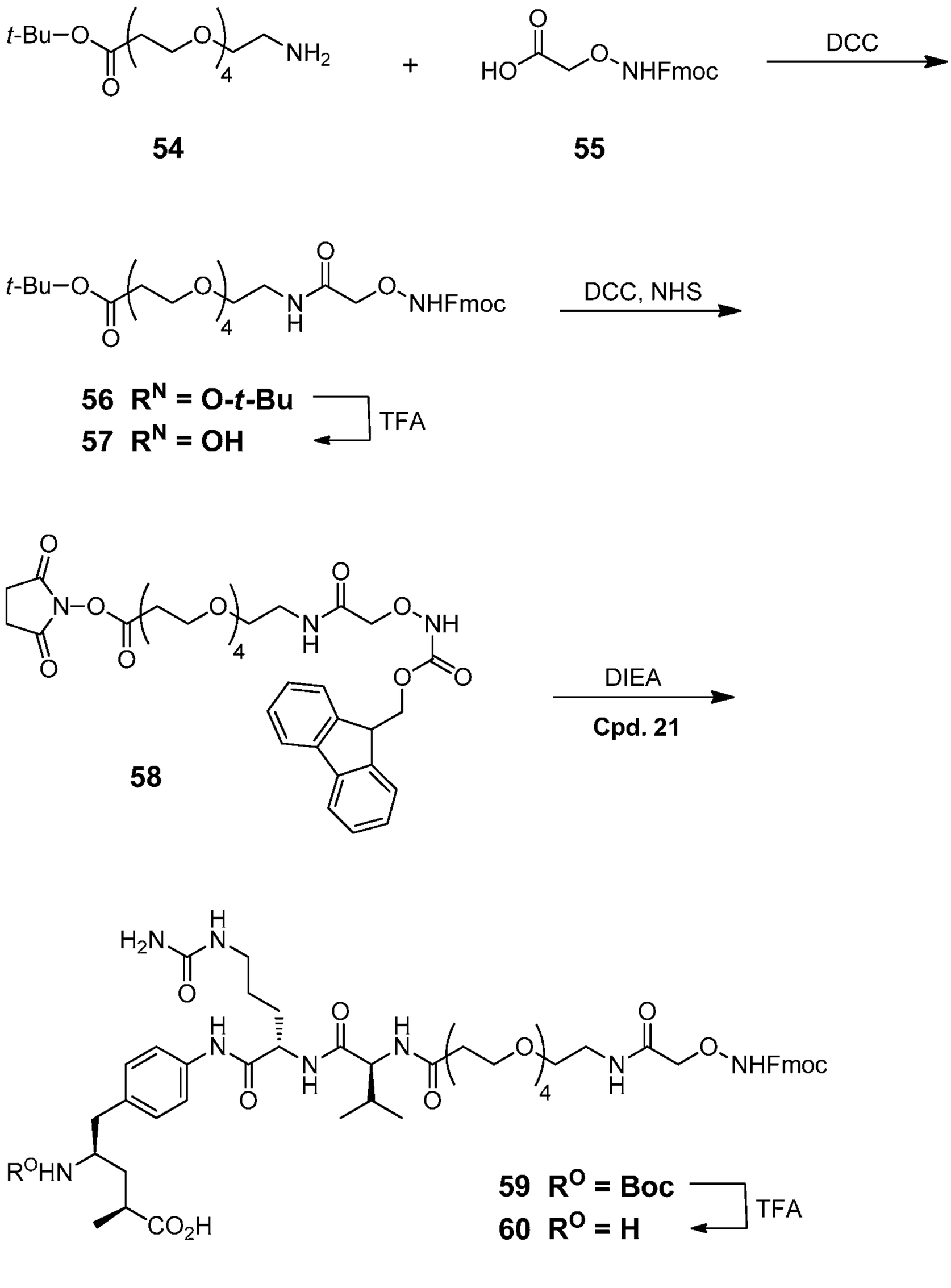

t-Bu–O
O
O
4
NH2
54
+
HO
O
O
NHFmoc
55
DCC
DCC, NHS
56 R^N = O-t-Bu
57 R^N = OH
TFA
N–O
NH
58
DIEA
Cpd. 21
H2N
R^OHN
CO2H
59 R^O = Boc
60 R^O = H
TFA

Fig. 9b

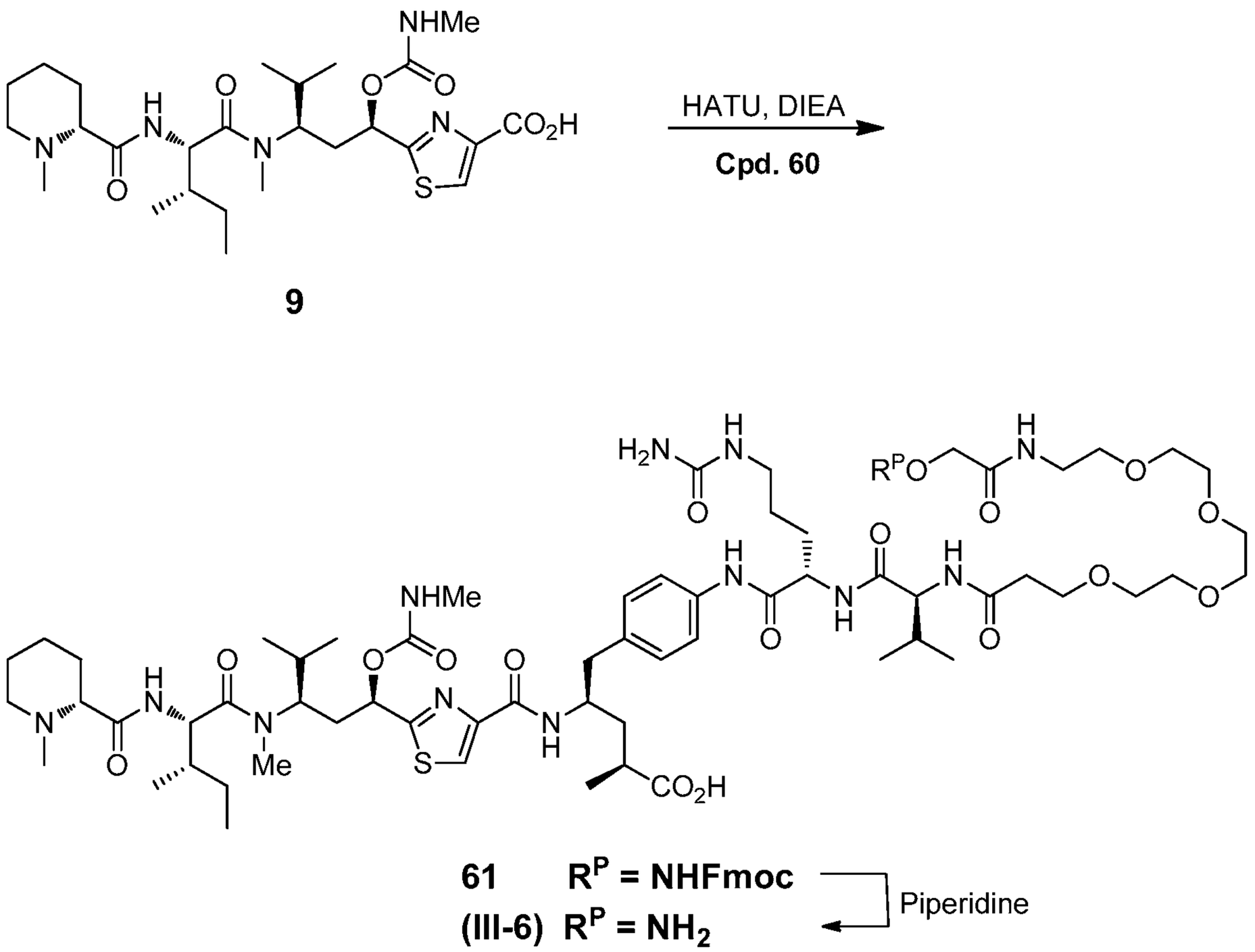

NHMe
HATU, DIEA
Cpd. 60
CO2H
9
H2N
RPO
NHMe
Me
CO2H
61 RP = NHFmoc
(III-6) RP = NH2
Piperidine

Fig. 10

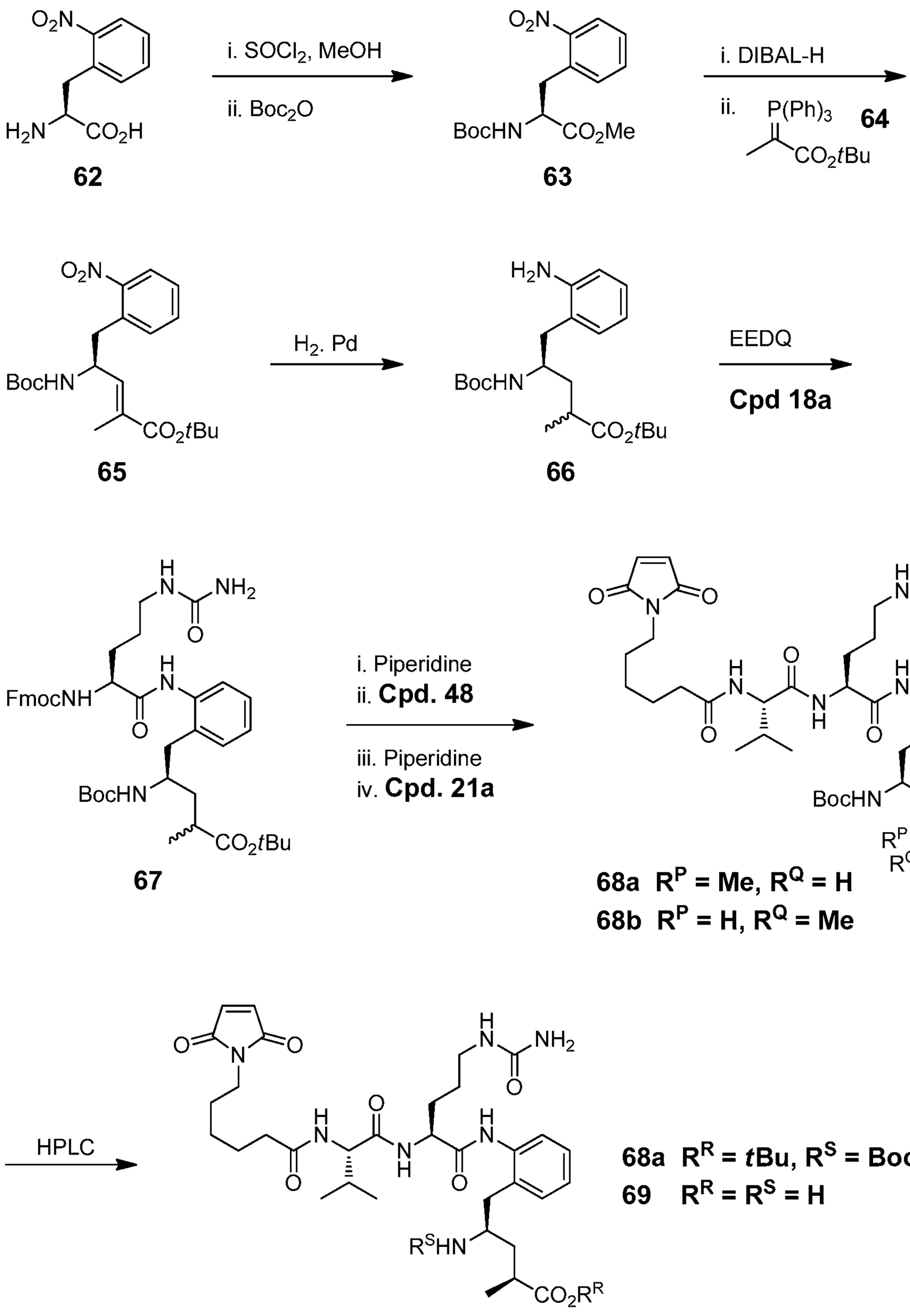
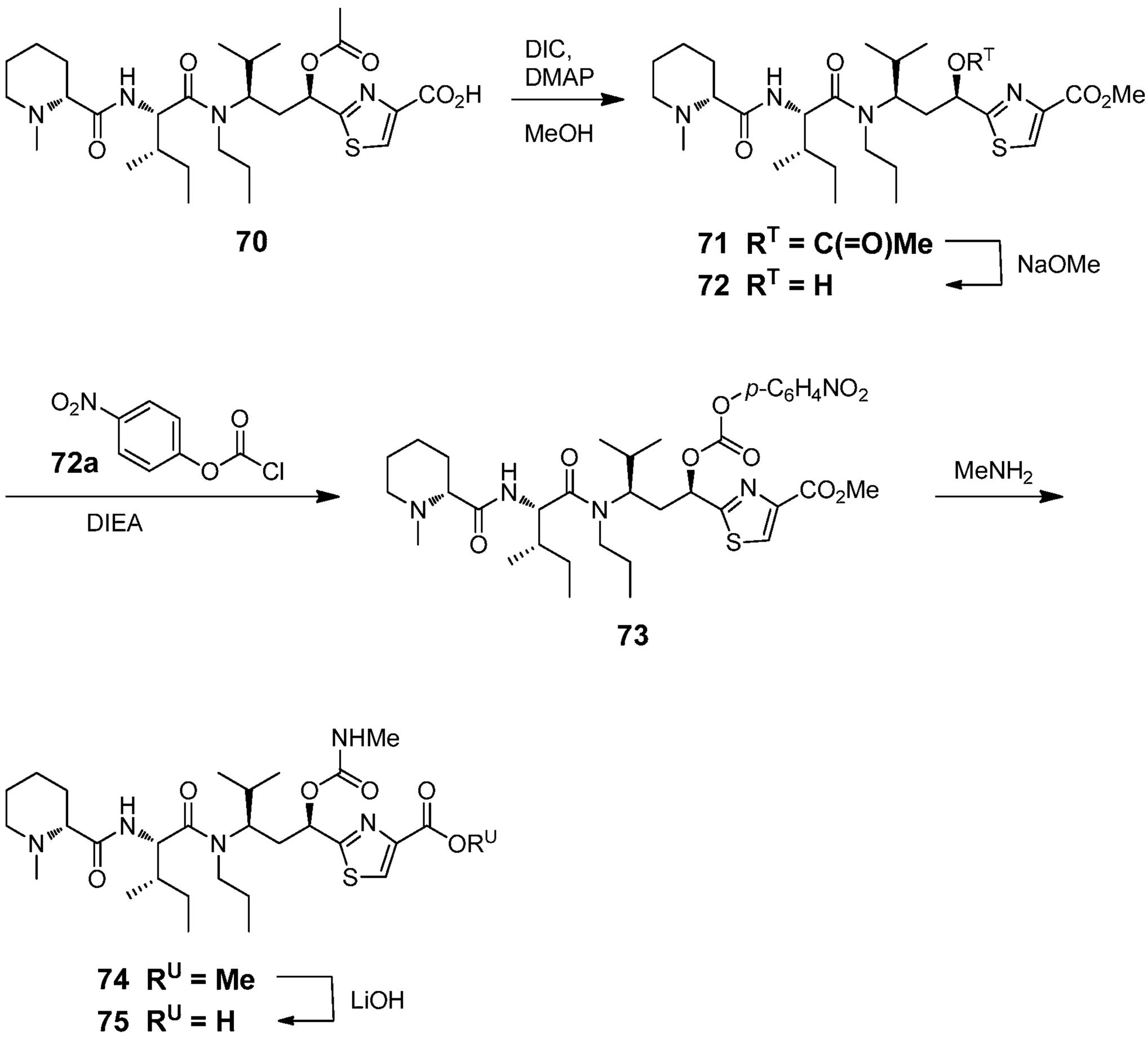

O2N
H2N
CO2H
62
i. SOCl2, MeOH
ii. Boc2O
O2N
BocHN
CO2Me
63
i. DIBAL-H
ii.
P(Ph)3
CO2tBu
64
O2N
BocHN
CO2tBu
65
H2. Pd
H2N
BocHN
CO2tBu
66
EEDQ
Cpd 18a
H
N
NH2
O
FmocNH
BocHN
CO2tBu
67
i. Piperidine
ii. Cpd. 48
iii. Piperidine
iv. Cpd. 21a
RP
RQ
68a RP = Me, RQ = H
68b RP = H, RQ = Me
HPLC
RSHN
CO2RR
68a RR = tBu, RS = Boc
69 RR = RS = H
TFA

Fig. 11


DIC,
DMAP
MeOH
CO2H
OR^T
CO2Me
71 R^T = C(=O)Me
72 R^T = H
NaOMe
O2N
72a
DIEA
p-C6H4NO2
MeNH2
73
NHMe
OR^U
74 R^U = Me
75 R^U = H
LiOH

70

Fig. 12

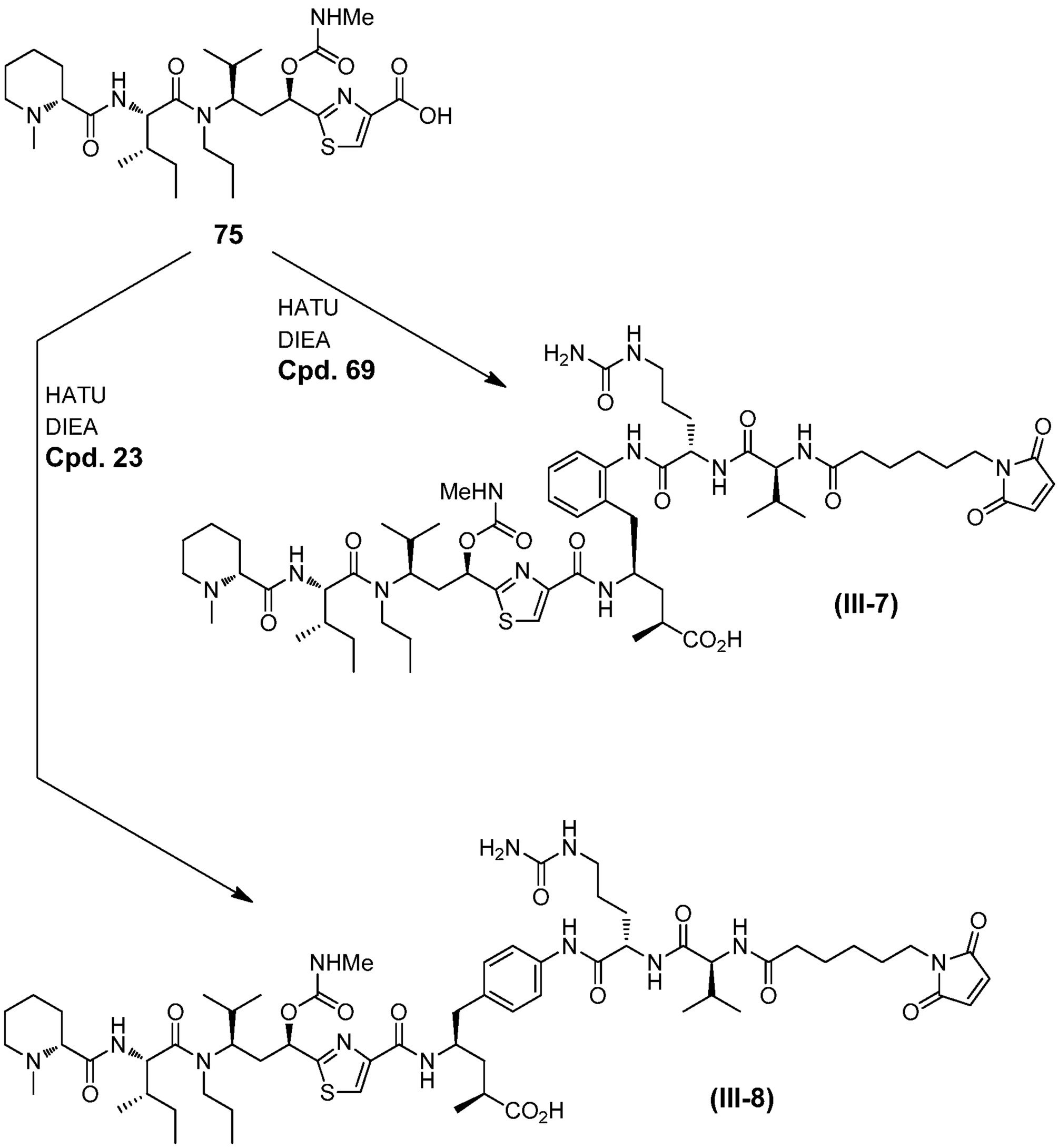

NHMe
O
O
O
H
N
O
N
N
N
OH
O
S
75
HATU
DIEA
Cpd. 69
HATU
DIEA
Cpd. 23
MeHN
NHMe
(III-7)
(III-8)

Fig. 13a

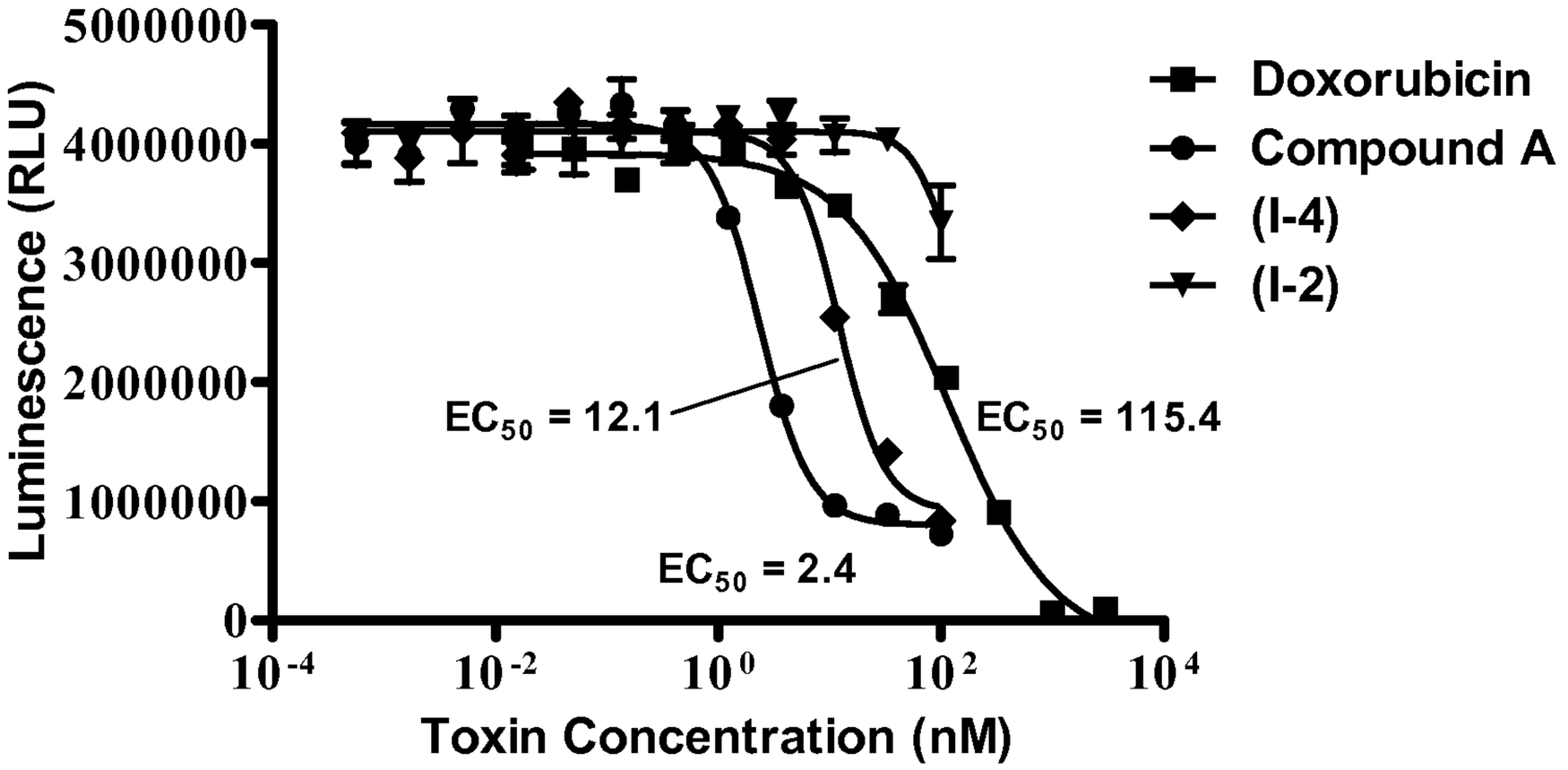


Fig. 13b

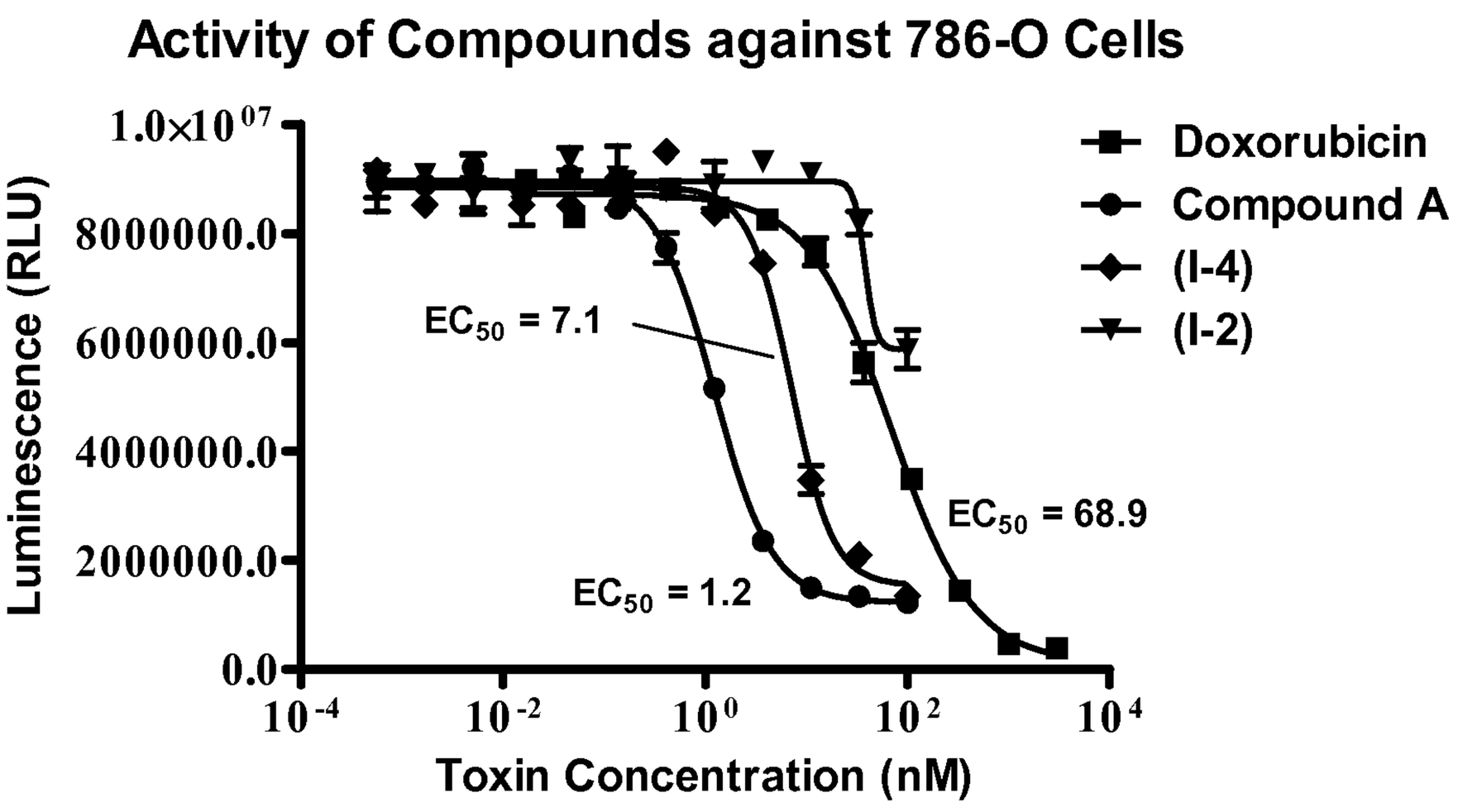
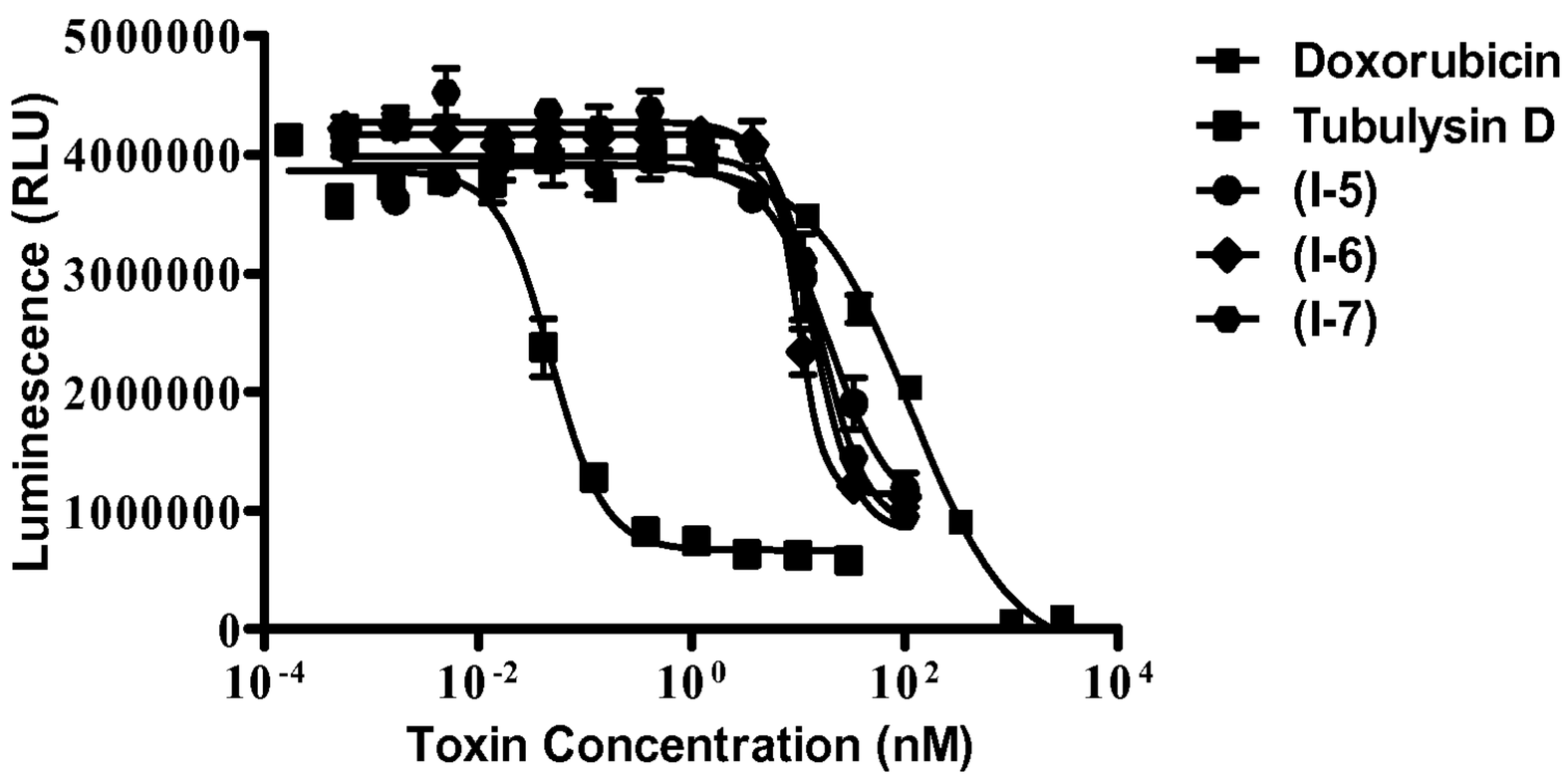
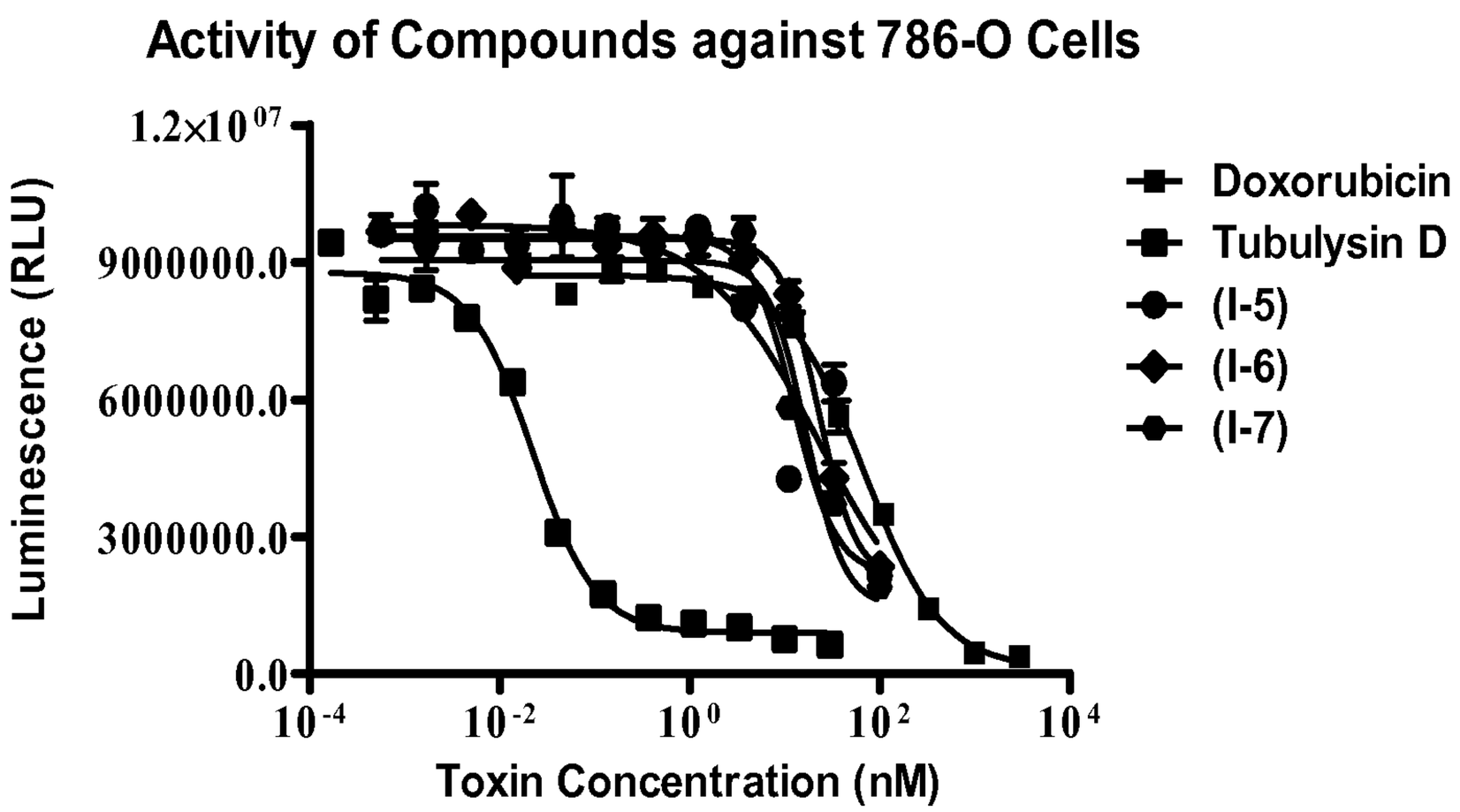


Fig. 13c



Compound	Doxorubicin	Tubulysin D	(I-5)	(I-6)	(I-7)
EC_{50} (nM)	115.4	0.05	19.5	9.9	15.4

Fig. 13d



Compound	Doxorubicin	Tubulysin D	(I-5)	(I-6)	(I-7)
EC_{50} (nM)	68.9	0.02	22.9	22.8	12.5

Fig. 14

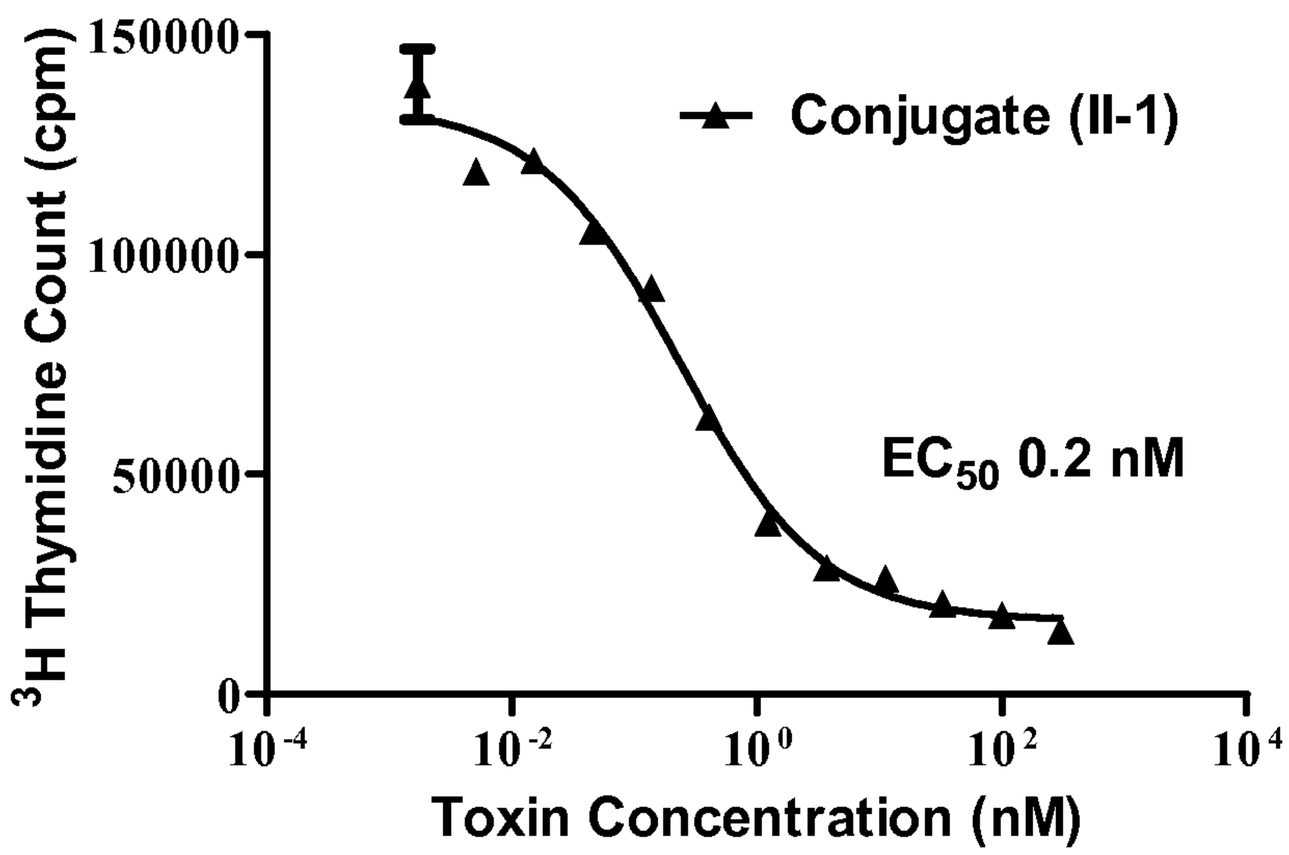

^{3}H Thymidine Incorporation Proliferation Assay
N87 Cells
^{3}H Thymidine Count (cpm)
150000
100000
50000
0
Conjugate (II-1)
EC_{50} 0.2 nM
10^{-4}
10^{-2}
10^{0}
10^{2}
10^{4}
Toxin Concentration (nM)

Fig. 15

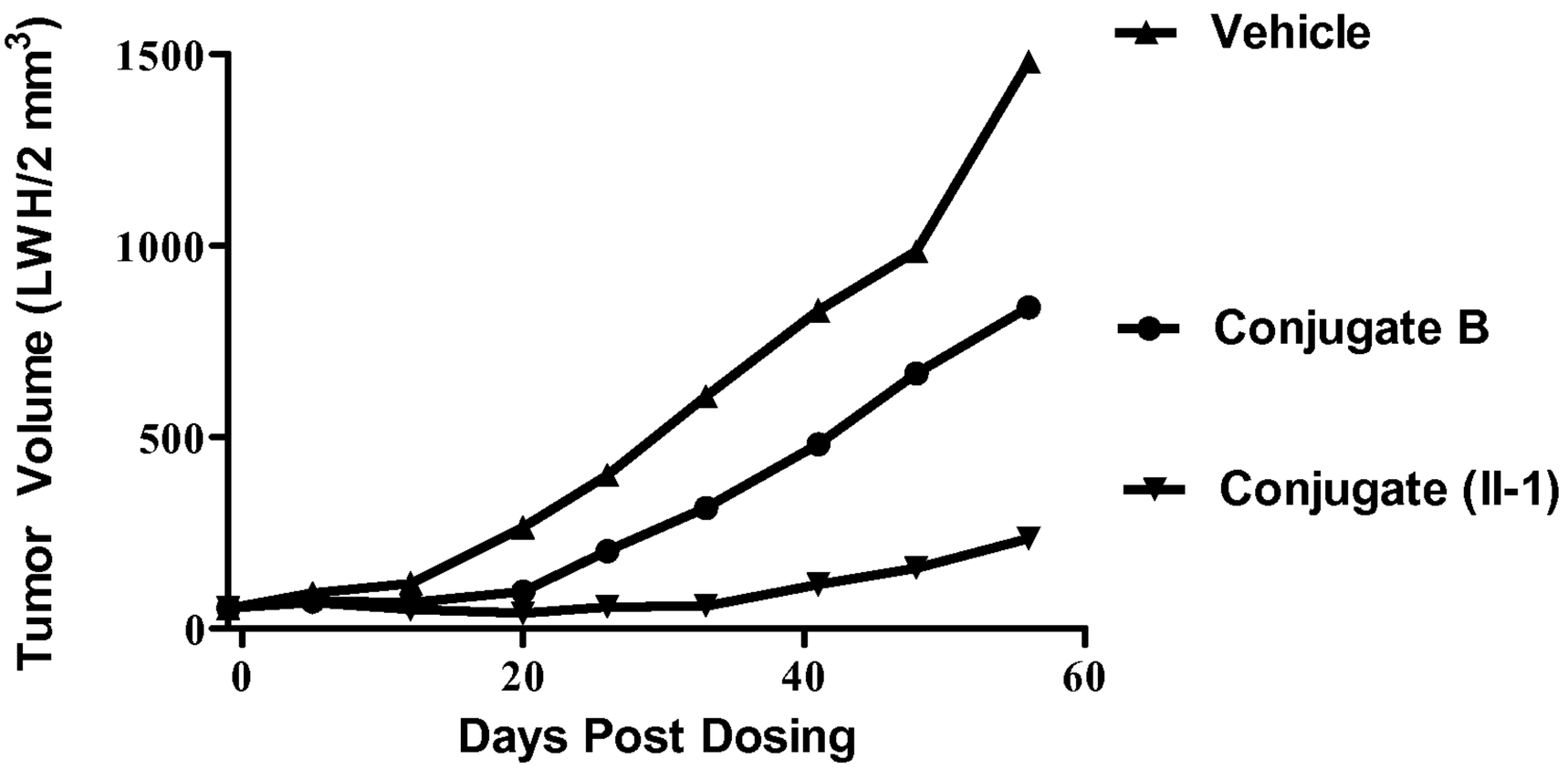

In Vivo Activity of Conjugates Against OVCAR3 Xenografts
Tumor Volume (LWH/2 mm^3)
1500
1000
500
0
0
20
40
60
Days Post Dosing
Vehicle
Conjugate B
Conjugate (II-1)

Fig. 16a

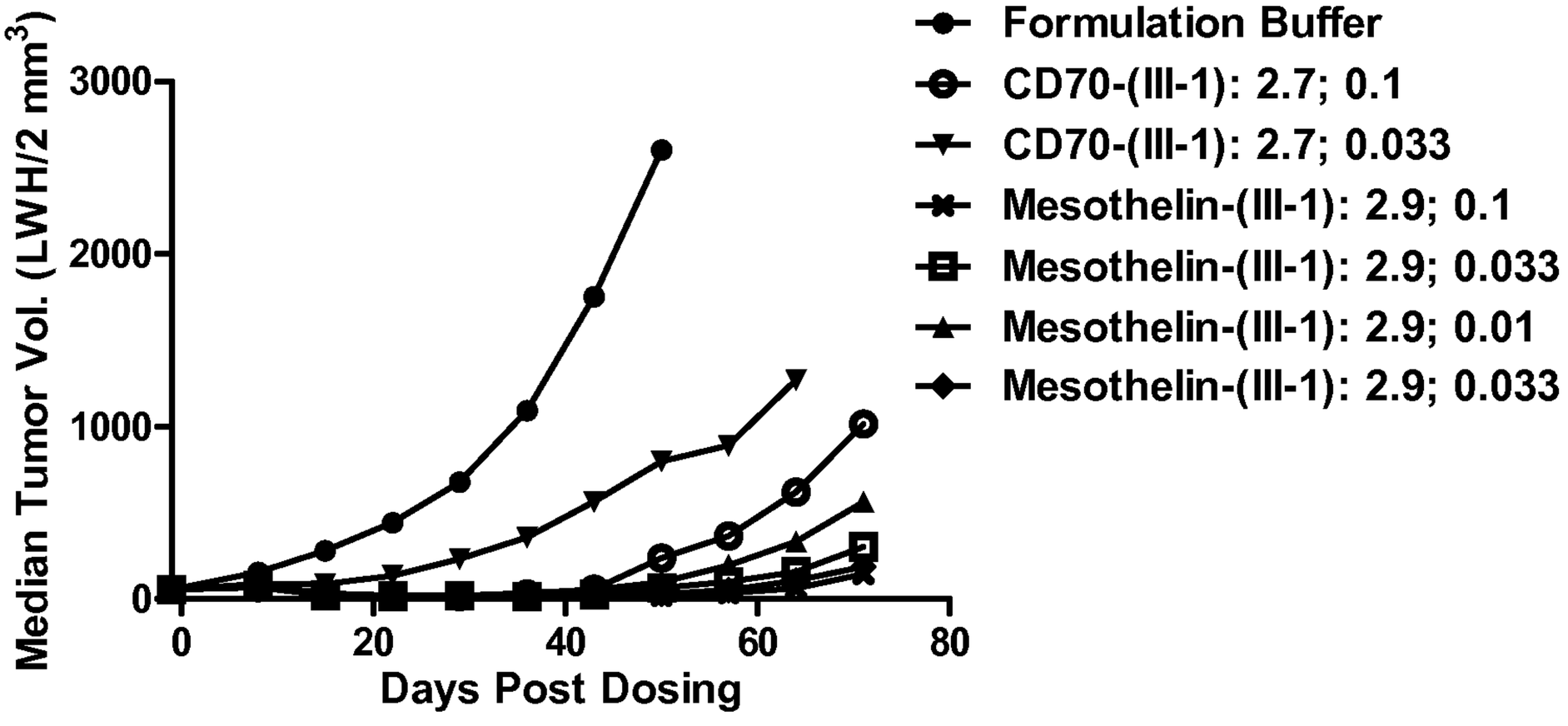


Fig. 16b

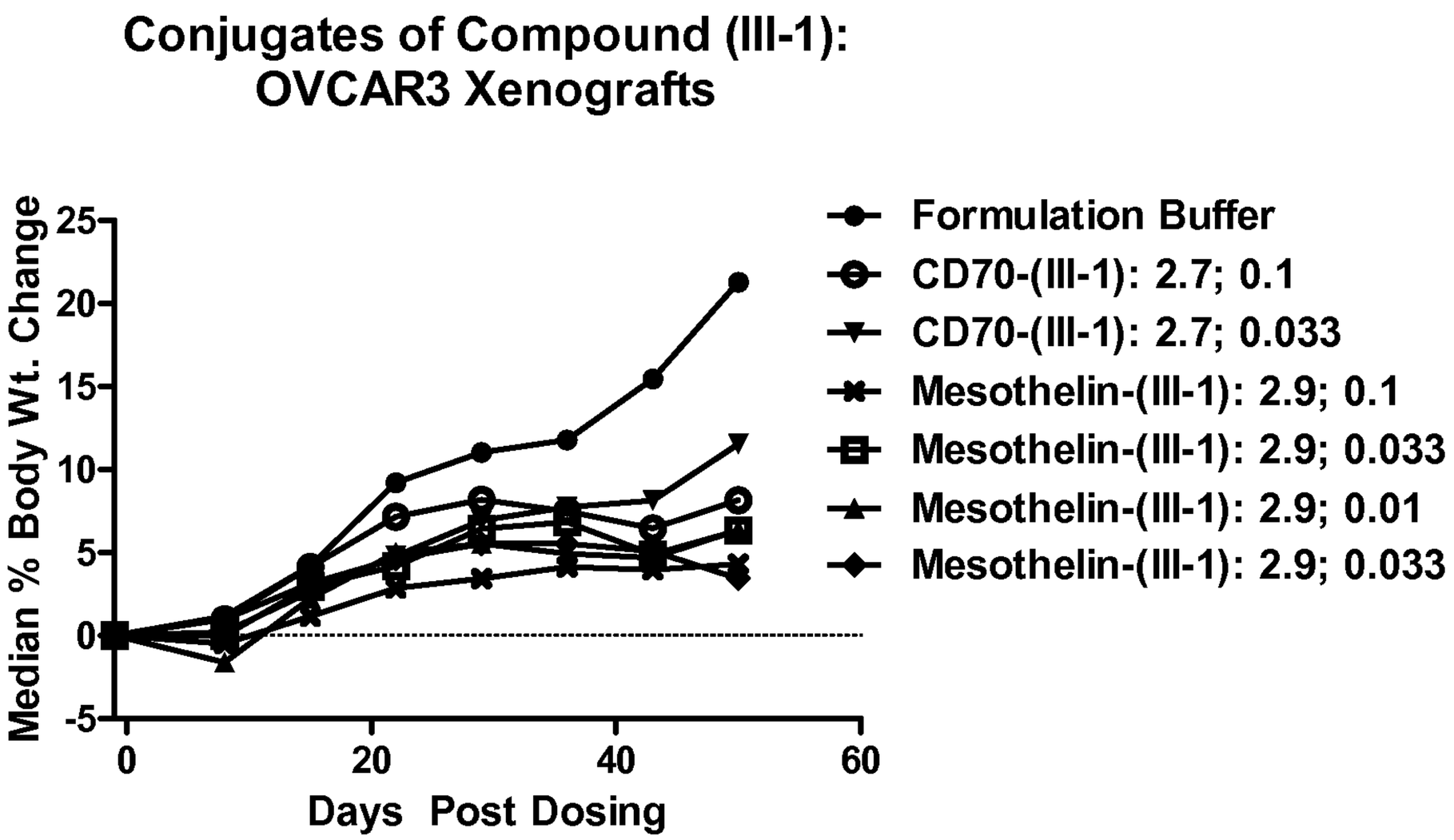


Fig. 17a

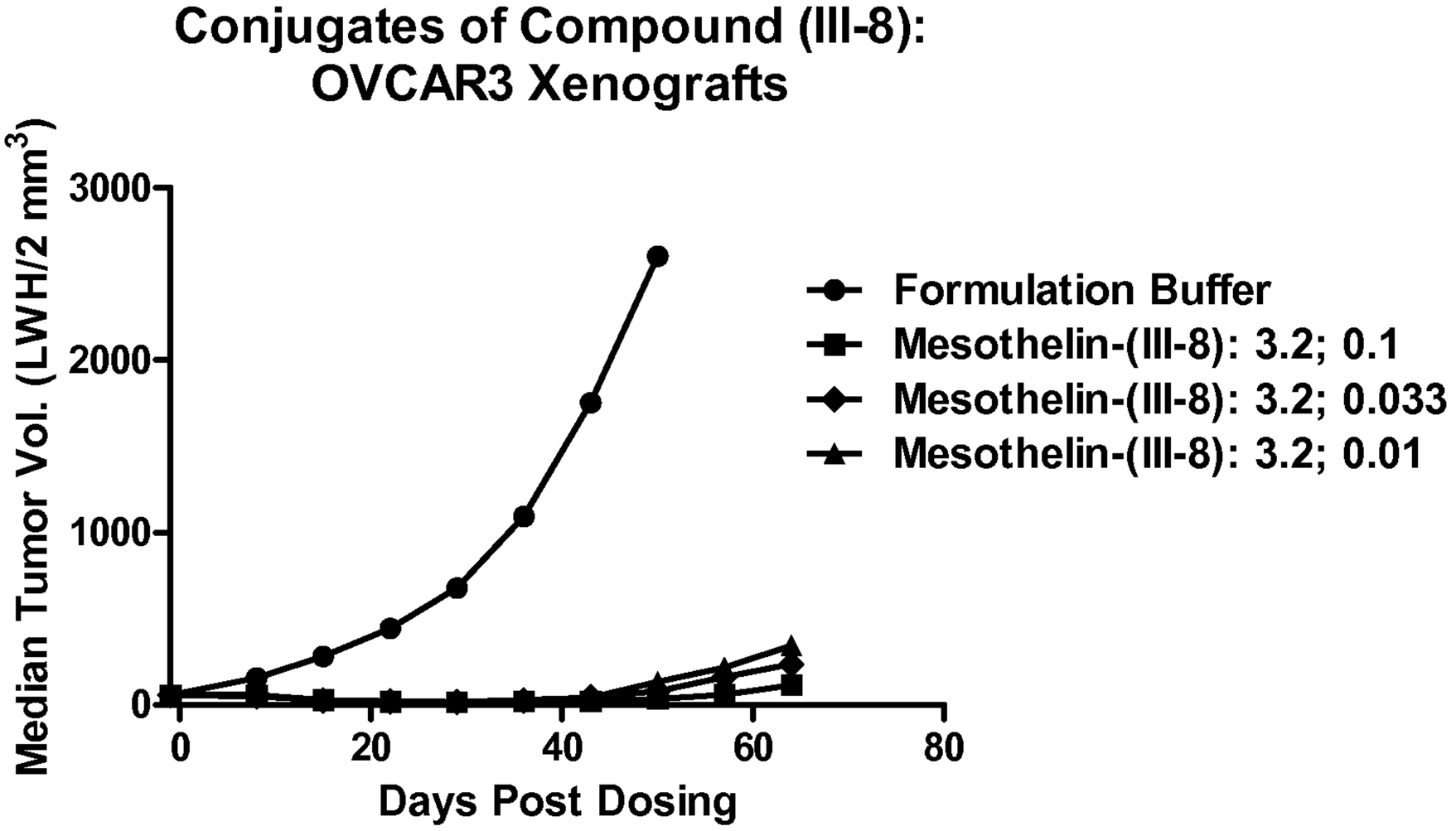


Fig. 17b

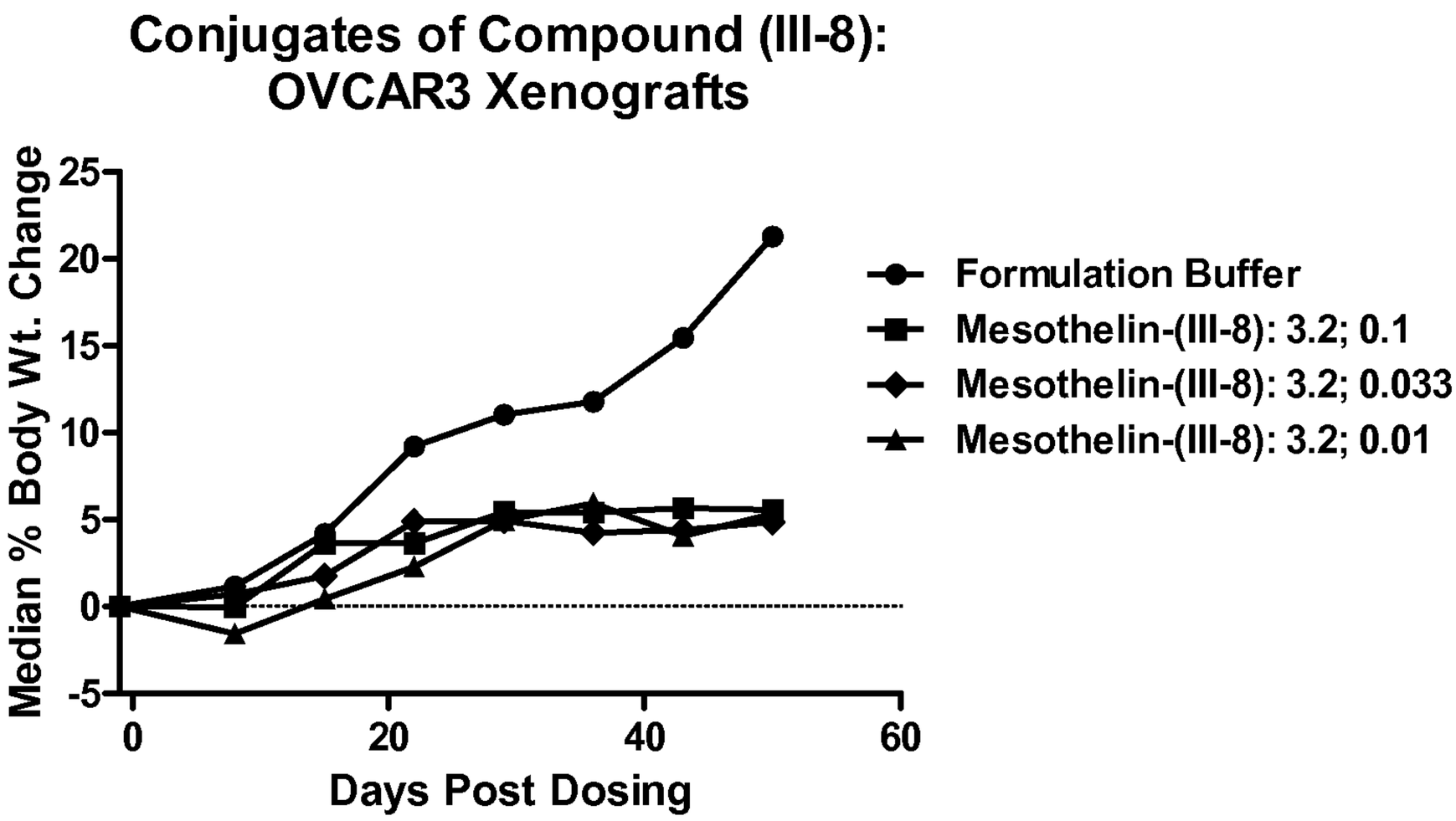


Fig. 18a

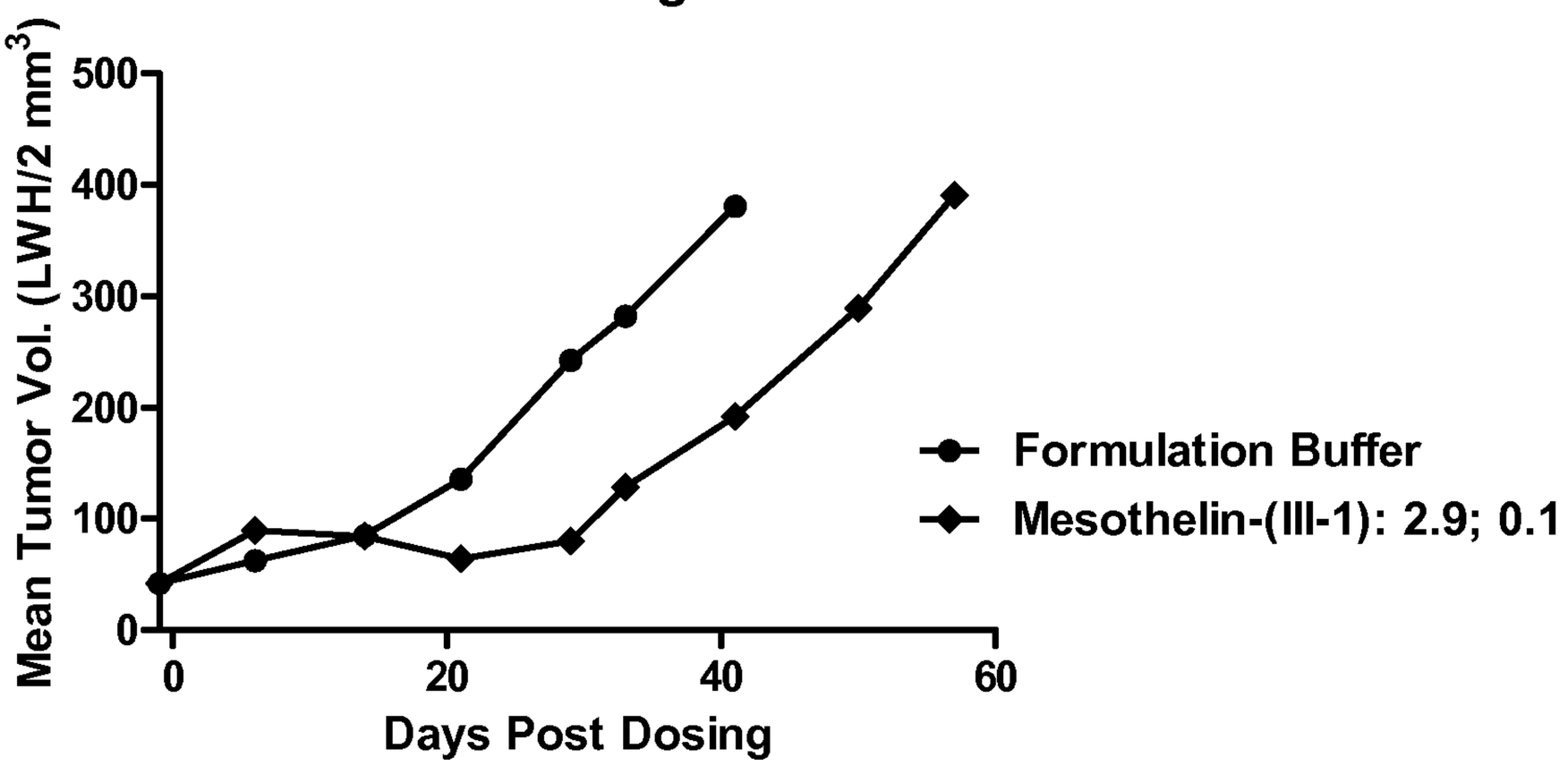


Fig. 18b

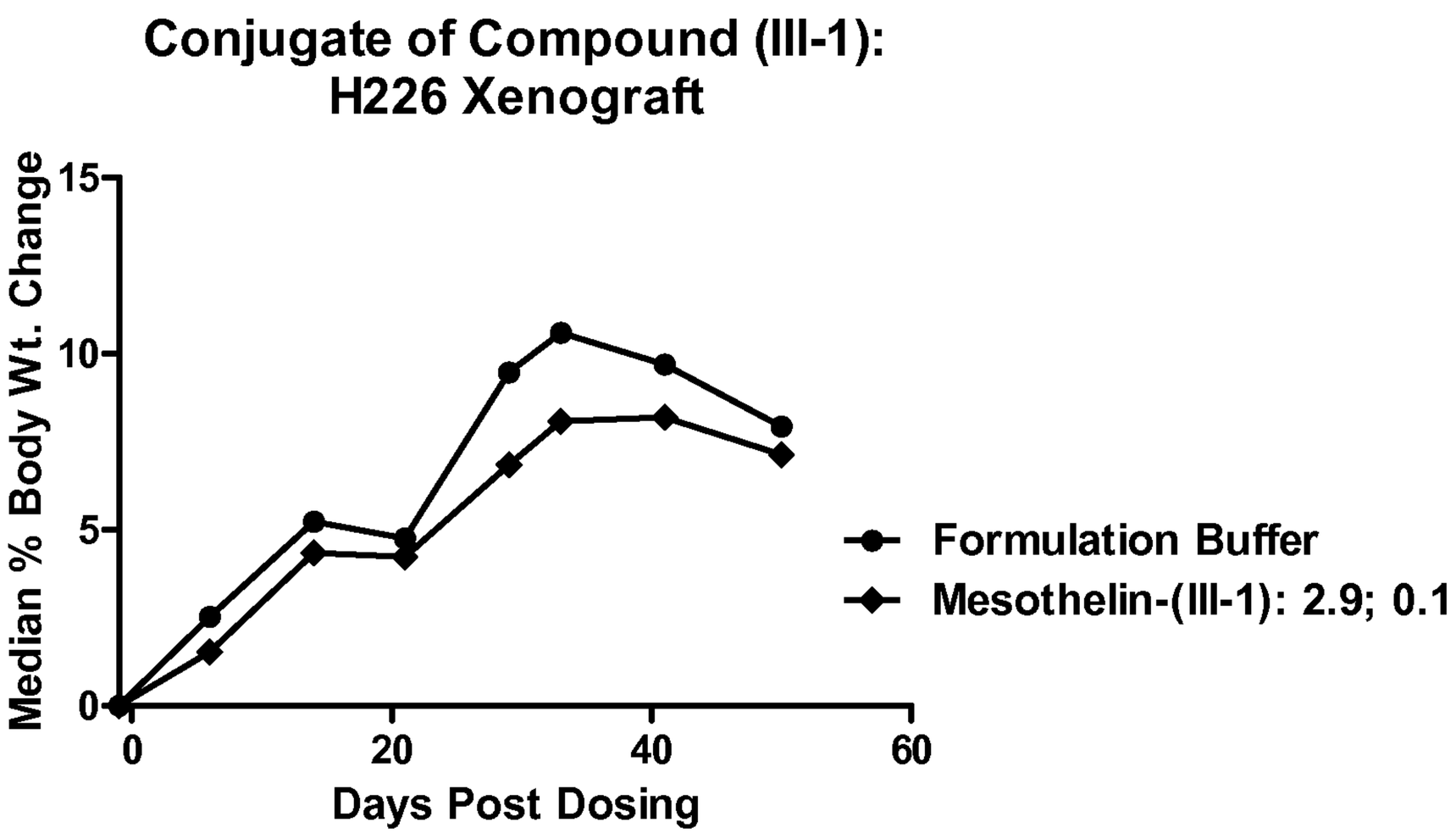


Fig. 19a

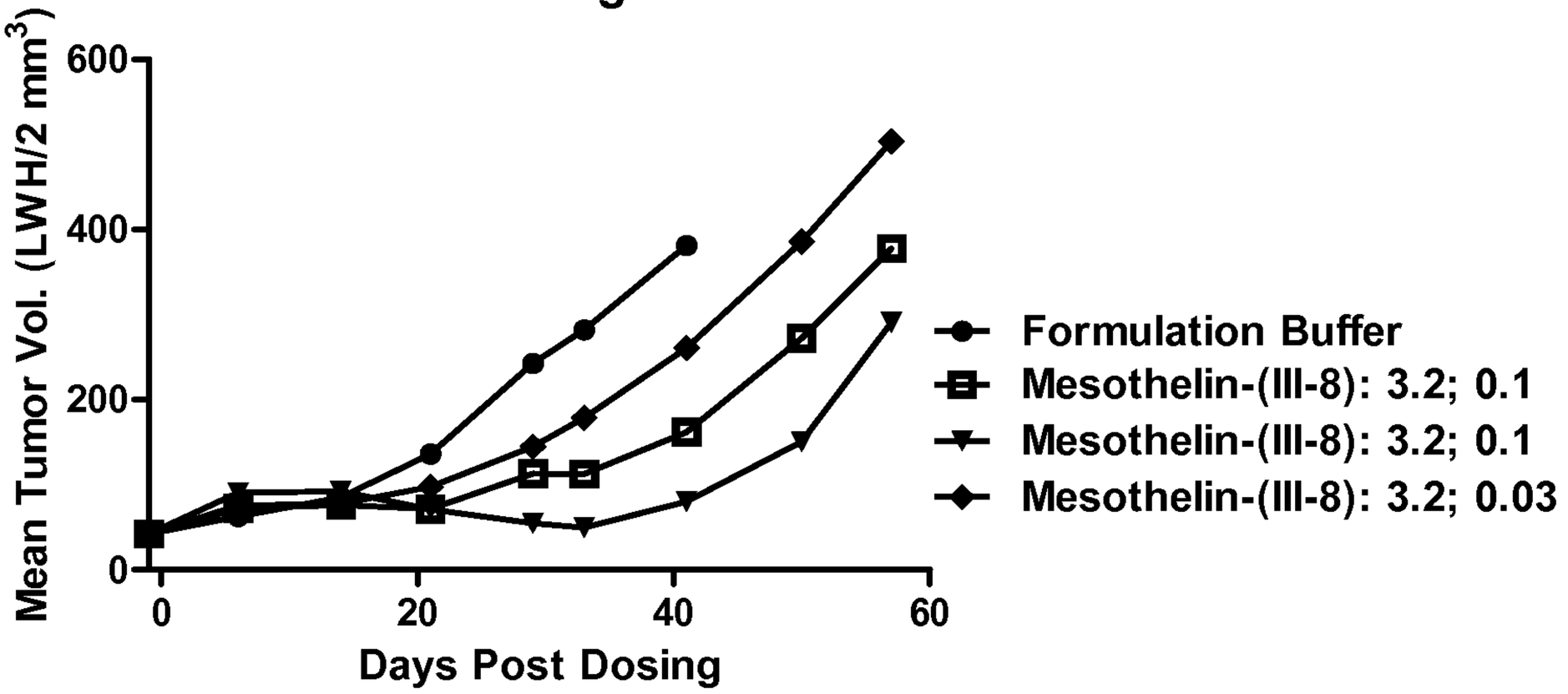


Fig. 19b

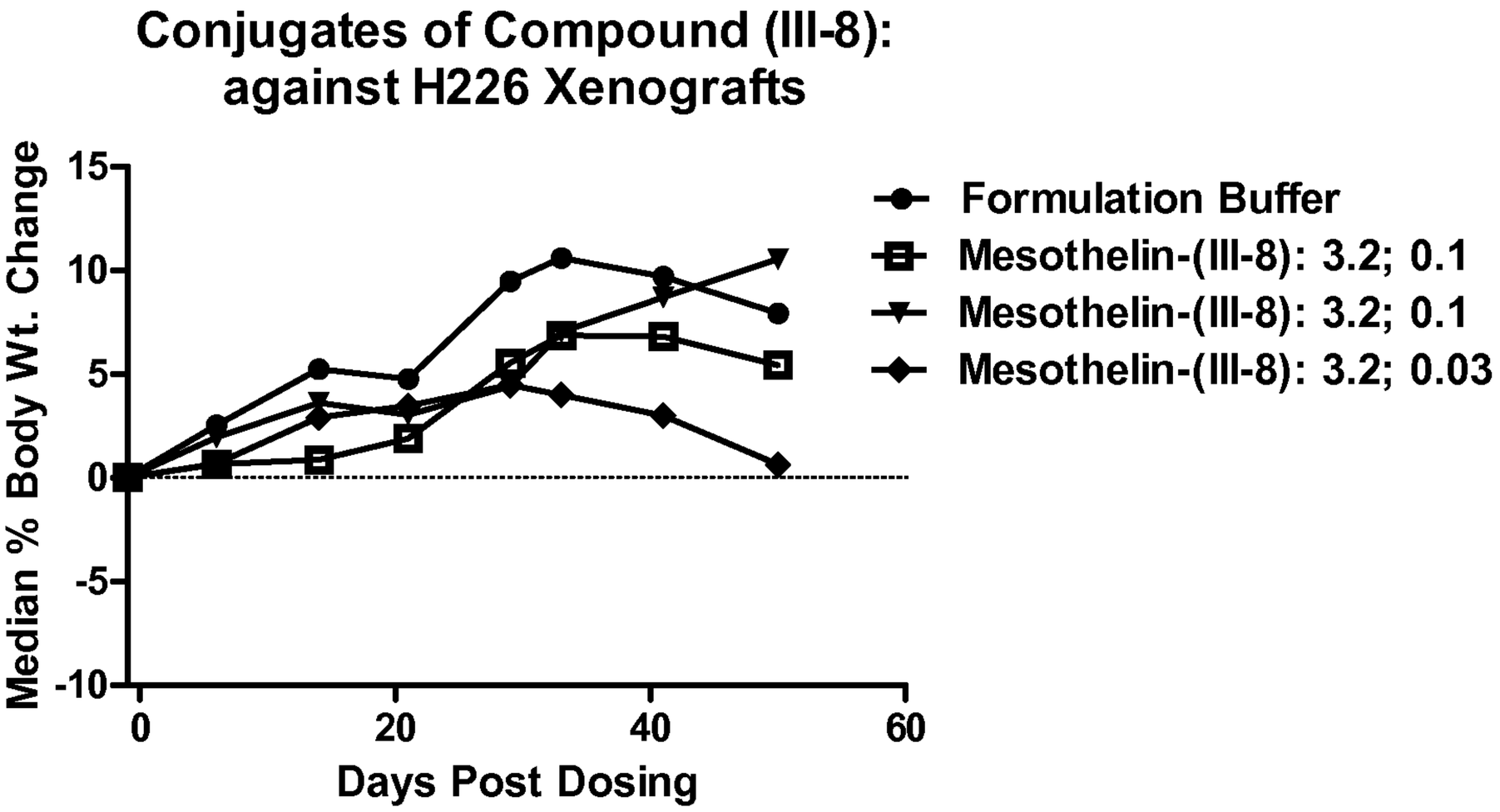


Fig. 20

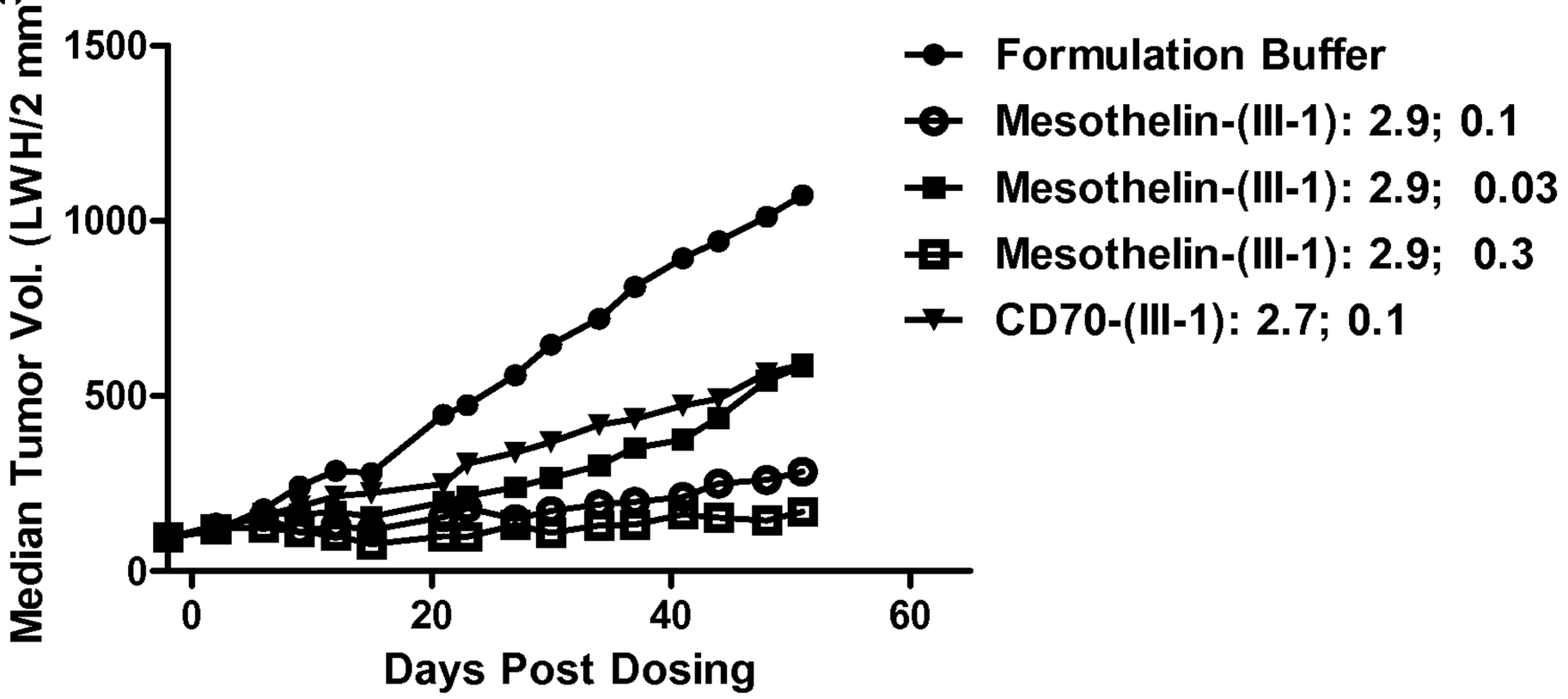
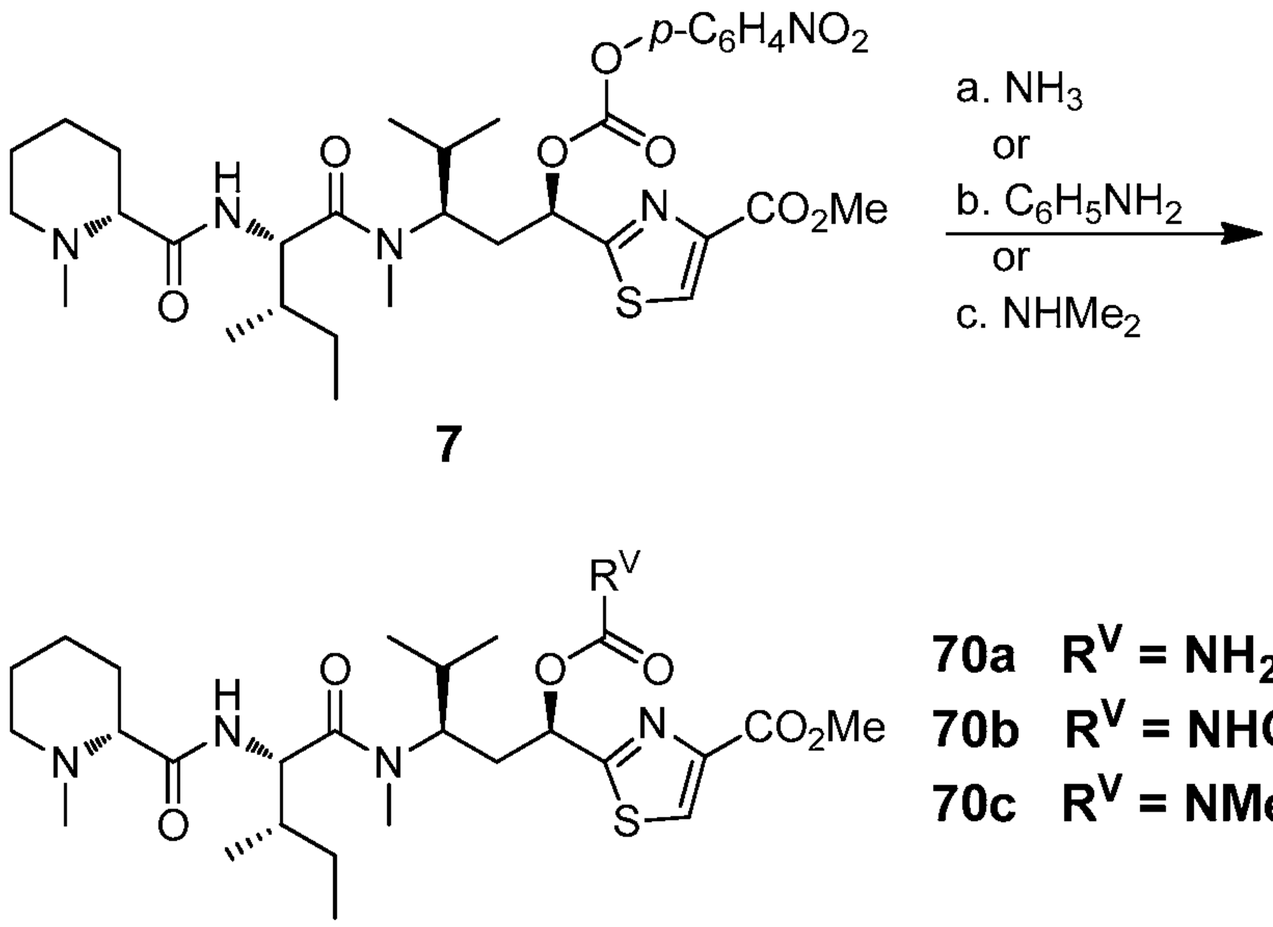
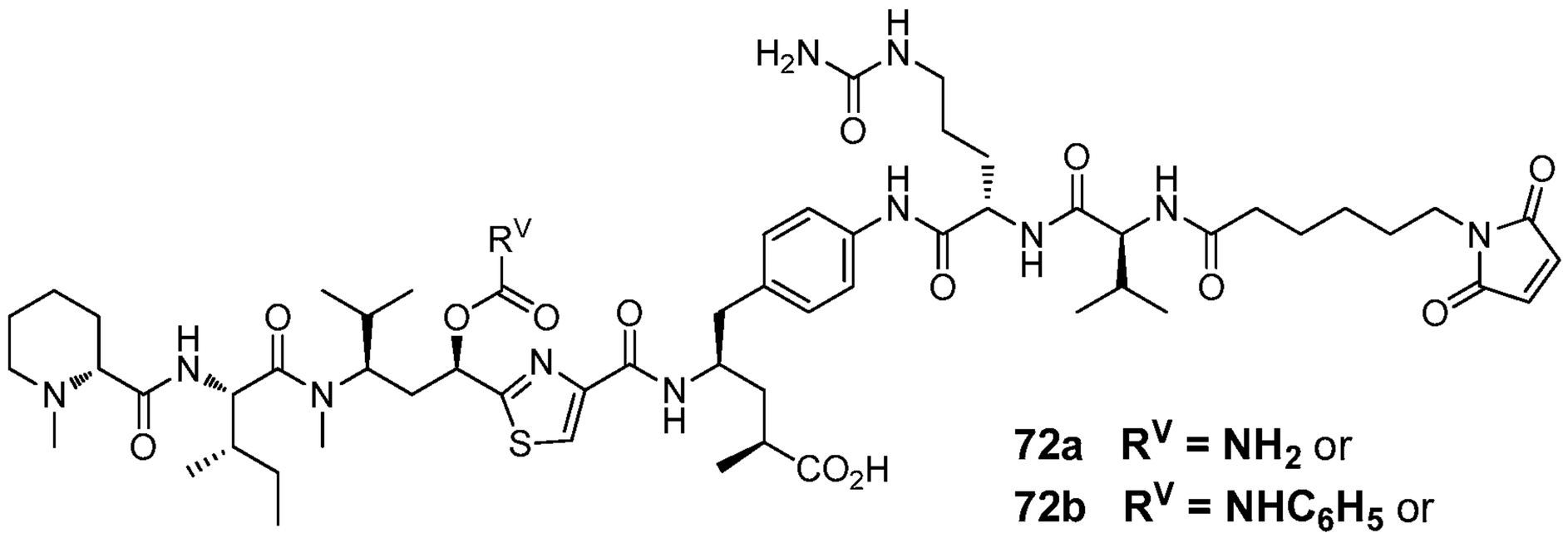

Conjugates of Compound (III-1):
N87 Xenografts
Median Tumor Vol. (LWH/2 mm3)
1500
1000
500
0
0
20
40
60
Days Post Dosing
Formulation Buffer
Mesothelin-(III-1): 2.9; 0.1
Mesothelin-(III-1): 2.9; 0.03
Mesothelin-(III-1): 2.9; 0.3
CD70-(III-1): 2.7; 0.1

Fig. 21

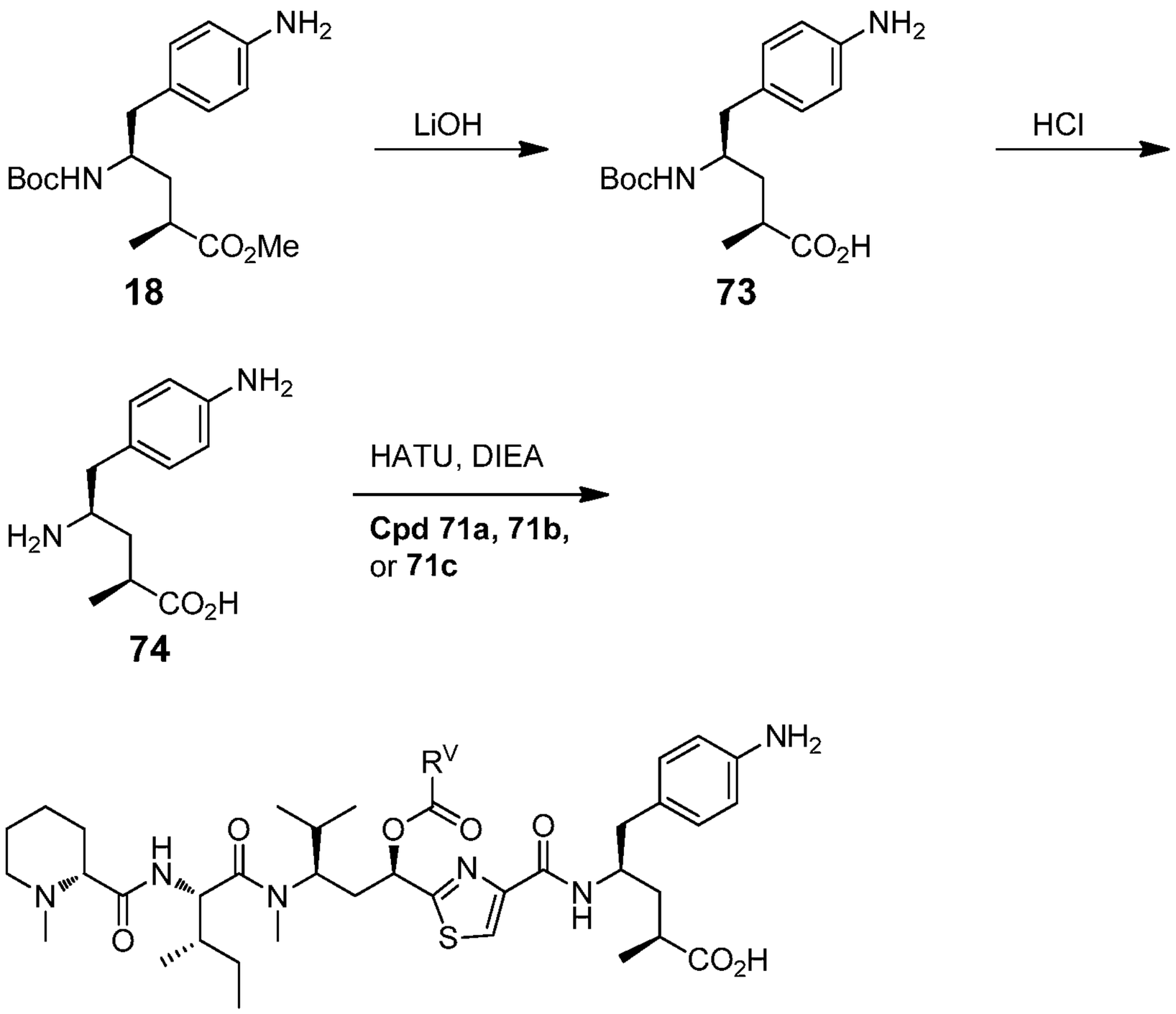


71a $R^V = NH_2$ or
71b $R^V = NHC_6H_5$ or
71c $R^V = NMe_2$



72a $R^V = NH_2$ or
72b $R^V = NHC_6H_5$ or
72c $R^V = NMe_2$

Fig. 22



18 **73**

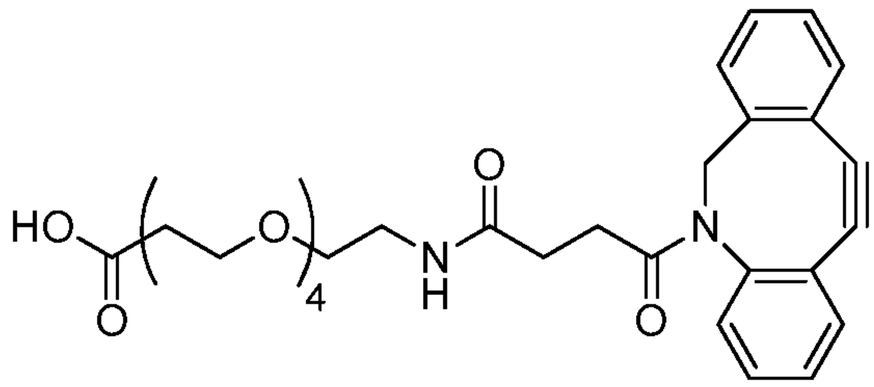
74

75a $R^V = NH_2$ or

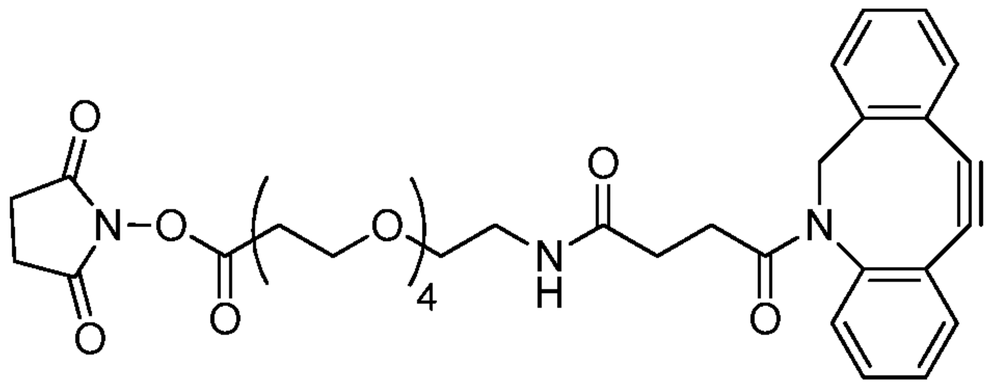
75b $R^V = NHC_6H_5$ or

75c $R^V = NMe_2$

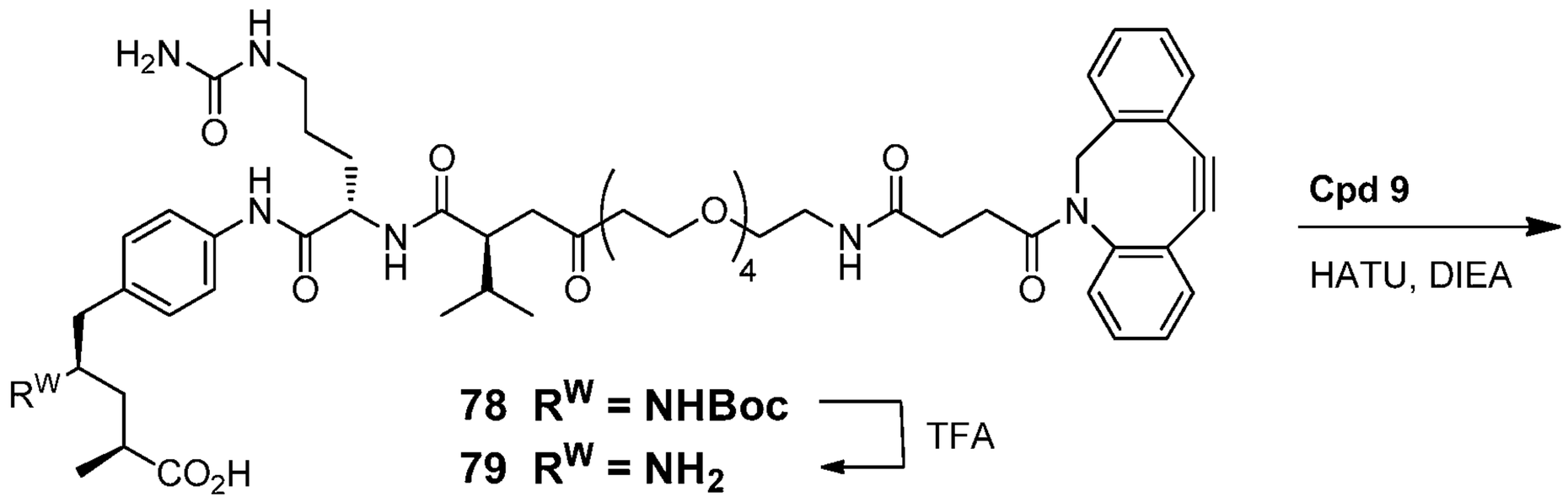
Fig. 23



76



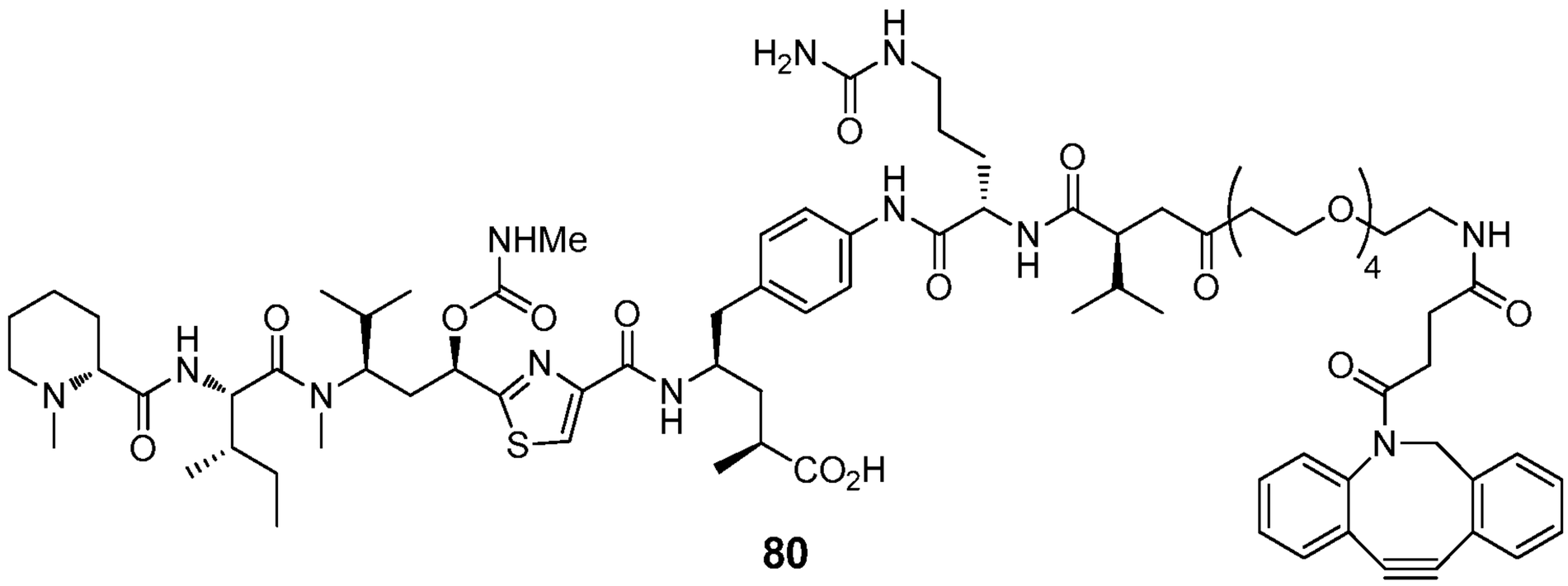
77



78 R^W = NHBoc

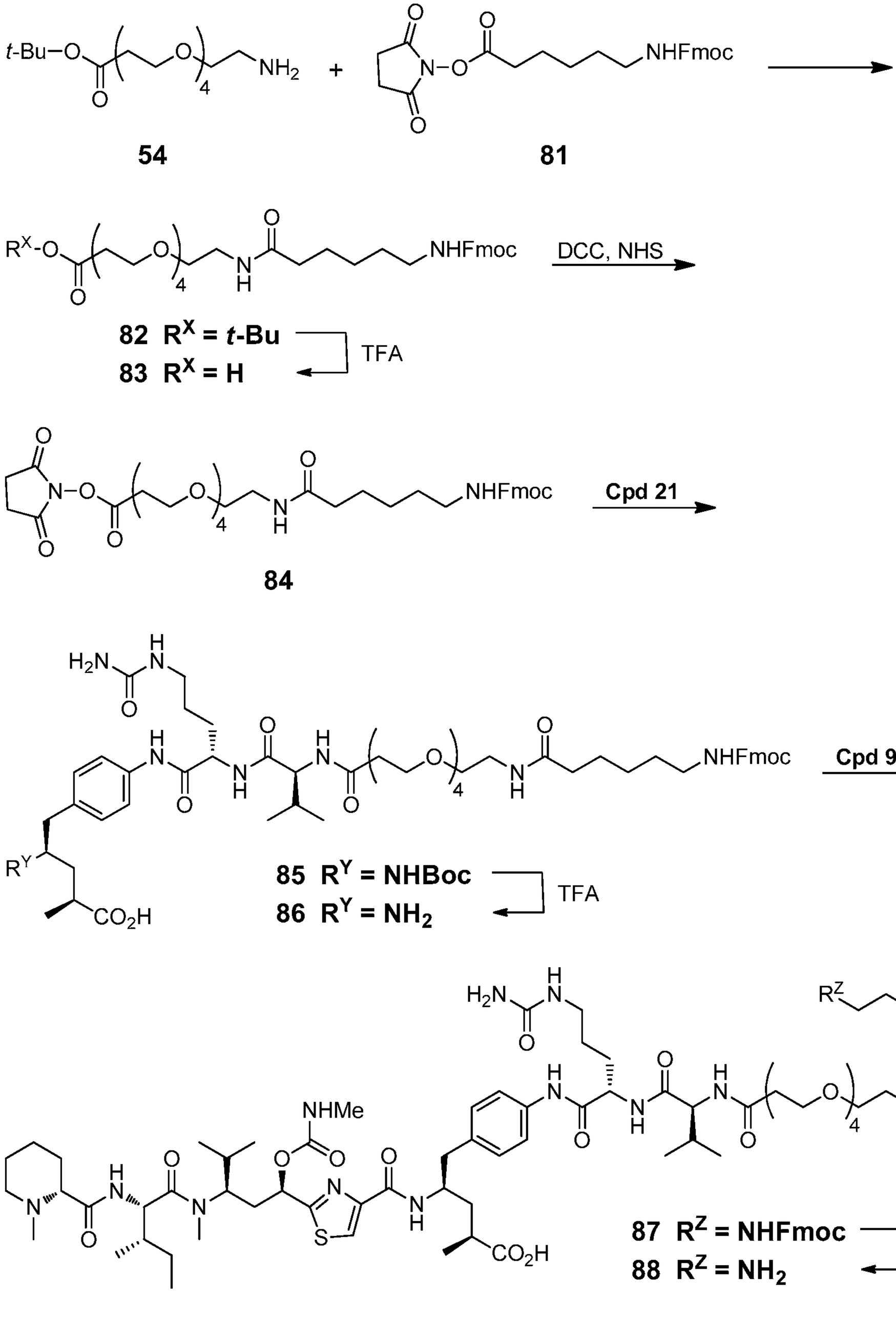
79 R^W = NH_2

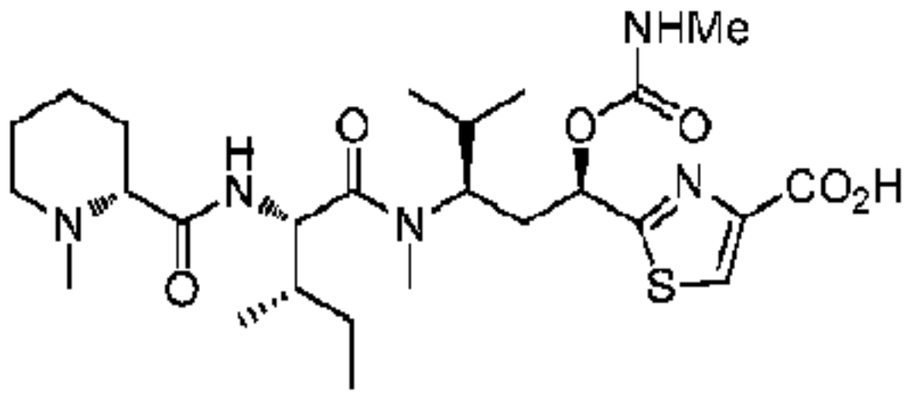
TFA



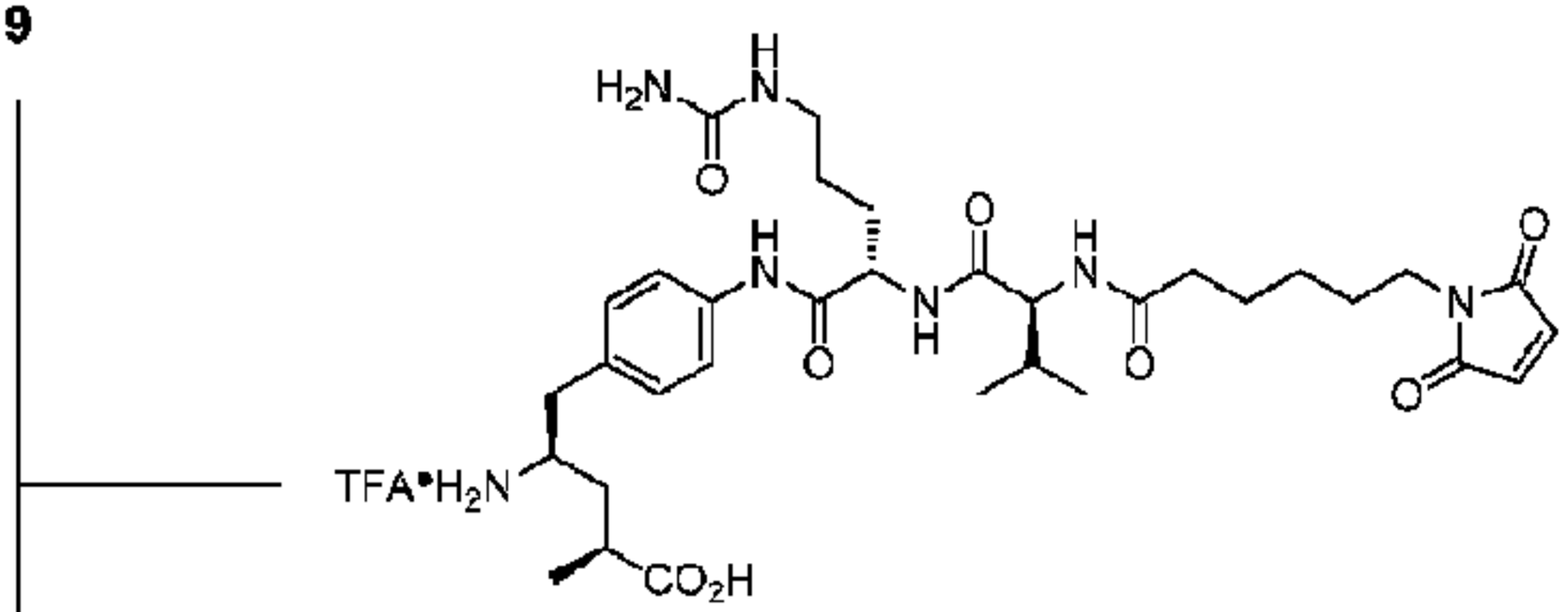
80

Fig. 24


t-Bu–O
NH2
54
81
NHFmoc
DCC, NHS
RX-O
82 RX = t-Bu
83 RX = H
TFA
84
Cpd 21
RY
85 RY = NHBoc
86 RY = NH2
Cpd 9
RZ
87 RZ = NHFmoc
88 RZ = NH2
CO2H
NHMe

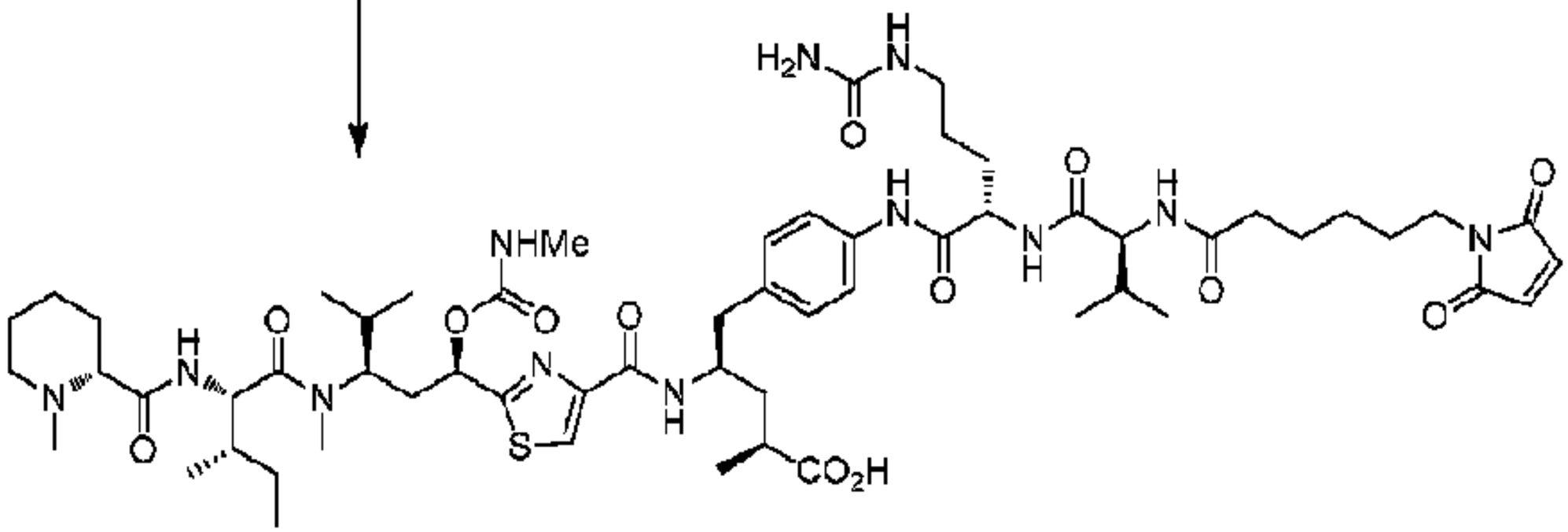


9



23

HATU,
DIEA



(III-1)