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(54) Title: SECO-CYCLOPROPAPYRROLINDOLE COMPOUNDS, ANTIBODY-DRUG CONJUGATES THEREOF, AND METHODS OF MAKING AND USE

(57) Abstract: seco-Cyclopropapyrroloindole compounds of formula (I) where Hal, R¹, R², and R³ are as defined in the application, are potent anti-cancer agents that can be used in antibody-drug conjugates.

SECO-CYCLOPROPAPYRROLOINDOLE COMPOUNDS, ANTIBODY-DRUG
CONJUGATES THEREOF, AND METHODS OF MAKING AND USE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit under 35 U.S.C. §119(e) of US Provisional Application Ser. No. 62/377,052, filed August 19, 2016; the disclosure of which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

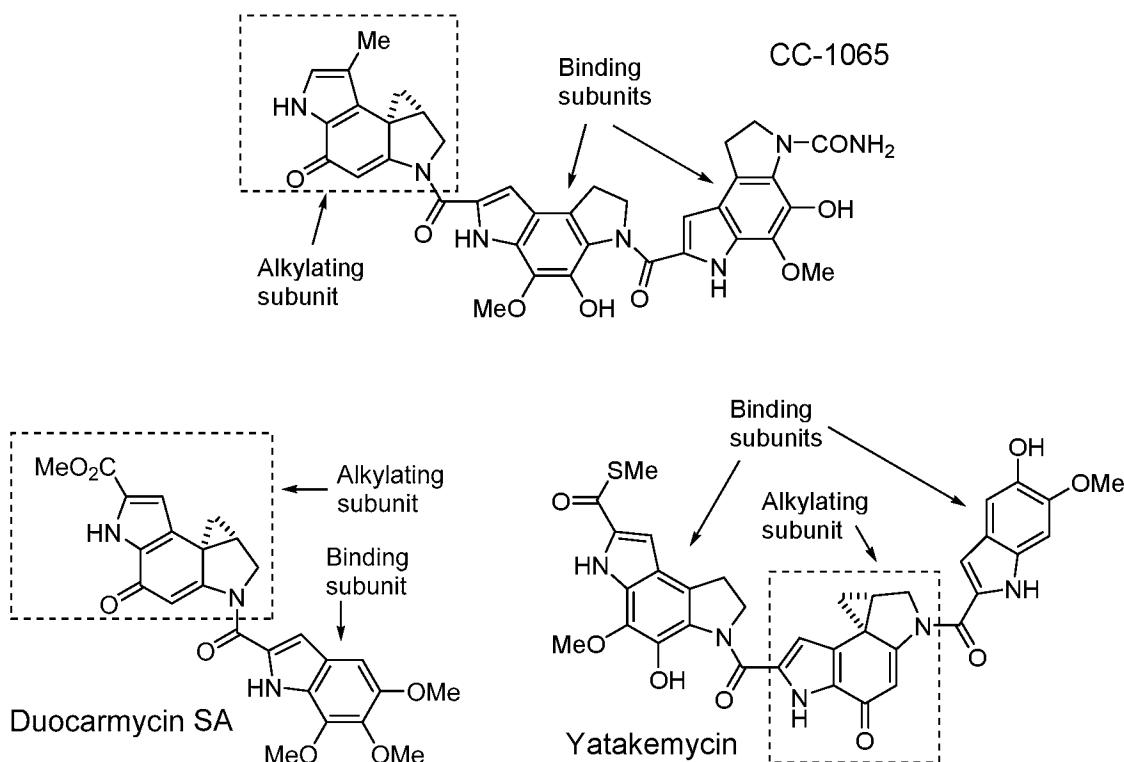
[0002] This invention relates to *seco*-cyclopropapyrroloindole compounds, conjugates thereof, and methods of making and using such compounds and conjugates.

[0003] Double helical DNA has two longitudinal spiral grooves running along its exterior, much like the stripes on a barbershop pole. The two grooves are not identical: one, called the major groove, is much wider than the other, called the minor groove.

[0004] The width of the minor groove is approximately equal to the thickness of a benzene ring. Many biologically active DNA-binding molecules are substantially planar polycyclic aromatic molecules having an arcuate footprint, such shape enabling them to slide snugly in the minor groove. One class of these molecules not only bind to DNA, but also alkylate it and are referred to as DNA minor groove binder-alkylators (“MGBAs”).

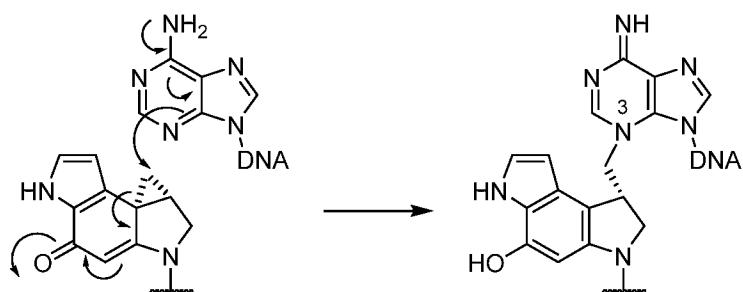
[0005] An MGBA subclass is represented by the natural products CC-1065, duocarmycin SA, and yatakemycin (Boger and Johnson 1995; Tichenor et al. 2007). (Full citations for the documents cited herein by first author or inventor and year are listed at the end of this specification.) They comprise an alkylating subunit and one or more binding subunits, the latter contributing to binding to DNA but being chemically unreactive towards it. In CC-1065 and duocarmycin SA, the alkylating subunit is at one end of the molecule and the binding subunit(s) are at the other end. In yatakemycin, the alkylating subunit is flanked by binding subunits.

Consonant with the overall MGBA architecture, the alkylating and binding subunits themselves are polycyclic and substantially planar. As the alkylating subunit has a cyclopropapyrroloindole (“CPI”) core structure, MGBAs in this subclass are eponymously named CPI compounds.



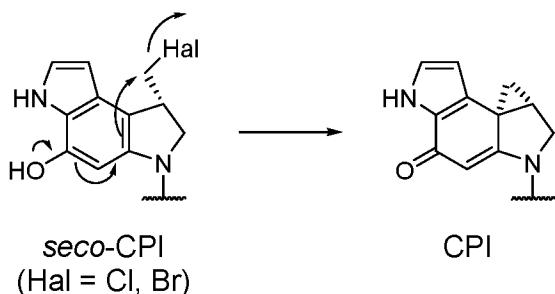
[0006] Upon binding to DNA, the CPI cyclopropyl ring is activated and alkylates DNA at an

5 adenine N3 nitrogen (Hurley *et al.* 1984). One theory proposed to explain the activation is that binding introduces further conformational strain into the already-strained cyclopropyl ring, increasing its reactivity (Boger 2001; Boger *et al.* 1997; Tichenor *et al.* 1997).



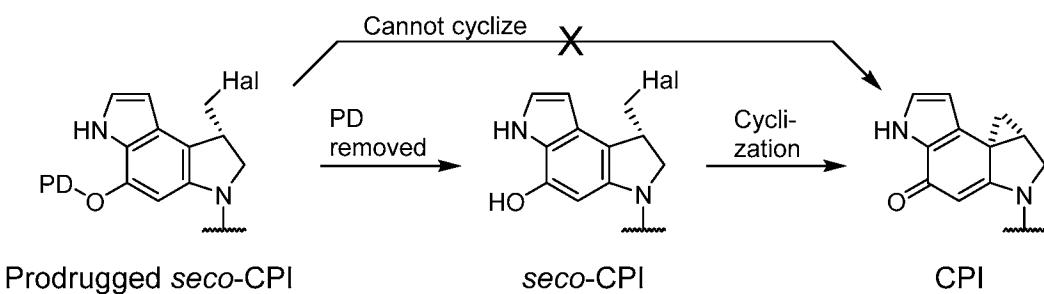
[0007] *Seco*-CPI compounds are variants of CPI compounds in which the cyclopropyl ring

10 has been opened and replaced with a halomethyl group. While *sec*-CPI compounds themselves do not alkylate DNA, they are readily convertible *in vitro* or *in vivo* to CPI compounds and their biological activity is essentially the same as the latter's (Li *et al.* 2012). Thus, *sec*-CPI compounds are of interest as synthetically convenient functional equivalents of CPI compounds or as intermediates for their synthesis (Boger *et al.* 2000).



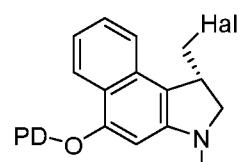
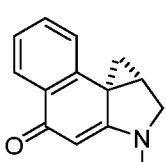
[0008] An advantage of a *seco*-CPI compound is that it can be prodrugged to control conversion to the CPI form. Attaching a prodrugging group PD to the phenolic hydroxyl group prevents conversion to the CPI form unless PD is cleaved off first. PD can be chosen such that it 5 is cleaved by an agent found at or near the site of intended biological action, such as a tumor, to reduce the risk of systemic toxicity. PD preferably is an enzymatically cleavable group, such as a carbamate, phosphate, glycoside, or glucuronide, which are cleavable by carboxyesterase, phosphatase, glycosidase, or glucuronidase, respectively. See, *e.g.*, Kobayashi *et al.* 1994; Lajiness *et al.* 2010; Sufi *et al.* 2013; Tietze *et al.* 2001; Zhang *et al.* 2014.

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[0009] Studies on analogs of CPI compounds led to the development of another MGBA subclass, in which the CPI pyrrole group is replaced by a benzene ring. Such compounds are called CBI compounds, after the cyclopropabenzindole (“CBI”) core of the alkylating subunit. Like CPI compounds, CBI compounds can exist in *seco* and prodrugged *seco* forms. The simpler CBI structure is more accessible synthetically and CBI compounds have been shown to be both stable and biologically potent (Lajiness *et al.* 2010; Boger *et al.* 1990 and 1999).



[0010] Both CPI and CBI compounds are potent cytotoxins, making them attractive candidates as anti-cancer agents. Substantial research efforts have been dedicated to synthesizing and evaluating such compounds and their *seco* variants for such use. See, *e.g.*, Boger 2003; Kobayashi *et al.* 1994; Li *et al.* 1992; Nagamura and Saito 1998; Nagamura *et al.* 1996; Tichenor *et al.* 2007; Tietze *et al.* 2008.

[0011] A type of anticancer agent that is generating strong interest is a conjugate, in which a drug is attached to a targeting agent that binds to a ligand on the cancer cell. The targeting agent thus directs the drug to the cancer cell, where it is released by one of several mechanisms to act on the cancer cell.

[0012] A common type of conjugate is an antibody-drug conjugate (ADC, also referred to as an immunoconjugate). In an ADC, a drug (also referred to as a therapeutic agent, cytotoxin, payload, or warhead) is covalently linked to an antibody whose antigen is a tumor associated antigen – *i.e.*, an antigen expressed by a cancer cell.

[0013] The moiety covalently linking the antibody and the drug is referred to as the linker.

Where each antibody has one drug attached to it, the structure of an ADC can be represented as:

[Antibody]-[Linker]-[Drug]

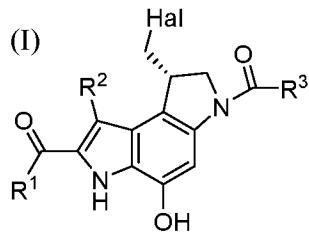
[0014] The antibody, upon binding to the antigen, delivers the ADC to the cancer site. There, cleavage of the linker or degradation of the antibody releases the drug. Frequently, the ADC is internalized by endocytosis into the target cell and release of the drug takes place inside it. While the ADC is circulating in the blood, the drug is held inactive because of its linkage to the antibody. Consequently, the drug in an ADC can be much more potent (cytotoxic) than an ordinary chemotherapy agent because its localized release reduces systemic toxicity. For a review on ADCs, see Schrama *et al.* 2006.

[0015] CPI and CBI compounds, along with their *seco* counterparts, have been proposed as the drug in an ADC. See, for example, Boyd *et al.* 2008; Chari *et al.* 1995; Chen *et al.* 2014; Ducry *et al.* 2010; Gangwar *et al.* 2011; Ng *et al.* 2006, 2009, and 2011; Sufi *et al.* 2013; Zhang *et al.* 2014; Zhao *et al.* 2010.

BRIEF SUMMARY OF THE INVENTION

[0016] The present invention provides novel and highly potent *seco*-CPI compounds, which are suitable for use as the drug in an ADC or other conjugate. These compounds have an amide group attached to the pyrrole group of the *seco*-CPI subunit and one or more binding subunits attached to its other end.

[0017] In one aspect, this invention provides a *seco*-CPI compound having a structure represented by formula (I):

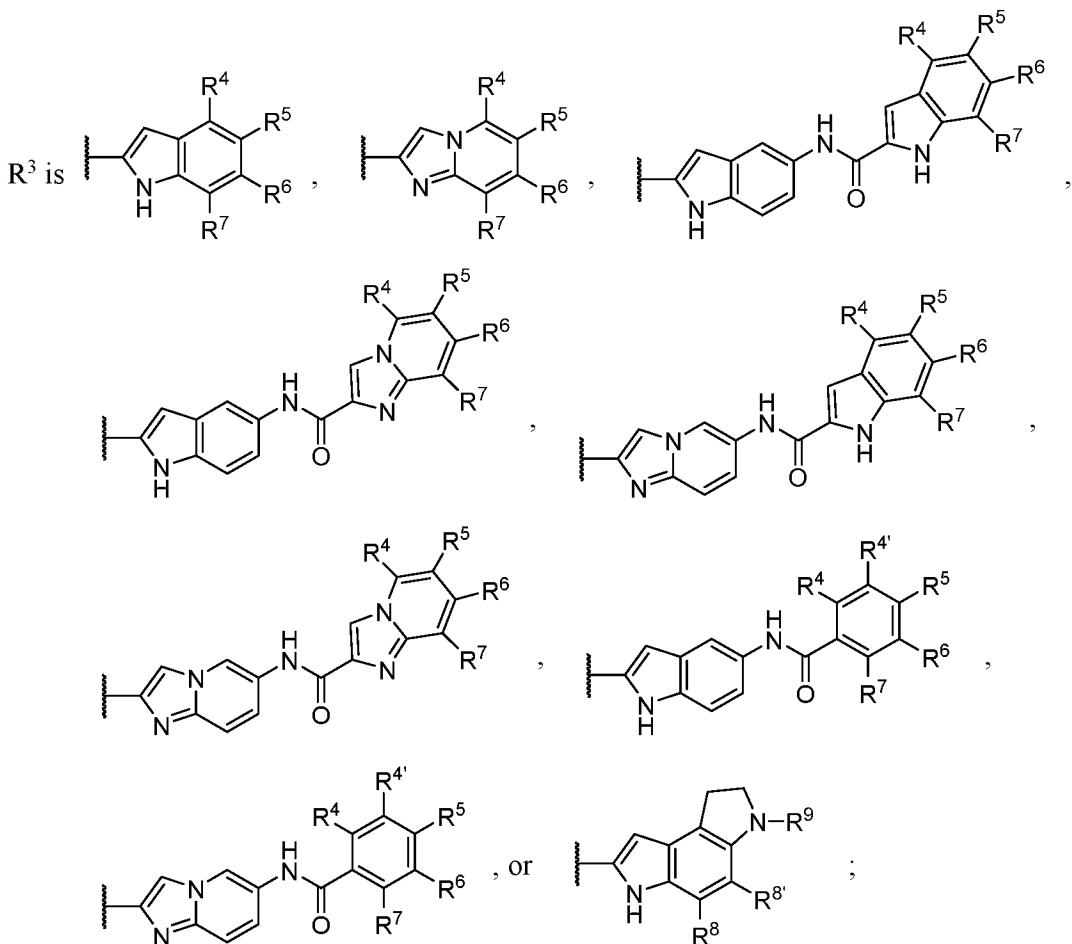


wherein

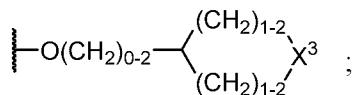
5 Hal is Cl or Br;

R¹ is (CH₂)₀₋₂—X¹, , or ;

R² is H, C₁-C₃ alkyl, CO₂H, CO₂(C₁-C₃ alkyl), C(=O)NH₂, C(=O)NH(C₁-C₃ alkyl), or C(=O)N(C₁-C₃ alkyl)₂;



R⁴, R^{4'}, R⁵, R⁶, or R⁷ are independently H, OMe, OH, a 6-membered aryl (preferably phenyl) group, a 5- or 6-membered heteroaryl group, NH₂, NHMe, NMe₂, NH(C₂-C₄ alkyl), N(C₂-C₄ alkyl)₂, NHC(=O)X¹, O(C₂-C₄ alkyl), O(CH₂)₀₋₂(C₃-C₆ cycloalkyl), O(CH₂)₀₋₂X¹, or



wherein a C₂-C₄ alkyl group may unsubstituted or substituted with OCH₂CH₂OH, OCH₂CH₂NH₂, NHCH₂CH₂OH, NHCH₂CH₂NH₂, OH, or NH₂ and an aryl or heteroaryl group may be substituted with C₁-C₂ alkyl, OH, NH₂, NH(C₁-C₂ alkyl), N(C₁-C₂ alkyl)₂, F, Cl, Br, NO₂, or CN;

10 with the proviso that at least one of R⁴, R^{4'}, R⁵, R⁶, and R⁷ is other than H;

R⁸ and R^{8'} are independently H, OH, O(C₁-C₃ alkyl), Cl, Br, F, O(CH₂)₂₋₄NH₂, or O(CH₂)₂₋₄OH; R⁹ is H, C(=O)(C₁-C₃ alkyl), C(=O)NH₂, C(=O)NH(C₁-C₃ alkyl), C(=O)(C₁-C₃ alkyl)₂, (CH₂)₂₋₄OH, (CH₂)₂₋₄O(C₁-C₃ alkyl), (CH₂)₂₋₄NH₂, (CH₂)₂₋₄NH(C₁-C₃ alkyl), or (CH₂)₂₋₄N(C₁-C₃ alkyl)₂;

15 each X¹ is independently a 6-membered aryl (preferably phenyl) or 5- to 6-membered heteroaryl group that is unsubstituted or substituted with C₁-C₃ alkyl, OH, O(C₁-C₃ alkyl), NH₂, NH(C₁-C₃ alkyl), N(C₁-C₃ alkyl)₂, F, Cl, Br, NO₂, or CN;

each X² is independently H, Me, or a C₂-C₄ alkyl group that may be unsubstituted or substituted with OCH₂CH₂OH, OCH₂CH₂NH₂, NHCH₂CH₂OH, NHCH₂CH₂NH₂, OH, or NH₂; and

20 each X³ is independently O, NH, N(C₁-C₃ alkyl), or S; or a pharmaceutically acceptable salt thereof.

[0018] In another aspect, this invention provides a *seco*-CPI compound of this invention attached to a linker, suitable for conjugation to a targeting moiety, especially an antibody.

[0019] In another aspect, this invention provides a conjugate of a *seco*-CPI compound of this invention conjugated via a linker to a targeting moiety, especially an antibody.

[0020] In another aspect, this invention provides 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 according to this invention, or a conjugate thereof with an antibody. In a preferred embodiment, the cancer treated is lung, gastric, ovarian, or colon cancer.

[0021] In another aspect, this invention provides a *seco*-CPI compound of this invention, or a conjugate thereof with an antibody, for use in treating a subject suffering from cancer. The cancer can be lung, gastric, ovarian or colon cancer.

BRIEF DESCRIPTION OF THE DRAWING(S)

5 **[0022]** **FIGs. 1A** and **1B** show a scheme for the synthesis of a *seco*-CPI core structure that can be used to make compounds of this invention.

10 **[0023]** **FIGs. 2, 4, 6, 7, 8, 9, 10, 11, and 12** show exemplary schemes for the synthesis of *seco*-CPI compounds of this invention. Those skilled in the art will appreciate that, in some instances, the schemes differ primarily in the order in which various segments of a compound are assembled or the order in which protecting groups are removed and that the order of performance of these steps can be a matter of individual preference.

[0024] **FIGs. 3A, 3B, and 5** show exemplary schemes for the synthesis of *seco*-CPI linker compounds of this invention. Again, those skilled in the art will appreciate that variations in the order of performance of certain steps may be subject to individual preference.

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DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

[0025] “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 5 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.

[0026] “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 10 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 15 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 20 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 25 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.

[0027] 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 30 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.

[0028] “Monoclonal antibody” or “monoclonal antibody composition” means a preparation of antibody molecules of single molecular composition, which displays a single binding

5 specificity and affinity for a particular epitope.

[0029] “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 10 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.

[0030] “Human monoclonal antibody” means an antibody displaying a single binding

15 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.

20 **[0031]** “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 “C₃ aliphatic,” “C₁₋₅ aliphatic,” “C_{1-C₅} aliphatic,” or “C₁ to C₅ aliphatic,” the latter three 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 25 the instance of unsaturated aliphatic moieties). A similar understanding is applied to the number of carbons in other types, as in C₂₋₄ alkene, C_{4-C₇} cycloaliphatic, etc. In a similar vein, a term such as “(CH₂)₁₋₃” is to be understood as shorthand for the subscript being 1, 2, or 3, so that such term represents CH₂, CH₂CH₂, and CH₂CH₂CH₂.

30 **[0032]** “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-C₄} 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 CH₂CH₂, CH₂CH₂CH₂, and CH₂CH₂CH₂CH₂.

[0033] “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, C₂-C₄ 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-

5 butadienyl (but-1,3-dienyl) and the like.

[0034] “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, C₂-C₄ alkynyl groups include ethynyl (acetylenyl), propargyl (prop-2-ynyl), 1-propynyl, but-2-ynyl, and the like.

10 [0035] “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.

20 [0036] “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. Preferred cycloaliphatic moieties consist of one ring, 5- to 6-membered in size. Similarly, “heterocycloalkyl,” “heterocycloalkenyl,” and

25 “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, tetrahydropyrananyl, tetrahydrothiopyrananyl, tetrahydrothiopyranyl sulfone, morpholinyl, thiomorpholinyl, thiomorpholinyl sulfoxide, thiomorpholinyl sulfone, 1,3-dioxolanyl, tetrahydro-1,1-dioxothienyl, 1,4-dioxanyl, thietanyl, and the like.

30 “Heterocycloalkylene” means a divalent counterpart of a heterocycloalkyl group.

[0037] “Alkoxy,” “aryloxy,” “alkylthio,” and “arylthio” mean –O(alkyl), -O(aryl), -S(alkyl), and -S(aryl), respectively. Examples are methoxy, phenoxy, methylthio, and phenylthio, respectively.

[0038] “Halogen” or “halo” means fluorine, chlorine, bromine or iodine, unless a narrower meaning is indicated.+

[0039] “Aryl” means a hydrocarbon moiety having a mono-, bi-, or tricyclic ring system (preferably monocyclic) 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.

[0040] “Heteroaryl” means a moiety having a mono-, bi-, or tricyclic ring system (preferably 5- to 7-membered monocyclic) 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 a heteroaryl group.

[0041] 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 C₁-C₅ 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. Where a moiety is identified as being “unsubstituted or substituted” or “optionally substituted,” in a preferred embodiment such moiety is unsubstituted.

[0042] “Arylalkyl,” (heterocycloaliphatic)alkyl,” “arylalkenyl,” “arylalkynyl,” “biarylalkyl,” and the like mean an alkyl, alkenyl, or alkynyl moiety, as the case may be, substituted with an 5 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 10 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).

[0043] 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 -OCF₃), -O(cycloalkyl), -O(heterocycloalkyl), -O(aryl), alkylthio, arylthio, =O, =NH, =N(alkyl), =NOH, =NO(alkyl), -C(=O)(alkyl), -C(=O)H, -CO₂H, -C(=O)NHOH, -C(=O)O(alkyl), -C(=O)O(hydroxyalkyl), -C(=O)NH₂, -C(=O)NH(alkyl), -C(=O)N(alkyl)₂, -OC(=O)(alkyl), 20 -OC(=O)(hydroxyalkyl), -OC(=O)O(alkyl), -OC(=O)O(hydroxyalkyl), -OC(=O)NH₂, -OC(=O)NH(alkyl), -OC(=O)N(alkyl)₂, azido, -NH₂, -NH(alkyl), -N(alkyl)₂, -NH(aryl), -NH(hydroxyalkyl), -NHC(=O)(alkyl), -NHC(=O)H, -NHC(=O)NH₂, -NHC(=O)NH(alkyl), -NHC(=O)N(alkyl)₂, -NHC(=NH)NH₂, -OSO₂(alkyl), -SH, -S(alkyl), -S(aryl), -S(cycloalkyl), -S(=O)alkyl, -SO₂(alkyl), -SO₂NH₂, -SO₂NH(alkyl), -SO₂N(alkyl)₂, and the like.

[0044] 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), -CO₂H, -C(=O)NHOH, -C(=O)O(alkyl), -C(=O)O(hydroxyalkyl), -C(=O)NH₂, -C(=O)NH(alkyl), -C(=O)N(alkyl)₂, -OC(=O)(alkyl), 30 -OC(=O)(hydroxyalkyl), -OC(=O)O(alkyl), -OC(=O)O(hydroxyalkyl), -OC(=O)NH₂, -OC(=O)NH(alkyl), -OC(=O)N(alkyl)₂, azido, -NH₂, -NH(alkyl), -N(alkyl)₂, -NH(aryl), -NH(hydroxyalkyl), -NHC(=O)(alkyl), -NHC(=O)H, -NHC(=O)NH₂, -NHC(=O)NH(alkyl), -NHC(=O)N(alkyl)₂, -NHC(=NH)NH₂, -OSO₂(alkyl), -SH, -S(alkyl), -S(aryl), -S(=O)alkyl,

-S(cycloalkyl), -SO₂(alkyl), -SO₂NH₂, -SO₂NH(alkyl), and -SO₂N(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)NH₂, -OC(=O)NH(alkyl), -OC(=O)N(alkyl)₂, azido, -NH₂, -NH(alkyl), -N(alkyl)₂, -NH(aryl), -NHC(=O)(alkyl), -NHC(=O)H, -NHC(=O)NH₂, -NHC(=O)NH(alkyl), -NHC(=O)N(alkyl)₂, and -NHC(=NH)NH₂. Especially preferred are phenyl, cyano, halo, hydroxyl, nitro, C₁-C₄alkyoxy, O(C₂-C₄ alkylene)OH, and O(C₂-C₄ alkylene)halo.

[0045] 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, -CO₂H, -C(=O)NHOH, -C(=O)O(alkyl), -C(=O)O(hydroxyalkyl), -C(=O)NH₂, -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)NH₂, -OC(=O)NH(alkyl), -OC(=O)N(alkyl)₂, azido, -NH₂, -NH(alkyl), -N(alkyl)₂, -NH(aryl), -NH(hydroxyalkyl), -NHC(=O)(alkyl), -NHC(=O)H, -NHC(=O)NH₂, -NHC(=O)NH(alkyl), -NHC(=O)N(alkyl)₂, -NHC(=NH)NH₂, -OSO₂(alkyl), -SH, -S(alkyl), -S(aryl), -S(cycloalkyl), -S(=O)alkyl, -SO₂(alkyl), -SO₂NH₂, -SO₂NH(alkyl), and -SO₂N(alkyl)₂. More preferred substituents are alkyl, alkenyl, halo, haloalkyl, hydroxyl, hydroxyalkyl, cyano, nitro, alkoxy, -O(hydroxyalkyl), -C(=O)(alkyl), -C(=O)H, -CO₂H, -C(=O)NHOH, -C(=O)O(alkyl), -C(=O)O(hydroxyalkyl), -C(=O)NH₂, -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)NH₂, -OC(=O)NH(alkyl), -OC(=O)N(alkyl)₂, azido, -NH₂, -NH(alkyl), -N(alkyl)₂, -NH(aryl), -NH(hydroxyalkyl), -NHC(=O)(alkyl), -NHC(=O)H, -NHC(=O)NH₂, -NHC(=O)NH(alkyl), -NHC(=O)N(alkyl)₂, and -NHC(=NH)NH₂. Especially preferred are C₁-C₄ alkyl, cyano, nitro, halo, and C₁-C₄alkyoxy.

[0046] Where a range is stated, as in “C₁-C₅ alkyl” or “5 to 10%,” such range includes the end points of the range, as in C₁ and C₅ in the first instance and 5% and 10% in the second instance.

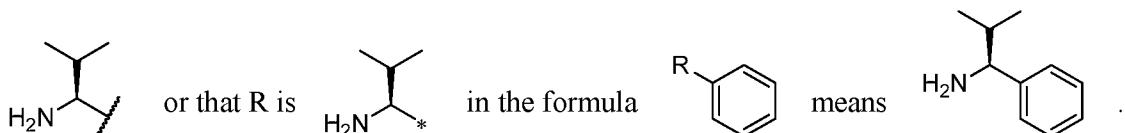
[0047] Unless particular stereoisomers are specifically indicated (e.g., by a bolded or dashed bond at a relevant stereocenter in a structural formula, by depiction of a double bond as having E or Z configuration in a structural formula, or by use stereochemistry-designating nomenclature), all stereoisomers 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.

[0048] 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.

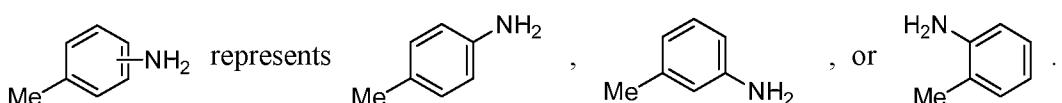
5 **[0049]** “Pharmaceutically acceptable ester” means an ester that hydrolyzes *in vivo* (for example in the human body) to produce the parent compound or a salt thereof or has *per se* activity similar to that of the parent compound. Suitable esters include C₁-C₅ alkyl, C₂-C₅ alkenyl or C₂-C₅ alkynyl esters, especially methyl, ethyl or n-propyl.

10 **[0050]** “Pharmaceutically acceptable salt” means a salt of a compound suitable for pharmaceutical formulation. Where a compound has one or more basic groups, the salt can be an acid addition salt, such as a sulfate, hydrobromide, tartrate, mesylate, maleate, citrate, phosphate, acetate, pamoate (embonate), hydroiodide, nitrate, hydrochloride, lactate, methylsulfate, fumarate, benzoate, succinate, mesylate, lactobionate, suberate, tosylate, and the like. Where a compound has one or more acidic groups, the salt can be a salt such as a calcium salt, potassium 15 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.

20 **[0051]** In the formulae of this specification, a wavy line (~~~~) transverse to a bond or an asterisk (*) at the end of the bond denotes a covalent attachment site. For instance, a statement that R is



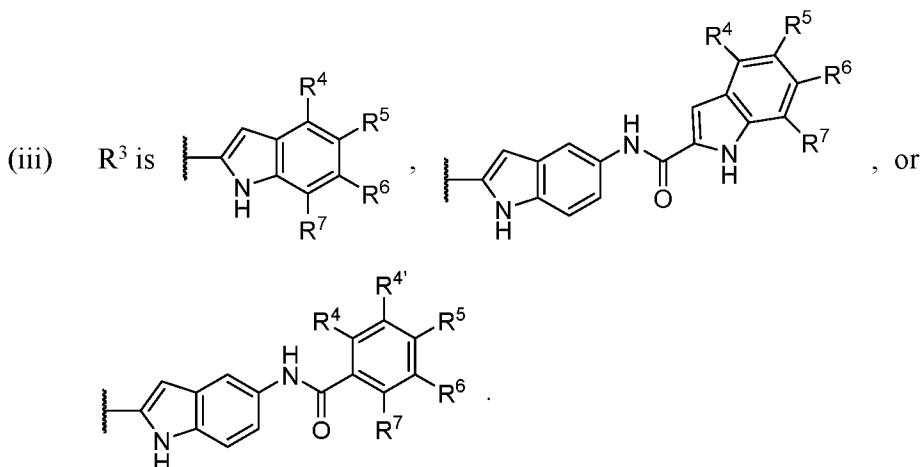
25 **[0052]** In the formulae of this specification, a bond traversing an aromatic ring between two carbons thereof means that the group attached to the bond may be located at any of the available positions of the aromatic ring. By way of illustration, the formula



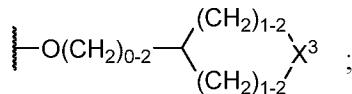
SECO-CPI COMPOUNDS

[0053] In formula (I) and other formulae where these variables are present, the following preferred embodiments apply, either individually or in combination with other preferred embodiments, unless the context indicates a different preferred embodiment or combination of preferred embodiments is applicable:

- (i) Hal is Cl.
- (ii) R² is H.



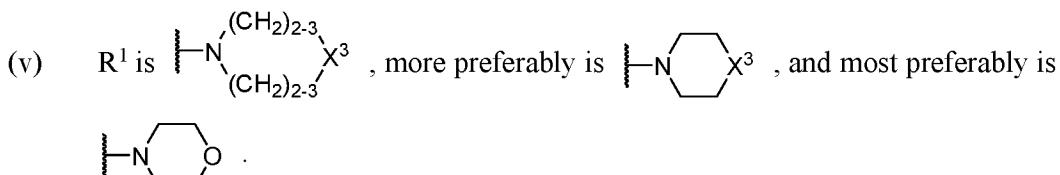
10 (iv) R⁵ is OMe, OH, phenyl, NH₂, NHMe, NMe₂, NH(C₂-C₄ alkyl), N(C₂-C₄ alkyl)₂, NHC(=O)X¹, O(C₂-C₄ alkyl), O(CH₂)₀₋₂(C₃-C₆ cycloalkyl), O(CH₂)₀₋₂X¹, or



wherein a C₂-C₄ alkyl group may unsubstituted or substituted with OCH₂CH₂OH, OCH₂CH₂NH₂, NHCH₂CH₂OH, NHCH₂CH₂NH₂, OH, or NH₂ and a phenyl

15 group may be substituted with C₁-C₂ alkyl, OH, NH₂, NH(C₁-C₂ alkyl), N(C₁-C₂ alkyl)₂, F, Cl, Br, NO₂, or CN;

(i.e., R⁵ is the one of R⁴, R^{4'}, R⁵, R⁶, or R⁷ that is other than H).

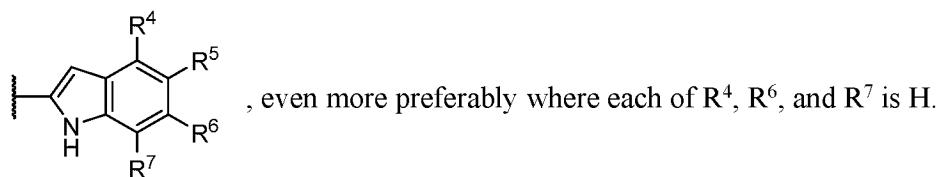


20 (vi) No more than three, and more preferably no more than two, of R⁴, R^{4'}, R⁵, R⁶, and R⁷ is other than H.

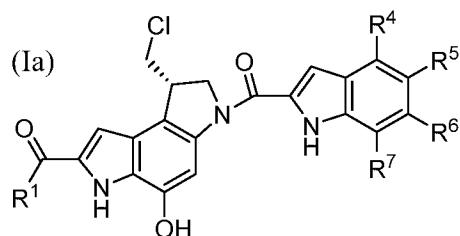
(vii) The aryl or heteroaryl group in X^1 is phenyl, pyrrolyl, furanyl, thienyl, imidazolyl, pyrazolyl, oxazolyl, isoxazolyl, thiazolyl, isothiazolyl, triazolyl, tetrazolyl, pyridyl, pyridazinyl, or pyrazinyl; more preferably phenyl and especially phenyl substituted with NH_2 or OH .

5

(viii) Where R^1 is  , at least one X^2 is other than H.



[0055] In a preferred aspect of the invention, compounds according to formula (I) have a structure represented by formula (Ia), where R^1 , R^4 , R^5 , R^6 , and R^7 are as defined in respect of formula (I) in the BRIEF SUMMARY OF THE INVENTION section hereinabove:



[0056] Examples of compounds according to formula (Ia) are listed in Table I, with R^4 , R^6 , and R^7 each being H unless otherwise noted.

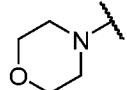
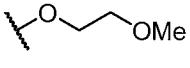
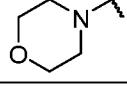
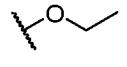
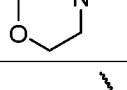
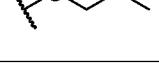
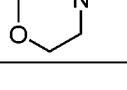
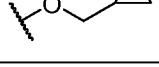
Table I – Compounds According to Formula (Ia)		
Cpd. No.	R^1	R^5
Ia-01		
Ia-02		
Ia-03		
Ia-04		

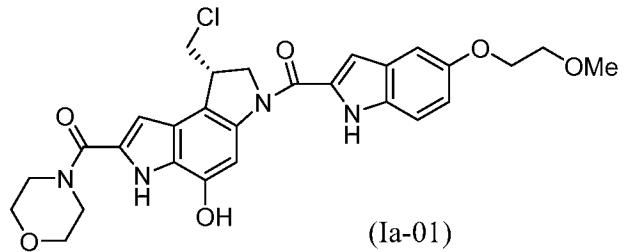
Table I – Compounds According to Formula (Ia)

Cpd. No.	R ¹	R ⁵
Ia-05		
Ia-06		
Ia-07		
Ia-08		
Ia-09		
Ia-10		
Ia-11		
Ia-12		
Ia-13		
Ia-14		
Ia-15		
Ia-16		
Ia-17		
Ia-18		

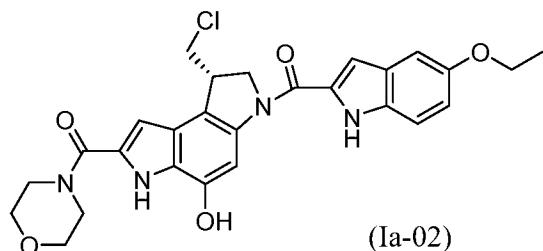
Table I – Compounds According to Formula (Ia)

Cpd. No.	R ¹	R ⁵
Ia-19		
Ia-20		
Ia-21		
Ia-22		
Ia-23		
Ia-24		
Ia-25		
Ia-26		
Ia-27		
Ia-28		
Ia-29		
Ia-30		 (R ⁴ = H, R ⁶ = R ⁷ = OMe)
Ia-31		

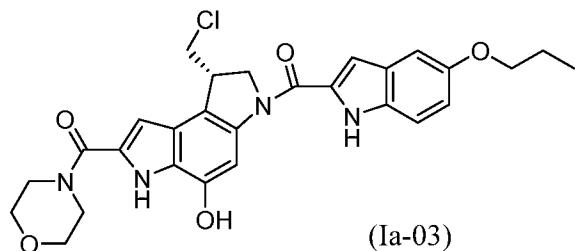
[0057] A preferred *sec*o-CPI compound is (Ia-01), whose complete structure is shown following:



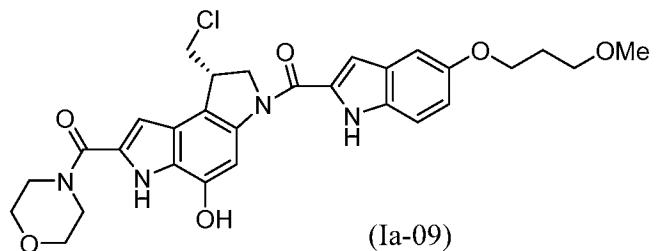
[0058] A preferred *sec*o-CPI compound is (Ia-02), whose complete structure is shown following:



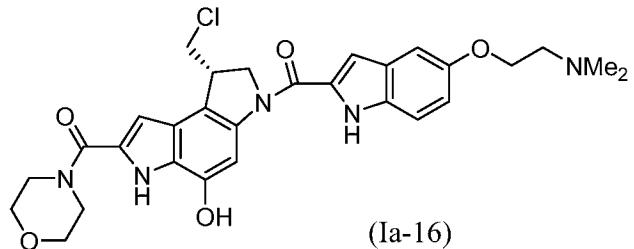
[0059] A preferred *sec*o-CPI compound is (Ia-03), whose complete structure is shown following:



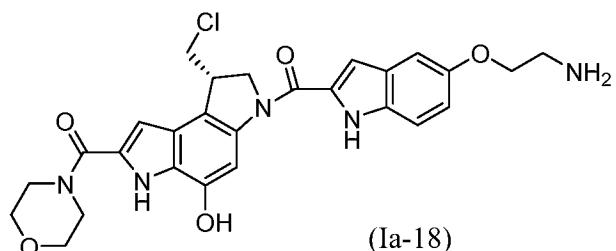
[0060] A preferred *sec*o-CPI compound is (Ia-09), whose complete structure is shown following:



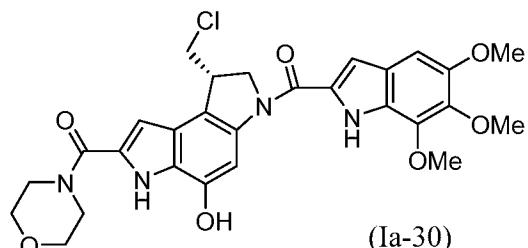
[0061] A preferred *sec*o-CPI compound is (Ia-16), whose complete structure is shown following:



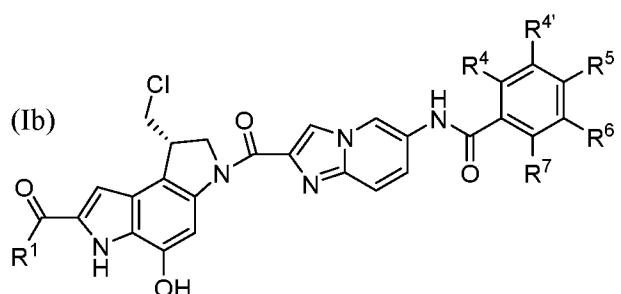
[0062] A preferred *sec*o-CPI compound is (Ia-18), whose complete structure is shown following:



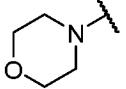
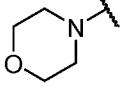
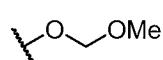
[0063] A preferred *sec*o-CPI compound is (Ia-30), whose complete structure is shown following:



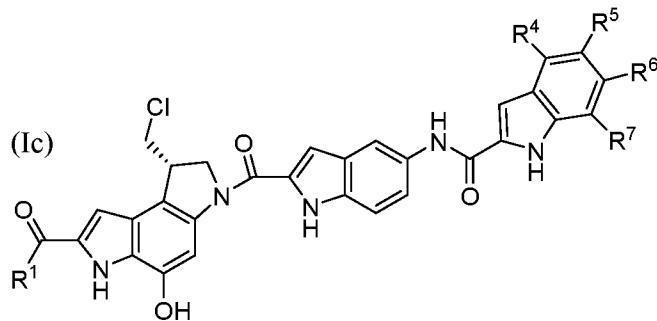
[0064] Another preferred embodiment of compounds according to formula (I) has a structure represented by formula (Ib), where R¹, R⁴, R⁵, R⁶, and R⁷ are as defined in respect of formula (I) in the BRIEF SUMMARY OF THE INVENTION section hereinabove:



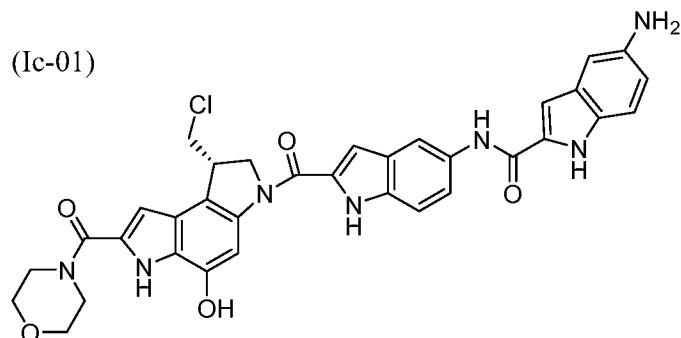
[0065] Examples of compounds according to formula (Ib) are listed in Table II (R⁴, R^{4'}, R⁶, and R⁷ each being H).

Table II – Compounds According to Formula (Ib)		
Cpd. No.	R ¹	R ⁵
Ib-01		
Ib-02		

5 [0066] Another preferred embodiment of compounds according to formula (I) has a structure represented by formula (Ic), where R¹, R⁴, R⁵, R⁶, and R⁷ are as defined in respect of formula (I) in the BRIEF SUMMARY OF THE INVENTION section hereinabove:



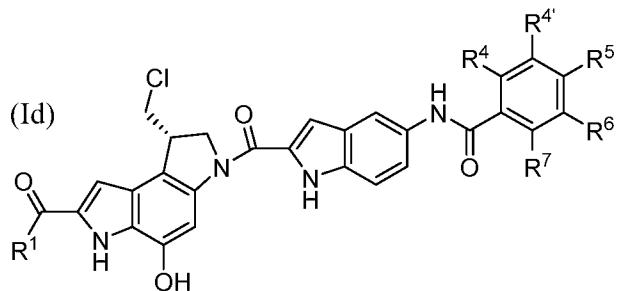
[0067] An example of a compound (Ic) is compound (Ic-01):



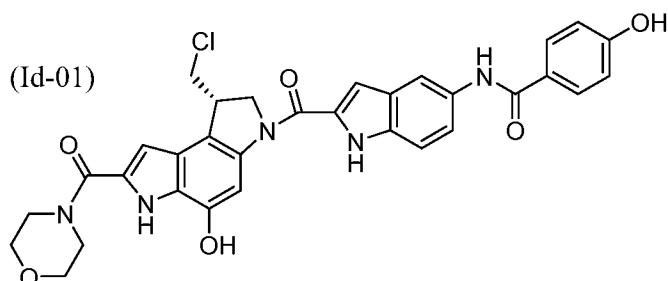
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[0068] Another embodiment of compounds (I) has a structure represented by formula (Id), where R¹, R⁴, R^{4'}, R⁵, R⁶, and R⁷ are as defined in respect of formula (I) in the BRIEF

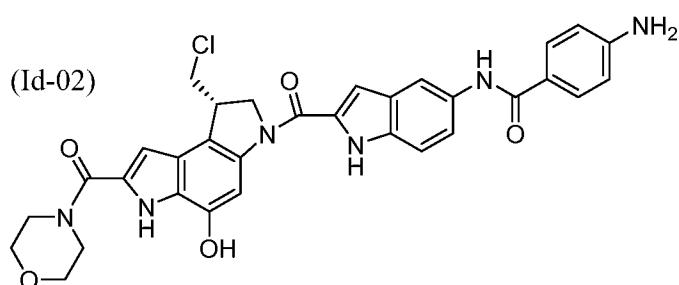
SUMMARY OF THE INVENTION section hereinabove:



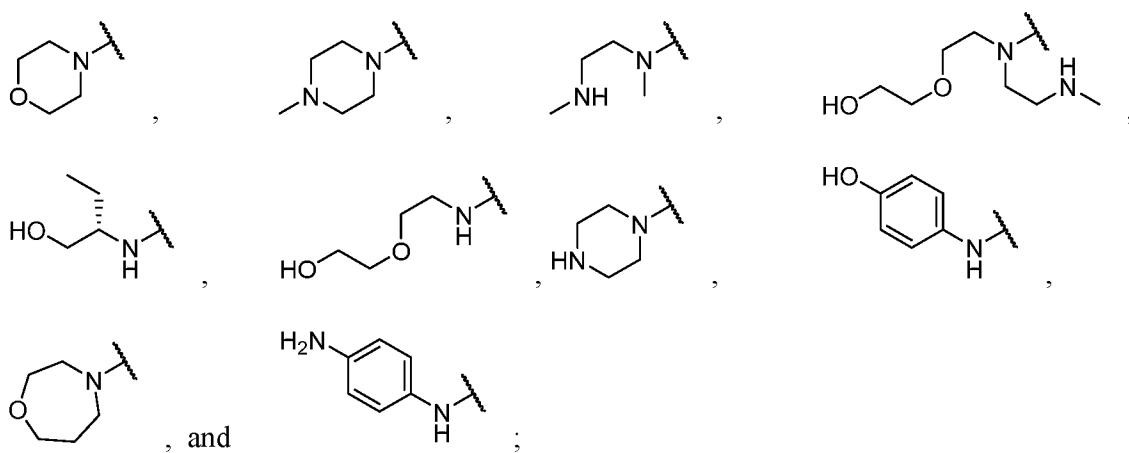
[0069] Examples of compounds (Id) are (Id-01) and (Id-02):



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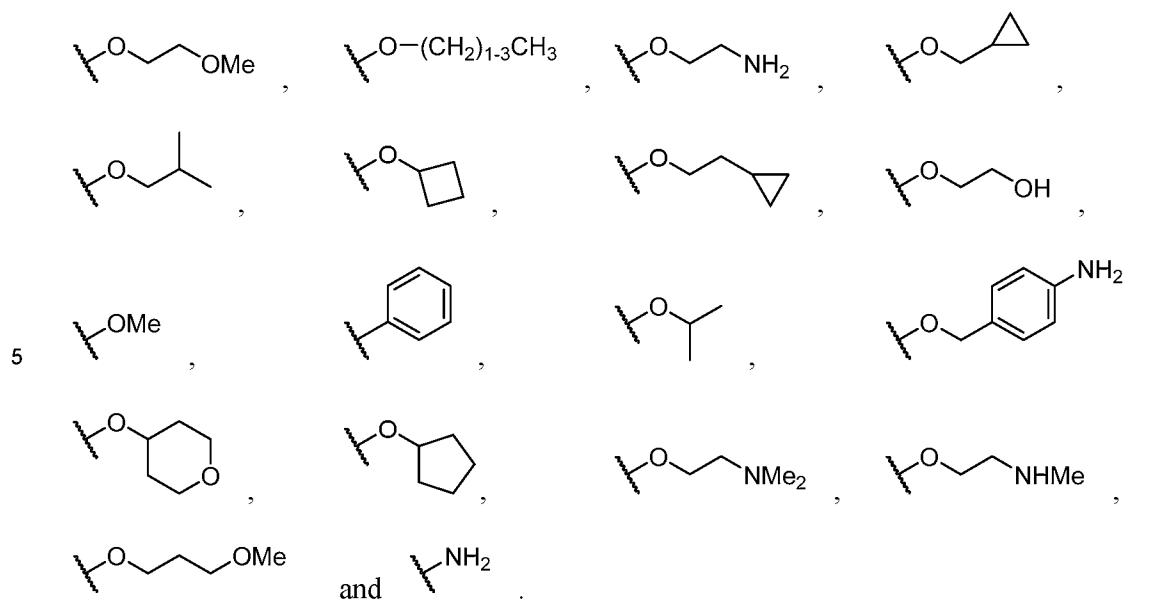


[0070] Specific preferred embodiments of R¹ are



and specific preferred embodiments of R⁵ are

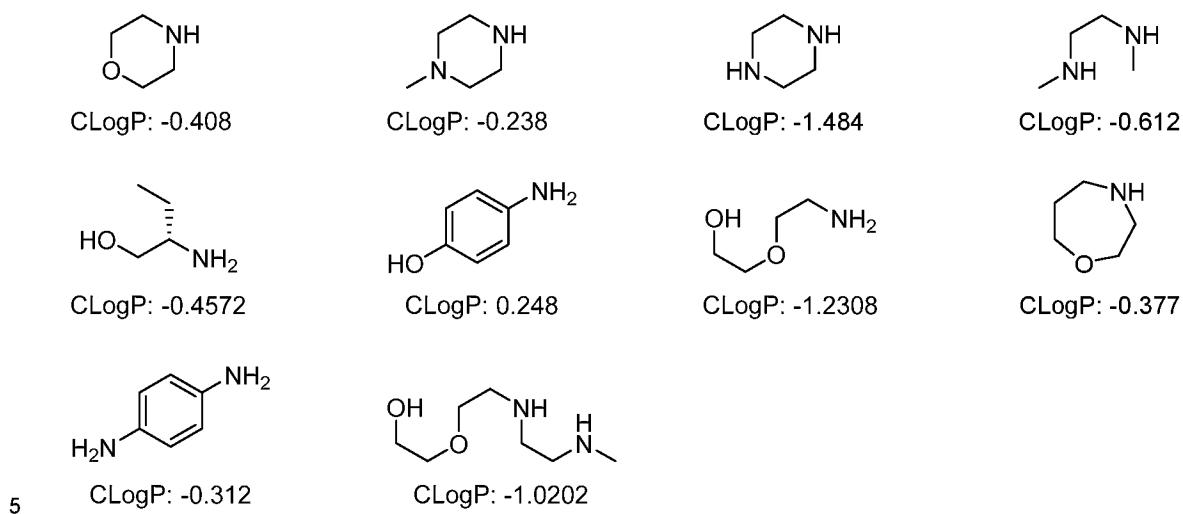
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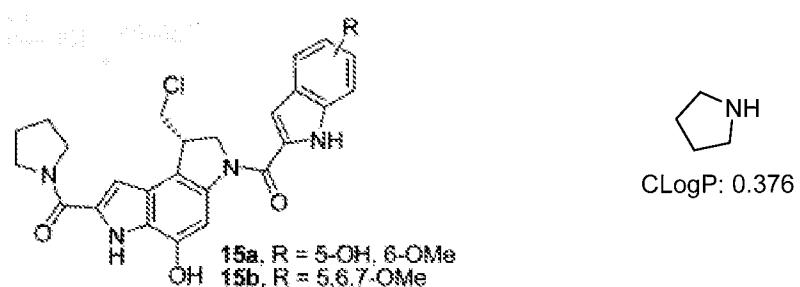
10 [0071] Without being bound by theory, it is believed that the R¹ groups of this invention are hydrophilic, advantageously improving their properties. Having a low CLogP R¹ group can be advantageous because it increases the aqueous solubility of a *seco*-CPI compound and its linker construct. As noted in Examples 1 and 2 below, conjugation to an antibody is performed in an essentially aqueous medium and a more soluble *seco*-CPI compound-linker compound can be 15 more efficiently conjugated. Further, in the event of unintended premature cleavage of an ADC releasing *seco*-CPI compound into the blood, the increased hydrophilicity reduces the likelihood that the compound will permeate through a cell membrane and cause off-target toxicity. (Entry of an ADC into a cell is by a different process: endocytosis triggered by binding of the antibody to its antigen.)

20 [0072] The open valence in each group R¹ – see, *e.g.*, Table I – is at a nitrogen, so that it forms an amide bond with the carboxyl group of the *seco*-CPI core. Their hydrophilicity can be estimated via the hydrophilicity of the corresponding amines R¹H.

25 [0073] The hydrophilicity (or, conversely, the hydrophobicity) of a compound can be estimated from its CLogP, which is a value calculatable from its structure: lower values indicate a more hydrophilic molecule while higher values indicating a more hydrophobic molecule. CLogP values for the amines R¹H corresponding to the R¹ groups in Table I are shown following. The CLogP values are all less than 0.300, ranging from -1.484 to 0.248. (CLogP values were calculated using CHEMBIODRAW® Ultra software (Version 14.0.0.126) from PerkinElmer. (The same software was used to calculate CLogP values cited elsewhere in this 30 specification.)



[0074] For comparison, Tichenor *et al.* 2007 disclose compounds **15a** and **15b** having a pyrrolodinyl amide attached to a *seco*-CPI core, but the CLogP value for the corresponding amine (pyrroline) is significantly higher, 0.376.



10 [0075] Thus, in one embodiment, the groups R¹ are those whose corresponding amine R¹H have a CLogP value of less than 0.300, as calculated using the aforementioned CHEMBIODRAW™ software.

CONJUGATES

General

15 [0076] *Seco*-CPI compounds of this invention can be used as therapeutic agents *per se*, but preferably are used in conjugates. More preferably, the targeting moiety in the conjugate is an antibody or antigen binding portion thereof and its antigen is a tumor associated antigen, *i.e.*, one that is expressed by a cancer cell. Preferably, the tumor associated antigen is uniquely expressed or overexpressed by the cancer cell, compared to a normal cell. The tumor associated antigen can 20 be located on the surface of the cancer cell or secreted by the cancer cell into its environs.

[0077] Thus, another embodiment of this invention is a conjugate comprising *seco*-CPI compound of this invention and a ligand, represented by formula (II)



where Z is a targeting moiety, D is a *seco*-CPI compound of this invention, and $-(X^D)_a C(X^Z)_b-$ 5 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 D; X^D and X^Z are spacer moieties (or “spacers”) that space apart D and C and C and Z, respectively; subscripts a, b, and c are independently 0 or 1 (that is, the presence of X^D , X^Z and C are optional). 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 10 and Z are more fully described hereinbelow.

[0078] By binding to a target tissue or cell where its antigen or receptor is located, Z directs the conjugate there. Cleavage of group C at the target tissue or cell releases 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 D 15 is achieved at the site of intended action, reducing the dosage needed. Also, D is normally biologically inactive (or significantly less active) in its conjugated state, thereby reducing undesired toxicity against non-target tissue or cells.

[0079] As reflected by the subscript m, each Z can conjugate with more than one D, depending on the number of sites Z has available for conjugation and the experimental 20 conditions employed. Those skilled in the art will appreciate that, while each individual Z is conjugated to an integer number of Ds, a preparation of the conjugate may analyze for a non-integer ratio of D to Z, reflecting a statistical average. This ratio is referred to as the substitution ratio (“SR”) or the drug-antibody ratio (“DAR”).

Targeting moiety Z

[0080] Preferably, targeting moiety Z is an antibody. For convenience and brevity and not by way of limitation, the detailed discussion in this specification about Z and its conjugates is 25 written in the context of its being an antibody, but those skilled in the art will understand that other types of Z can be conjugated, *mutatis mutandis*. For example, conjugates with folic acid as the targeting moiety can target cells having the folate receptor on their surfaces (Leamon *et al.*, 30 Cancer Res. 2008, 68 (23), 9839). For the same reasons, the detailed discussion in this specification is primarily written in terms of a 1:1 ratio of Z to D (m = 1).

[0081] Preferably, Z is an antibody against a tumor associated antigen, allowing the selective targeting of cancer cells. Examples of such antigens include: mesothelin, prostate specific membrane antigen (PSMA), CD19, CD22, CD30, CD70, B7H3, B7H4 (also known as O8E), protein tyrosine kinase 7 (PTK7), glypican-3, RG1, fucosyl-GM1, CTLA-4, and CD44. The

5 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 8,609,816 B2 (2013; B7H4, also known as 08E; in particular antibodies 2A7, 1G11, and 2F9); Rao-Naik *et al.*, 8,097,703 B2 (2012; CD19; in particular antibodies 5G7, 13F1, 46E8,

10 21D4, 21D4a, 47G4, 27F3, and 3C10); King *et al.*, US 8,481,683 B2 (2013; CD22; in particular antibodies 12C5, 19A3, 16F7, and 23C6); Keler *et al.*, US 7,387,776 B2 (2008; CD30; in

15 particular antibodies 5F11, 2H9, and 17G1); Terrett *et al.*, US 8,124,738 B2 (2012; CD70; in

particular antibodies 2H5, 10B4, 8B5, 18E7, and 69A7); Korman *et al.*, US 6,984,720 B1 (2006; CTLA-4; in particular antibodies 10D1, 4B6, and 1E2); Vistica *et al.*, US 8,383,118 B2 (2013,

15 fucosyl-GM1, in particular antibodies 5B1, 5B1a, 7D4, 7E4, 13B8, and 18D5); Korman *et al.*, US 8,008,449 B2 (2011; PD-1; in particular antibodies 17D8, 2D3, 4H1, 5C4, 4A11, 7D3, and 5F4); Huang *et al.*, US 2009/0297438 A1 (2009; PSMA. in particular antibodies 1C3, 2A10,

20 2F5, 2C6); Cardarelli *et al.*, US 7,875,278 B2 (2011; PSMA; in particular antibodies 4A3, 7F12, 8C12, 8A11, 16F9, 2A10, 2C6, 2F5, and 1C3); Terrett *et al.*, US 8,222,375 B2 (2012; PTK7; in

25 particular antibodies 3G8, 4D5, 12C6, 12C6a, and 7C8); Terrett *et al.*, US 8,680,247 B2 (2014; glypican-3; in particular antibodies 4A6, 11E7, and 16D10); Harkins *et al.*, US 7,335,748 B2

(2008; RG1; in particular antibodies A, B, C, and D); Terrett *et al.*, US 8,268,970 B2 (2012; mesothelin; in particular antibodies 3C10, 6A4, and 7B1); Xu *et al.*, US 2010/0092484 A1

(2010; CD44; in particular antibodies 14G9.B8.B4, 2D1.A3.D12, and 1A9.A6.B9); Deshpande

30 *et al.*, US 8,258,266 B2 (2012; IP10; in particular antibodies 1D4, 1E1, 2G1, 3C4, 6A5, 6A8, 7C10, 8F6, 10A12, 10A12S, and 13C4); Kuhne *et al.*, US 8,450,464 B2 (2013; CXCR4; in

particular antibodies F7, F9, D1, and E2); and Korman *et al.*, US 7,943,743 B2 (2011; PD-L1; in

particular antibodies 3G10, 12A4, 10A5, 5F8, 10H10, 1B12, 7H1, 11E6, 12B7, and 13G4); the

disclosures of which are incorporated herein by reference.

35 [0082] In addition to being an antibody, Z can also be an antibody fragment (such as Fab, Fab', F(ab')₂, Fd, or Fv) 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.

[0083] Any one of several different reactive groups on Z can be a conjugation site, including ϵ -amino groups in lysine residues, pendant carbohydrate moieties, carboxylic acid groups on aspartic or glutamic acid side chains, cysteine-cysteine disulfide groups, and cysteine thiol groups. For reviews on antibody reactive groups suitable for conjugation, see, e.g., Garnett, *Adv.*

5 *Drug Delivery Rev.* **2001**, 53, 171-216 and Dubowchik and Walker, *Pharmacology & Therapeutics* **1999**, 83, 67-123, the disclosures of which are incorporated herein by reference.

[0084] Most antibodies have multiple lysine residues, which can be conjugated via their ϵ -amino groups via amide, urea, thiourea, or carbamate bonds.

10 [0085] In another embodiment, 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* 15 83, 2632-2636 (1986); the disclosure of which is incorporated herein by reference.

[0086] In another embodiment, Z can be conjugated via an aspartic or glutamic acid side chain carboxylic acid, for example by conversion to a carbohydrazide, which is then reacted with an aldehyde-bearing linker. See Fisch *et al.*, *Bioconjugate Chemistry* **1992**, 3, 147-153.

20 [0087] A thiol (-SH) group in the side chain of a cysteine can be used to form a conjugate by several methods. It can be used to form a disulfide bond between it and a thiol group on the linker. Another method is via its Michael addition to a maleimide group on the linker.

25 [0088] Typically, although antibodies have cysteine residues, they lack free thiol groups because all their cysteines are engaged in intra- or inter-chain disulfide bonds. To generate a free thiol group, a native disulfide group can be reduced. See, e.g., Packard *et al.*, *Biochemistry* **1986**, 25, 3548; King *et al.*, *Cancer Res.* **1994**, 54, 6176; and Doronina *et al.*, *Nature Biotechnol.* **2003**, 21, 778. Alternatively, a cysteine having a free -SH group can be introduced by mutating the antibody, substituting a cysteine for another amino acid or inserting one into the polypeptide chain. See, for example, Eigenbrot *et al.*, US 7,521,541 B2 (2009); Chilkoti *et al.*, *Bioconjugate Chem.* **1994**, 5, 504; Urnovitz *et al.*, US 4,698,420 (1987); Stimmel *et al.*, *J. Biol. Chem.* **2000**, 30, 275, 30445; Bam *et al.*, US 7,311,902 B2 (2007); Kuan *et al.*, *J. Biol. Chem.* **1994**, 269, 7610; Poon *et al.*, *J. Biol. Chem.* **1995**, 270, 8571; Junutula *et al.*, *Nature Biotechnology* **2008**, 26, 925 and Rajpal *et al.*, US Provisional Application No. 62/270245, filed Dec. 21, 2015. In yet another approach, a cysteine is added to the C-terminus of the heavy or light chain. See, e.g., Liu *et al.*,

US 8,865,875 B2 (2014); Cumber *et al.*, *J. Immunol.* **1992**, 149, 120; King *et al.*, *Cancer Res.* **1994**, 54, 6176; Li *et al.*, *Bioconjugate Chem.* **2002**, 13, 985; Yang *et al.*, *Protein Engineering* **2003**, 16, 761; and Olafson *et al.*, *Protein Engineering Design & Selection* **2004**, 17, 21. The disclosures of the documents cited in this paragraph are incorporated herein by reference.

5 *Linkers and Their Components*

[0089] As noted above, the linker comprises up to three elements: a cleavable group C and optional spacers X^Z and X^D.

[0090] Group C is cleavable under physiological conditions. Preferably it is relatively stable while the conjugate is in circulation in the blood, but is readily cleaved once the conjugate 10 reaches its site of intended action, near, at, or within the target cell. Preferably, the conjugate is internalized by a target cell upon binding of an antibody Z to an antigen displayed on the surface of the target cell. Subsequently, cleavage of C occurs in a vesicular body of the target cell (an early endosome, a late endosome, or, especially, a lysosome).

[0091] In one embodiment, C is a pH sensitive group. The pH in blood plasma is slightly 15 above neutral, while the pH inside a lysosome is acidic, circa 5. Thus, an acid-sensitive C will cleave at a rate several orders of magnitude faster inside a lysosome than in blood. Examples of acid-sensitive groups are 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.* **1981**, 102, 1048; and Yang *et al.*, *Proc. Natl Acad. Sci (USA)* **1988**, 85, 1189; the 20 disclosures of which are incorporated herein by reference.

[0092] In another embodiment, 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 25 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. See, e.g., Thorpe *et al.*, *Cancer Res.* **2008**, 48, 6396-6403; 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; 30 Boyd *et al.*, US 7,691,962 B2 (2010); and Sufi *et al.*, US 8,461,117 B2 (2013); the disclosures of which are incorporated herein by reference.

[0093] A preferred group C is a peptide that is cleaved selectively by a protease inside the target cell, as opposed to by a protease in the serum. Typically, the peptide comprises from 1 to 20 amino acids, preferably from 1 to 6 amino acids, more preferably from 2 to 3 amino acids.

The amino acid(s) can be natural and/or non-natural α -amino acids. Natural amino acids are

5 those encoded by the genetic code, as well as amino acids derived therefrom, *e.g.*, hydroxyproline, γ -carboxyglutamate, citrulline, and O-phosphoserine. In this specification, the term “amino acid” also includes amino acid analogs and mimetics. Analogs are compounds having the same general $\text{H}_2\text{N}(\text{R})\text{CHCO}_2\text{H}$ 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, 10 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 amino acid can be of the “L” stereochemistry of the genetically encoded amino acids, as well as of the enantiomeric “D” stereochemistry.

[0094] Preferably, C contains an amino acid sequence that is a cleavage recognition

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

20 **[0095]** 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 a cancer, *e.g.*, a protease released by nearby dying cancer cells or a tumor-associated protease secreted by cancer cells. Exemplary extracellular tumor-associated proteases are plasmin, matrix metalloproteases (MMP), thimet oligopeptidase (TOP) and CD10. *See, e.g.*, Trouet *et al.*, US 7,402,556 B2 (2008); Dubois *et al.*, US 7,425,541 B2 (2008); and Bebbington *et al.*, US 6,897,034 B2 (2005).

25 **[0096]** For conjugates designed to be internalized by a cell, 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 30 cathepsin B. Exemplary cathepsin B cleavable peptides include Val-Ala, Val-Cit, Val-Lys, Lys-Val-Ala, Asp-Val-Ala, Val-Ala, Lys-Val-Cit, Ala-Val-Cit, Val-Gly, Val-Gln, and Asp-Val-Cit. (Herein, amino acid sequences are written in the N-to-C direction, as in $\text{H}_2\text{N-AA}^2\text{-AA}^1\text{-CO}_2\text{H}$, unless the context clearly indicates otherwise.) *See* Dubowchik *et al.*, *Biorg. Med. Chem. Lett.*

1998, 8, 3341; Dubowchik *et al.*, *Bioorg. Med. Chem. Lett.* 1998, 8, 3347; and Dubowchik *et al.*, *Bioconjugate Chem.* 2002, 13, 855; the disclosures of which are incorporated by reference.

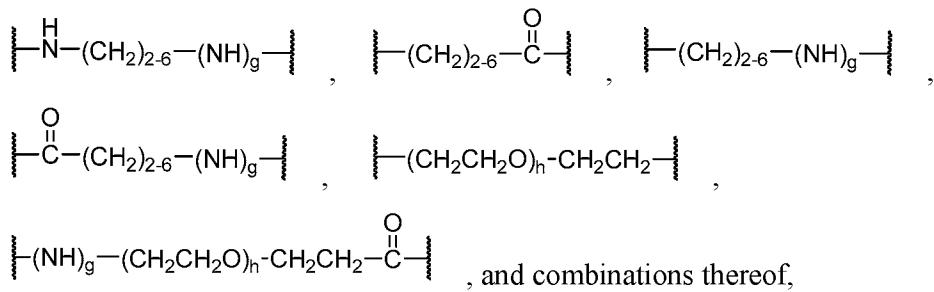
[0097] Another enzyme that can be utilized for cleaving peptidyl linkers is legumain, a lysosomal cysteine protease that preferentially cleaves at Ala-Ala-Asn.

5 [0098] In one embodiment, Group C is a peptide comprising a two-amino acid sequence -AA²-AA¹- wherein AA¹ is lysine, arginine, or citrulline and AA² is phenylalanine, valine, alanine, leucine or isoleucine. In another embodiment, C consists of a sequence of one to three 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. More 10 preferably, it is a two to three amino acid peptide from the foregoing group.

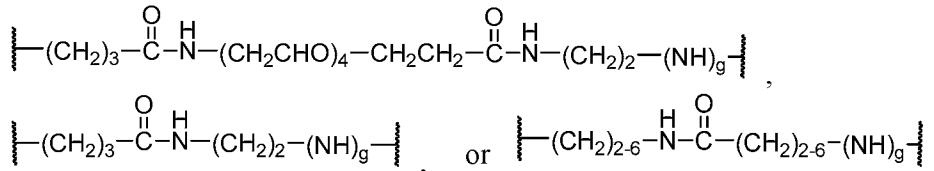
[0099] The preparation and design of cleavable groups C consisting of a single amino acid is disclosed in Chen *et al.*, US 8,664,407 B2 (2014), the disclosure of which is incorporated herein by reference.

15 [00100] Group C can be bonded directly to Z or D; *i.e.* spacers X^Z or X^D, as the case may be, can be absent.

[00101] When present, spacer X^Z provides spatial separation between C and 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 20 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:



25 where the subscript g is 0 or 1 and the subscript h is 1 to 24, preferably 2 to 4. These segments can be combined, such as illustrated below:



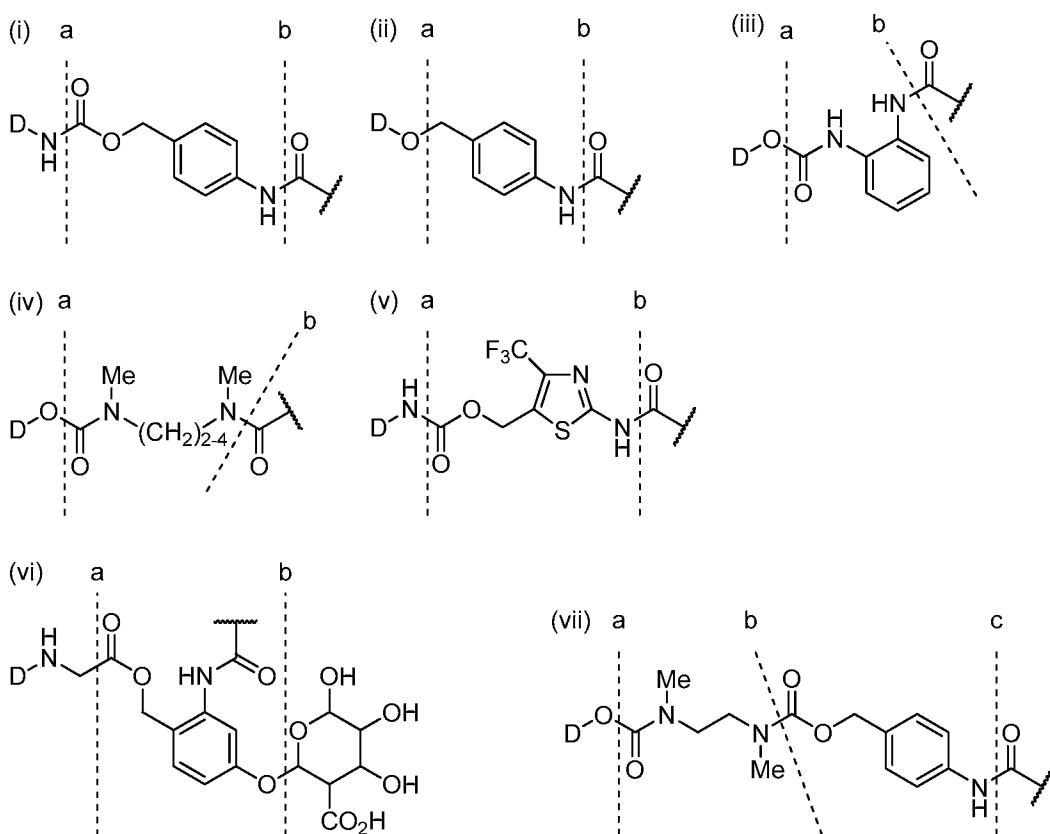
[00102] Spacer X^D, if present, provides spatial separation between C and D, lest the latter interfere sterically or electronically with cleavage of the former. Spacer X^D also can serve to introduce additional molecular mass and chemical functionality into a conjugate. Generally, the additional mass and functionality will affect the serum half-life and other properties of the conjugate. Thus, through judicious selection of spacer groups, the serum half-life of a conjugate can be modulated. Spacer X^D also can be assembled from modular segments, analogously to the description above for spacer X^Z.

[00103] 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.

[00104] The linker can perform other functions in addition to covalently linking the antibody and the drug. For instance, the linker can contain a poly(ethylene glycol) (“PEG”) group. Since the conjugation step typically involves coupling a drug-linker to an antibody in an aqueous medium, a PEG group may enhance the aqueous solubility of the drug-linker. Also, a PEG group may enhance the solubility or reduce aggregation in the resulting ADC. Where a PEG group is present, it may be incorporated into either spacer X^Z or X^D, or both. The number of repeat units in a PEG group can be from 2 to 20, preferably between 4 and 10.

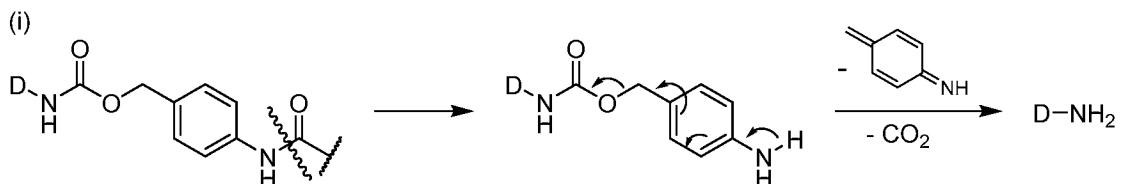
[00105] Either spacer X^Z or X^D, or both, can comprise a self-immolating moiety. A self-immolating moiety is a moiety that (1) is bonded to C and either Z or D and (2) has a structure such that cleavage from group C initiates a reaction sequence resulting in the self-immolating moiety disbonding itself from Z or D, as the case may be. In other words, reaction at a site distal from Z or 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 D, the biological activity of D may be impaired. The use of a self-immolating moiety is especially desirable where cleavable group C is a polypeptide, in which instance the self-immolating moiety typically is located adjacent thereto, in order to prevent D from sterically or electronically interfering with peptide cleavage.

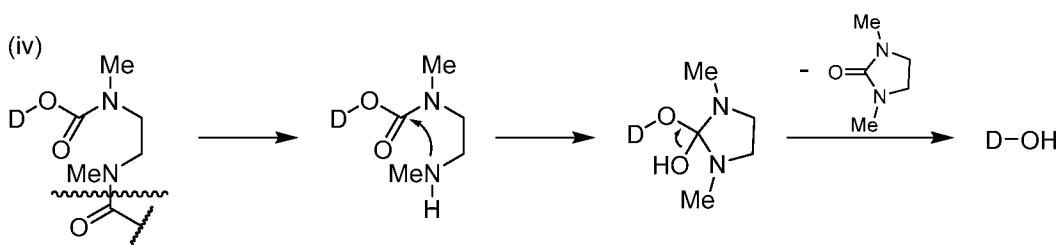
[00106] Exemplary self-immolating moieties (i)-(v) bonded to a hydroxyl or amino group of D are shown below:



[00107] The self-immolating moiety is the structure between dotted lines a and b (or dotted lines b and c), with adjacent structural features shown to provide context. Self-immolating moieties (i) and (v) are bonded to a D-NH₂ (*i.e.*, conjugation is via an amino group), while self-immolating moieties (ii), (iii), and (iv) are bonded to a D-OH (*i.e.*, conjugation is via a hydroxyl or carboxyl group). Cleavage of the bond at dotted line b by an enzyme – a peptidase in the instance of structures (i)-(v) and a β -glucuronidase in the instance of structure (vi) – initiates a self-immolating reaction sequence that results in the cleavage of the bond at dotted line a and the consequent release of D-OH or D-NH₂, as the case may be. By way of illustration, self-immolating mechanisms for structures (i) and (iv) are shown below:

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[00108] In other words, cleavage of a first chemical bond at one part of a self-immolating group initiates a sequence of steps that results in the cleavage of a second chemical bond – the one connecting the self-immolating group to the drug – at a different part of the self-immolating group, thereby releasing the drug.

[00109] In some instances, self-immolating groups can be used in tandem, as shown by structure (vii). In such case, cleavage at dotted line c triggers self-immolation of the moiety between dotted lines b and c by a 1,6-elimination reaction, followed by self-immolation of the moiety between dotted lines a and b by a cyclization-elimination reaction. For additional disclosures regarding self-immolating moieties, see Carl *et al.*, *J. Med. Chem.* **1981**, 24, 479; Carl *et al.*, WO 81/01145 (1981); Dubowchik *et al.*, *Pharmacology & Therapeutics* **1999**, 83, 67; Firestone *et al.*, US 6,214,345 B1 (2001); Toki *et al.*, *J. Org. Chem.* **2002**, 67, 1866; Doronina *et al.*, *Nature Biotechnology* **2003**, 21, 778 (*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; Jeffrey *et al.*, US 8,039,273; and Senter *et al.*, US 2003/0096743 A1; the disclosures of which are incorporated by reference.

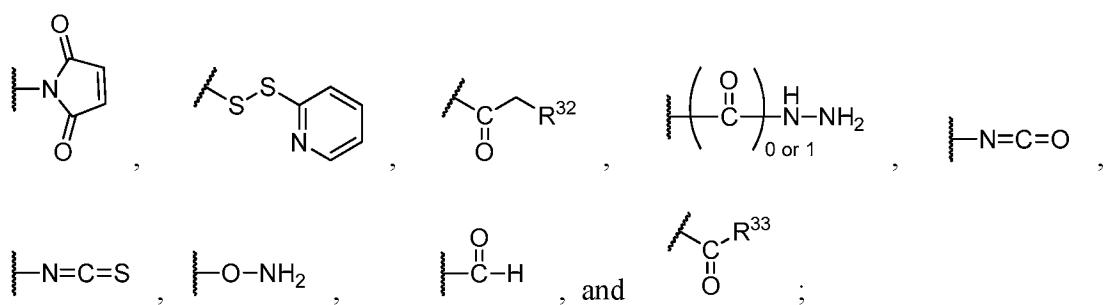
[00110] In another embodiment, Z and D are linked by a non-cleavable linker, *i.e.*, C is absent. Metabolism of D eventually reduces the linker to a small appended moiety that does not interfere with the biological activity of D.

20 Conjugation Techniques

[00111] Conjugates of this invention preferably are made by first preparing a compound comprising D and linker $(X^D)_a(C)_c(X^Z)_b$ (where X^D , C, X^Z , a, b, and c are as defined for formula (II)) to form drug-linker compound represented by formula (III):



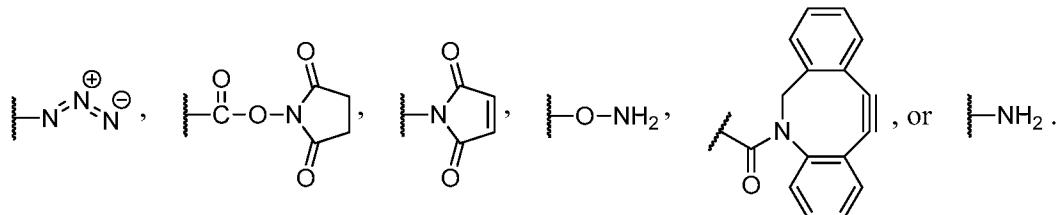
25 where R^{31} is a functional group suitable for reacting with a complementary functional group on Z to form the conjugate. Examples of suitable groups R^{31} include amino, azide, thiol, cyclooctyne,



where R^{32} is Cl, Br, F, mesylate, or tosylate and R^{33} is Cl, Br, I, F, OH, -O-N-succinimidyl,

5 -O-(4-nitrophenyl), -O-pentafluorophenyl, or -O-tetrafluorophenyl. Chemistry generally usable for the preparation of suitable moieties $D-(X^D)_aC(X^Z)_b-R^{31}$ 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 10 7,847,105 B2 (2010); Gangwar *et al.*, US 7,968,586 B2 (2011); Sufi *et al.*, US 8,461,117 B2 (2013); and Chen *et al.*, US 8,664,407 B2 (2014); the disclosures of which are incorporated herein by reference.

[00112] Preferably reactive functional group $-R^{31}$ is -NH₂, -OH, -CO₂H, -SH, maleimido, cyclooctyne, azido (-N₃), hydroxylamino (-ONH₂) or N-hydroxysuccinimido. Especially preferred functional groups $-R^{31}$ are:



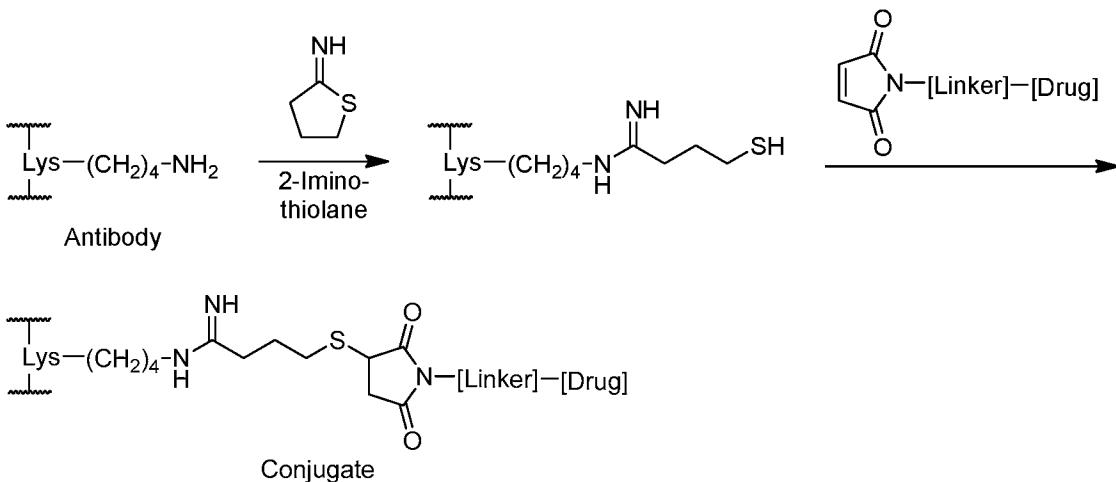
[00113] An -OH group can be esterified with a carboxy group on the antibody, for example, on an aspartic or glutamic acid side chain.

[00114] A -CO₂H group can be esterified with a -OH group or amidated with an amino group 20 (for example on a lysine side chain) on the antibody.

[00115] An N-hydroxysuccinimide group is functionally an activated carboxyl group and can conveniently be amidated by reaction with an amino group (e.g., from lysine).

[00116] 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 sulphydryl 25 functionality), in a Michael addition reaction.

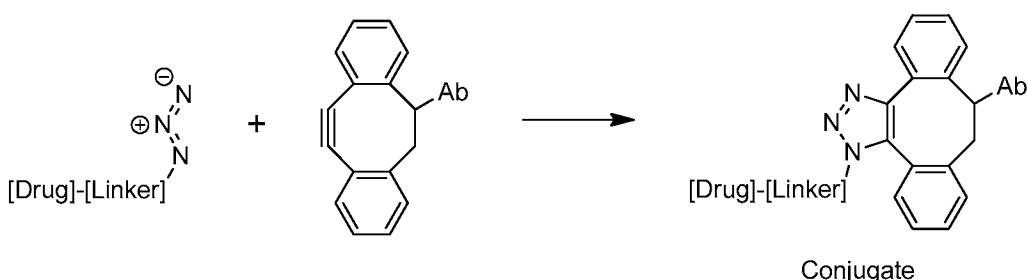
[00117] Where an antibody does not have a cysteine –SH available for conjugation, an ϵ -amino group in the side chain of a lysine residue can be reacted with 2-iminothiolane or N-succinimidyl-3-(2-pyridyldithio)propionate (“SPDP”) to introduce a free thiol (-SH) group – creating a cysteine surrogate, as it were. The thiol group can react with a maleimide or other nucleophile acceptor group to effect conjugation. The mechanism is illustrated below with 2-iminothiolane.



[00118] Typically, a thiolation level of two to three thiols per antibody is achieved. For a representative procedure, see Cong *et al.*, US 8,980,824 B2 (2015), the disclosure of which is incorporated herein by reference.

[00119] In a reversed arrangement, an antibody Z can be modified with N-succinimidyl 4-(maleimidomethyl)-cyclohexanecarboxylate (“SMCC”) or its sulfonated variant sulfo-SMCC, both of which are available from Sigma-Aldrich, to introduce a maleimide group thereto. Then, conjugation can be effected with a drug-linker compound having an –SH group on the linker.

[00120] An alternative conjugation method employs copper-free “click chemistry,” in which an azide group adds across a strained cyclooctyne to form an 1,2,3-triazole ring. See, *e.g.*, Agard *et al.*, *J. Amer. Chem. Soc.* **2004**, 126, 15046; Best, *Biochemistry* **2009**, 48, 6571, the disclosures of which are incorporated herein by reference. The azide can be located on the antibody and the cyclooctyne on the drug-linker moiety, or vice-versa. A preferred cyclooctyne group is dibenzocyclooctyne (DIBO). Various reagents having a DIBO group are available from Invitrogen/Molecular Probes, Eugene, Oregon. The reaction below illustrates click chemistry conjugation for the instance in which the DIBO group is attached to the antibody (Ab):

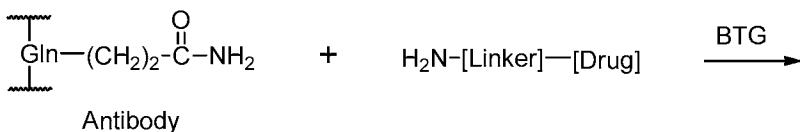


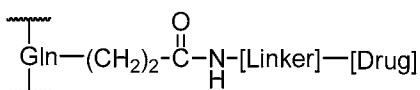
[00121] Yet another conjugation technique involves introducing a non-natural amino acid into an antibody, with the non-natural amino acid providing a functionality for conjugation with a reactive functional group in the drug moiety. For instance, the non-natural amino acid p-

5 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-acetylphenylalanine can be a conjugation site via the formation of an oxime with a hydroxylamino group on the linker-drug moiety. Alternatively, the non-natural amino acid p-azidophenylalanine can be incorporated into 10 an antibody to provide an azide functionality for conjugation via click chemistry, as discussed above. 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. The foregoing disclosures are 15 incorporated herein by reference. Thus, in one embodiment, an antibody that is used for making a conjugate with a drug of this invention has one or more amino acids replaced by a non-natural amino acid, which preferably is p-acetylphenylalanine or p-azidophenylalanine, more preferably p-acetylphenylalanine.

[00122] Still another conjugation technique uses the enzyme transglutaminase (preferably bacterial transglutaminase from *Streptomyces mobaraensis* or BTG), per Jeger *et al.*, *Angew. Chem. Int. Ed.* **2010**, 49, 9995. BTG forms an amide bond between the side chain carboxamide

20 of a glutamine (the amine acceptor) and an alkyleneamino group (the amine donor), which can be, for example, the ϵ -amino group of a lysine or a 5-amino-n-pentyl group. In a typical conjugation reaction, the glutamine residue is located on the antibody, while the alkyleneamino group is located on the linker-drug moiety, as shown below:





Conjugate

[00123] The positioning of a glutamine residue on a polypeptide chain has a large effect on its susceptibility to BTG mediated transamidation. None of the glutamine residues on an antibody are normally BTG substrates. However, if the antibody is deglycosylated – the glycosylation site

5 being asparagine 297 (N297; numbering per EU index as set forth in Kabat *et al.*, "Sequences of proteins of immunological interest, 5th ed., Pub. No. 91-3242, U.S. Dept. Health & Human Services, NIH, Bethesda, Md., 1991; hereinafter "Kabat") of the heavy chain – nearby glutamine 295 (Q295) is rendered BTG susceptible. An antibody can be deglycosylated enzymatically by treatment with PNGase F (Peptide-N-Glycosidase F). Alternatively, an antibody can be

10 synthesized glycoside free by introducing an N297A mutation in the constant region, to eliminate the N297 glycosylation site. Further, it has been shown that an N297Q substitution not only eliminates glycosylation, but also introduces a second glutamine residue (at position 297) that too is an amine acceptor. Thus, in one embodiment, an antibody that is conjugated to a drug of this invention is deglycosylated. In another embodiment, the antibody has an N297Q

15 substitution. Those skilled in the art will appreciate that deglycosylation by post-synthesis modification or by introducing an N297A mutation generates two BTG-reactive glutamine residues per antibody (one per heavy chain, at position 295), while an antibody with an N297Q substitution will have four BTG-reactive glutamine residues (two per heavy chain, at positions 295 and 297).

20 **[00124]** An antibody can also be rendered susceptible to BTG-mediated conjugation by introducing into it a glutamine containing peptide, or "tag," as taught, for example, in Pons *et al.*, US 2013/0230543 A1 (2013) and Rao-Naik *et al.*, PCT Application No. PCT/US2016/020192, filed Mar. 1, 2016.

25 **[00125]** In a complementary approach, the substrate specificity of BTG can be altered by varying its amino acid sequence, such that it becomes capable of reacting with glutamine 295 in an unmodified antibody, as taught in Rao-Naik *et al.*, US Provisional Application No. 62/236274, filed Oct. 2, 2015.

30 **[00126]** Lastly, while the most commonly available bacterial transglutaminase is that from *S. mobaraensis*, transglutaminase from other bacteria, having somewhat different substrate specificities, can be considered, such as transglutaminase from *Streptovorticillium ladakanum*

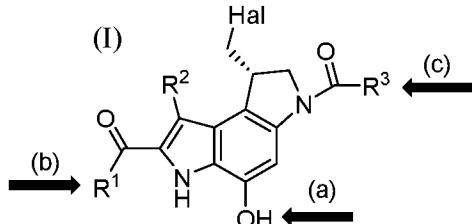
(Hu *et al.*, US 2009/0318349 A1 (2009), US 2010/0099610 A1 (2010), and US 2010/0087371 A1 (2010)).

[00127] 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 R³¹ in formula (III), or vice-versa.

Seco-CPI-Linker Compounds

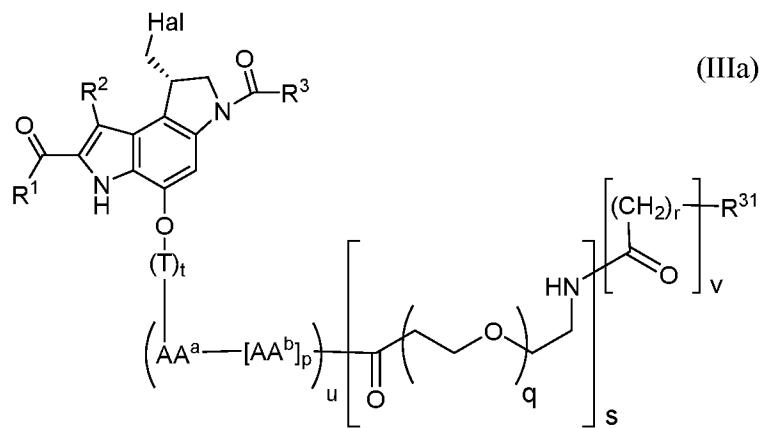
[00128] An ADC of a *seco*-CPI compound of this invention includes a linker attached to a functional group on the *seco*-CPI compound, which linker is attached to the antibody. Reflecting the diversity of conjugation techniques known in the art, the *seco*-CPI compounds of this invention can be elaborated into many different *seco*-CPI compound-linker compounds suitable for conjugation to an antibody.

[00129] Generally, there are three different sites for attachment of a linker to a *seco*-CPI compound of this invention, as illustrated below:



[00130] In type (a) *seco*-CPI-linker compounds, the linker is attached to the phenolic hydroxyl of the binding subunit and, while attached, acts as a prodrugging group preventing cyclization to the CPI structure. In type (b) *seco*-CPI-linker compounds, the linker is attached via a suitable functionality in the group R¹, such as an amino, carboxy, or hydroxyl group. In type (c) *seco*-CPI-linker compounds, the linker is attached via a suitable functionality in the group R³, such as an amino, carboxy, or hydroxyl group. In types (b) and (c), the phenolic hydroxyl group optionally can be prodrugged, as discussed above.

[00131] In one embodiment, type (a) *seco*-CPI-linker compounds can be represented by the formula (IIIa):



wherein

T is a self-immolating group;

t is 0 or 1;

5 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;

10 u is 0 or 1;

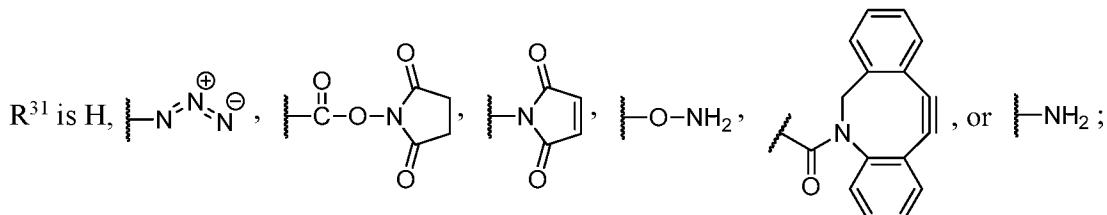
p is 1, 2, 3, or 4;

q is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 (preferably 2, 3, 4, or 8);

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

s is 0 or 1;

15 v is 0 or 1;

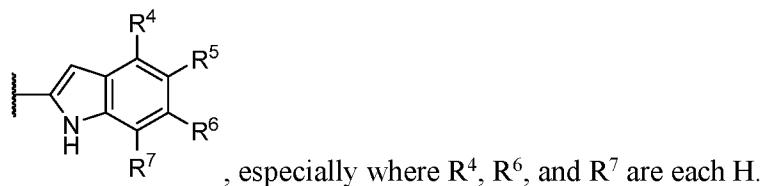


with the provisos that R^{31} can be H only if s is 1 and v is 0 and that v can be 0 only if s is 1 and R^{31} is H ;

and

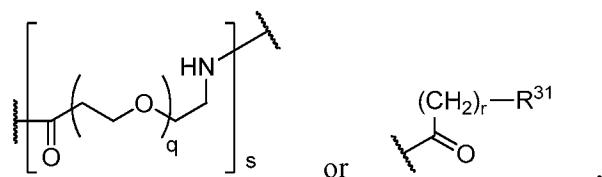
20 R^1 , R^2 , and R^3 are as defined in the BRIEF SUMMARY OF THE INVENTION section hereinabove in respect of formula (I).

[00132] In one embodiment of *seco*-CPI linkers according to formula (IIIa), R³ is



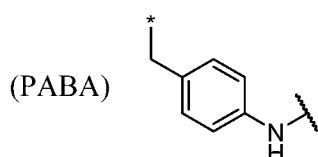
[00133] In one embodiment, u is 1 in formula (IIIa).

[00134] The moiety -AA^a-[AA^b]_p- represents a polypeptide whose length is determined by the value of p (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 an amine nitrogen of the *seco*-CPI compound. Conversely, the last AA^b is at the amino terminus of the polypeptide and its α -amino group forms a peptide bond with

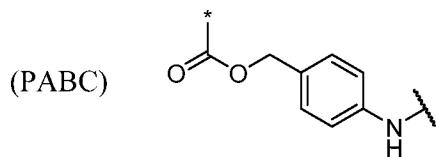


depending on whether s is 1 or 0, respectively. Preferred polypeptides -AA^a-[AA^b]_p- are Val-Ala, Val-Cit, Val-Lys, Lys-Val-Ala, Asp-Val-Ala, Val-Ala, Lys-Val-Cit, Ala-Val-Cit, Val-Gly, Val-Gln, and Asp-Val-Cit, written in the conventional N-to-C direction, as in H₂N-Val-Cit-CO₂H). More preferably, the polypeptide is Val-Cit, Val-Lys, or Val-Ala. Preferably, a polypeptide -AA^a-[AA^b]_p- is cleavable by an enzyme found inside the target (cancer) cell, for example a cathepsin and especially cathepsin B.

[00135] As indicated by the subscript t equals 0 or 1, a self-immolating group T is optionally present. A preferred self-immolating group T is a *p*-aminobenzyl alcohol (PABA) group, which has the structure is shown below (phenolic oxygen not shown), with an asterisk (*) denoting the end bonded to the phenolic oxygen of the *seco*-CPI compound and a wavy line (~~~~) denoting the end bonded to the polypeptide -AA^a-[AA^b]_p-.

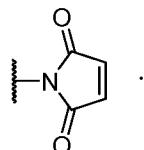


[00136] Another preferred self-immolating group T is a *p*-aminobenzyl oxy carbonyl (PABC) group, which has the structure shown below:



[00137] In some instances a PABA group may be preferable over a PABC group, as being more hydrolytically stable.

[00138] In a preferred embodiment, the group R³¹ is

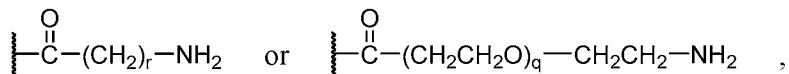


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[00139] In another preferred embodiment, the group R³¹ is

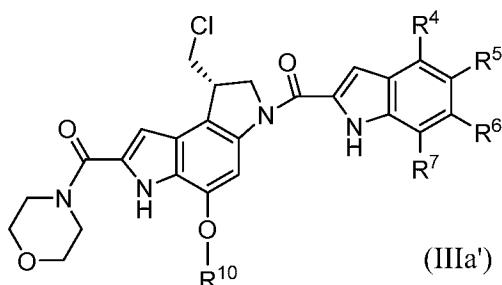


or R³¹ is H while v is 0 and s is 1. In each case, the result is a *seco*-CPI-linker compound that has an amino-terminal group, of the structure



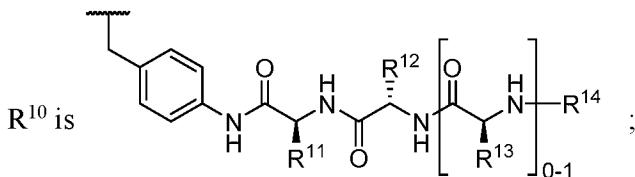
respectively, which is suitable as an amine donor for a transglutaminase-mediated conjugation.

[00140] Preferred type (a) *seco*-CPI-linker compounds are according to formula (IIIa')

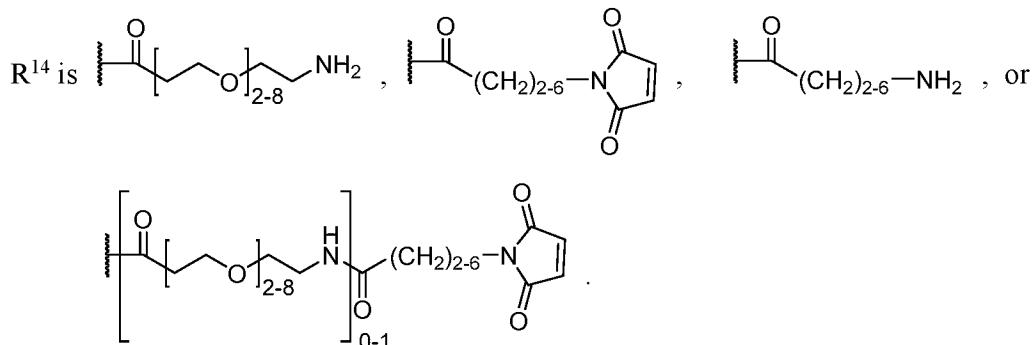


where

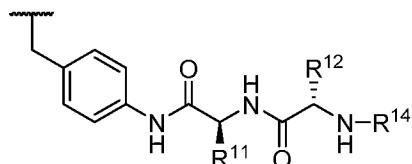
15 R⁴, R⁵, R⁶, and R⁷ are as defined in respect of formula (I) the BRIEF SUMMARY OF THE INVENTION section hereinabove;



R¹¹, R¹² and R¹³ are independently H, CH₃, CH(CH₃)₂, CH₂CO₂H, CH₂CH₂CO₂H, CH₂C(=O)NH₂, CH₂CH₂C(=O)NH₂, (CH₂)₄NH₂, (CH₂)₃NHC(=NH)NH₂ or (CH₂)₃NHC(=O)NH₂ (that is, R¹¹, R¹², and R¹³ correspond to the side chain residues of the amino acids glycine, valine, aspartic acid, glutamic acid, asparagine, glutamine, lysine, arginine, and citrulline); and



[00141] Preferably, the peptide in R^{10} is a dipeptide – *i.e.*, the suffix associated with the amino acid $HO_2CCH(R^{13})NH_2$ in the peptide is zero – corresponding to the formula



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[00142] Specific compounds according to formula (IIIa') are shown in Table III, where R⁴, R⁶, and R⁷ are H unless noted otherwise and R¹⁰ is A or B:

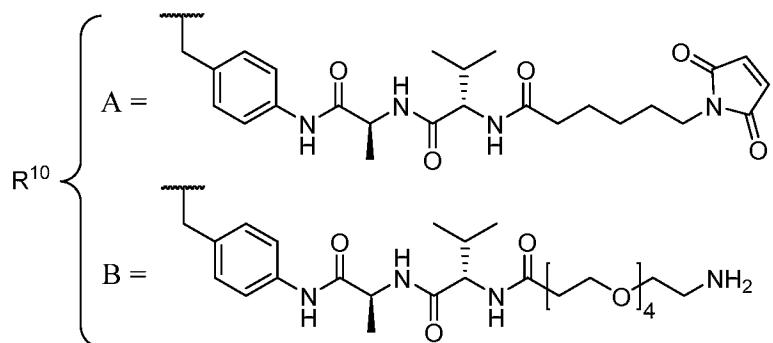
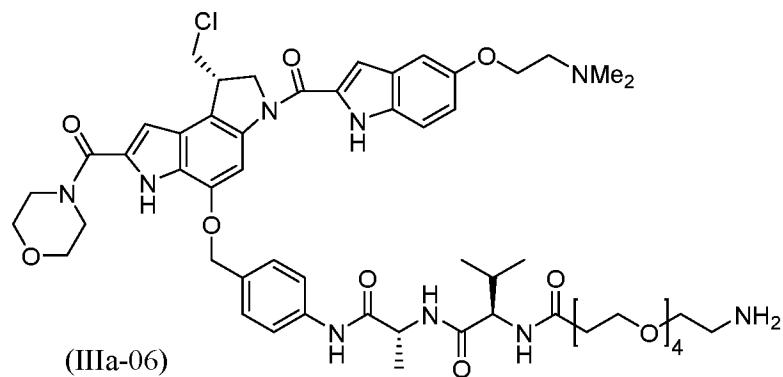


Table III – *seco*-CPI-Linker Compounds

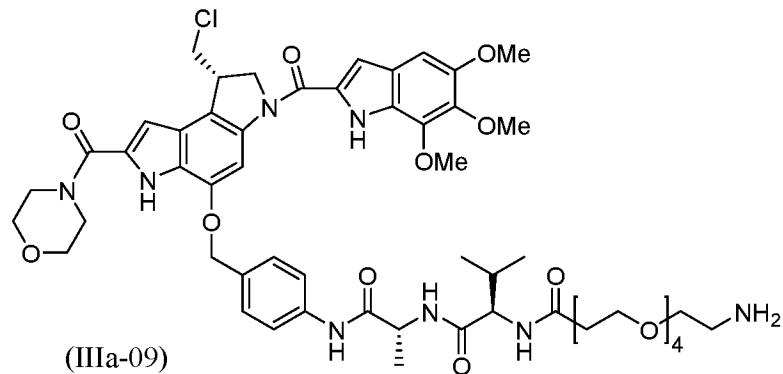
Table III - 3000 CPI-Linker Compounds		
seco-CPI-Linker	R ¹⁰	R ⁵
IIIa-01	A	

Table III – <i>seco</i> -CPI-Linker Compounds		
<i>seco</i> -CPI-Linker	R^{10}	R^5
IIIa-02	A	
IIIa-03	A	
IIIa-04	A	
IIIa-05	B	
IIIa-06	B	
IIIa-07	B	
IIIa-08	B	
IIIa-09	B	
IIIa-10	B	
IIIa-11	B	
IIIa-12	B	
IIIa-13	A	

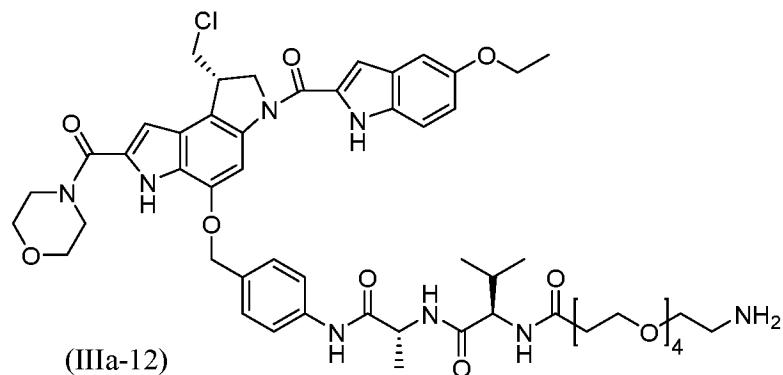
[00143] A preferred *seco*-CPI-linker compound is **(IIIa-06)**, whose complete structure is shown following:



[00144] A preferred *seco*-CPI-linker compound is **(IIIa-09)**, whose complete structure is shown following:



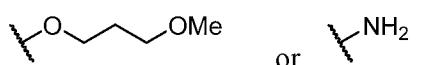
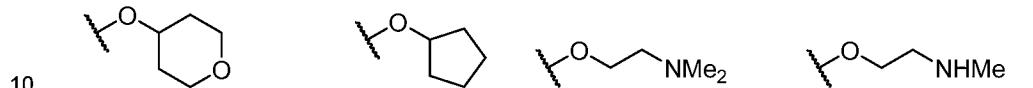
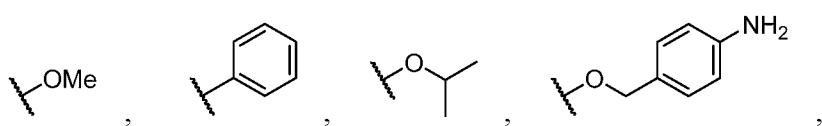
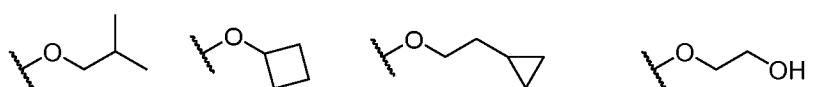
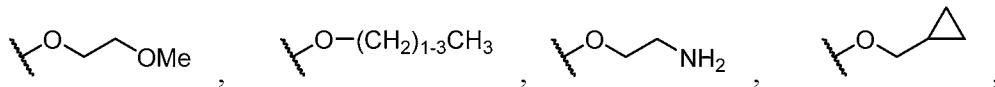
[00145] A preferred *seco*-CPI-linker compound is **(IIIa-12)**, whose complete structure is shown following:



[00146] *seco*-CPI-linker compounds having a A-type linkers are designed for conjugation via a Michael addition reaction with a thiol group on the antibody, as discussed above. Those having

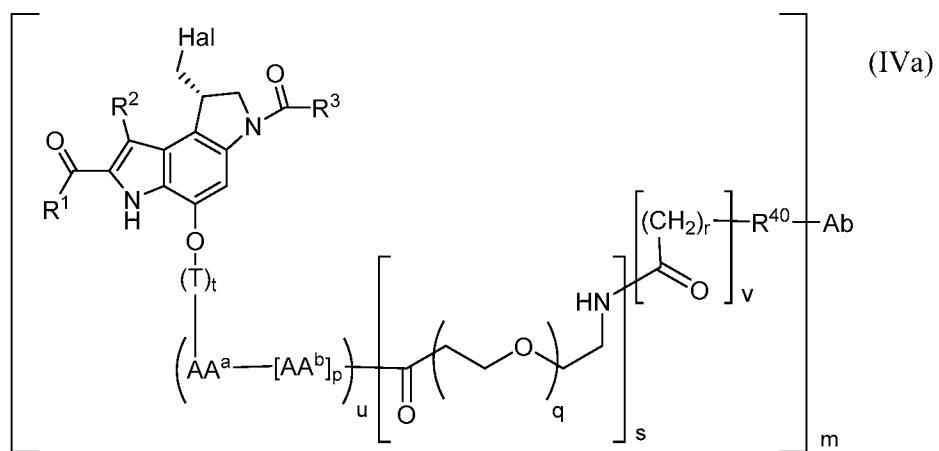
a B-type linker are designed for transglutaminase mediated conjugation, again as discussed above.

[00147] More preferably, in formula (IIIa') R⁴, R⁶, and R⁷ are each H; and R⁵ is



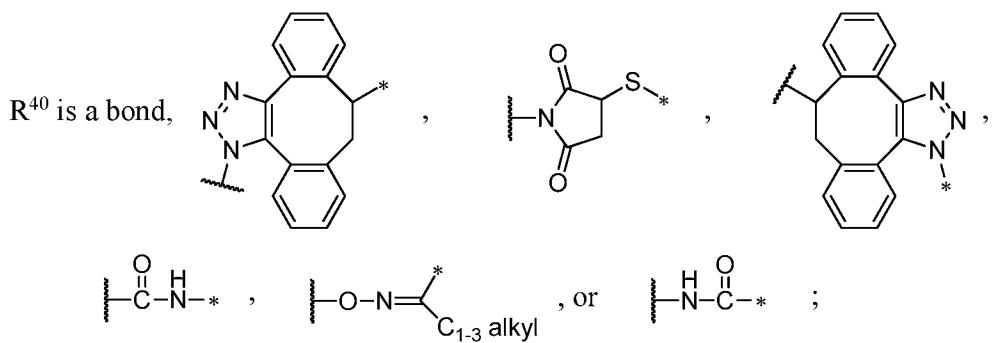
Conjugates

[00148] In one embodiment, conjugates of this invention are derived from type (a) *seco*-CPI-linker compounds and can be represented by the formula (IVa):



wherein

Ab is an antibody;



where the open valence of R^{40} that is bonded to Ab is denoted by an asterisk (*) and the open valence of R^{40} that is bonded to $(CH_2)_r$ is denoted by a wavy line (~~~~~);

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

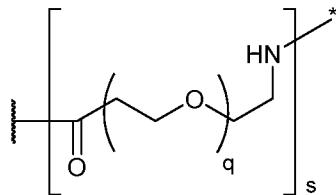
v is 0 or 1, with the provisos that v can be 0 only if s is 1 and R^{40} is a bond and that R^{40} can be a bond only if v is 0 and s is 1;

T, t, AA^a, AA^b, u, p, q, s, r, and X^A are as defined in respect of formula (IIIa); and

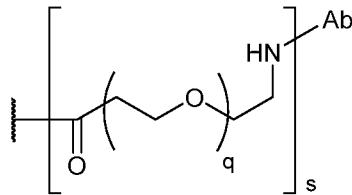
R¹, R², and R³ are as defined in the BRIEF SUMMARY OF THE INVENTION section

10 hereinabove.

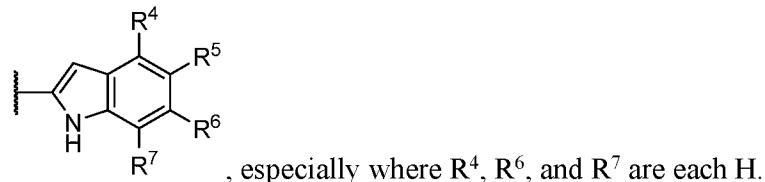
[00149] Where R^{40} is a bond, the nitrogen in



is bonded to directly antibody Ab, per the formula below:



15 **[00150]** In one embodiment of conjugates according to formula (IVa), R³ is



[00151] In a preferred embodiment, u is 1 in formula (IVa).

[00152] In formula (IVa), if the subscripts t and u are both 0, then the linker is of the non-cleavable type and relies on degradation of the antibody Ab to release the drug. The polyethylene glycol component optionally may be present (*i.e.*, s is 1) if its presence is beneficial, for example by increasing the solubility of the drug-linker compound during conjugation and does not 5 interfere with the biological activity of the drug.

PHARMACEUTICAL COMPOSITIONS

[00153] 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 10 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.

[00154] The pharmaceutical composition may comprise one or more excipients. Excipients that may be used include carriers, surface active agents, thickening or emulsifying agents, solid 15 binders, dispersion or suspension aids, solubilizers, colorants, flavoring agents, coatings, disintegrating agents, lubricants, sweeteners, preservatives, isotonic agents, and combinations thereof. The selection and use of suitable excipients is taught in Gennaro, ed., *Remington: The Science and Practice of Pharmacy*, 20th Ed. (Lippincott Williams & Wilkins 2003), the disclosure of which is incorporated herein by reference.

[00155] Preferably, a pharmaceutical composition is suitable for intravenous, intramuscular, 20 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 25 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 30 administration, for example, intranasally, orally, vaginally, rectally, sublingually or topically.

[00156] 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.

[00157] 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

5 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.

10 **[00158]** 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 15 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.

[00159] 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 20 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, or alternatively 0.1 to 5 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, 25 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.

[00160] A "therapeutically effective amount" of a compound of the invention preferably 30 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.

5 [00161] 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, poly-glycolic 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.

10 [00162] Administration can be 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 apparatus (US 4,447,233 and 4,447,224); and (5) osmotic devices (US 4,439,196 and 4,475,196); the disclosures of which are incorporated herein by reference.

15 [00163] 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; Ranade, *J. Clin. Pharmacol.* **1989**, 29, 685; Umezawa *et al.*, *Biochem. Biophys. Res. Commun.* **1988**, 153, 1038; Bloeman *et al.*, *FEBS Lett.* **1995**, 357, 140; Briscoe *et al.*, *Am. J. Physiol.* **1995**, 1233, 134; Schreier *et al.*, *J. Biol. Chem.* **1994**, 269, 9090; Keinanen and Laukkanen, *FEBS Lett.* **1994**, 346, 123; and Killion and Fidler, *Immunomethods* **1994**, 4, 273.

USES

25 [00164] 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, leiomyosarcoma, 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.

15 [00165] 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, immuno-modulators, 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, nivolumab, oxaliplatin, paclitaxel, procarbazine, suberoylanilide hydroxamic acid (SAHA), 6-thioguanidine, thioteplatin, teniposide, topotecan, trastuzumab, trichostatin A, vinblastine, vincristine, and vindesine.

EXAMPLES

[00166] 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 – Conjugation by Michael Addition

5 **[00167]** This general procedure is 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
10 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 room temperature (“RT,” circa 25 °C), the antibody is desalted into 50 mM HEPES, 5 mM Glycine, 2 mM DTPA, pH 5.5
15 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
20 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.

25 **[00168]** Typically a thiolation level of about two to 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 HEPES, 5 mM glycine, 2 mM DTPA, pH 5.5)). The thiolated material is maintained on ice while the number of thiols introduced is quantitated as described above.
30 **[00169]** After verification of the number of thiols introduced, the *seco*-CPI-linker moiety is added at a 2.5-fold molar excess per thiol. The conjugation reaction is allowed to proceed in conjugation buffer containing a final concentration of 25% propylene glycol and 5% trehalose.

Commonly, the drug-linker stock solution is dissolved in 100% DMSO. The stock solution is added directly to the thiolated antibody.

[00170] The conjugation reaction mixture is incubated at RT for 2 h with gentle stirring. A 10-fold molar excess of N-ethyl maleimide (100 mM Stock in DMSO) is then added to the conjugation mixture and stirred for an additional hour to block any unreacted thiols. The sample is then filtered via a 0.2 μ filter. The material is buffer exchanged via TFF VivaFlow 50 Sartorius 30 MWCO PES membrane into 10 mg/mL glycine, 20 mg/mL sorbitol, 15% acetonitrile (“ACN”) pH 5.0 (5X TFF buffer exchange volume), to remove any unreacted drug. The final formulation is carried out by TFF into 20 mg/mL sorbitol, 10 mg/mL glycine, pH 5.0.

10 *Example 2 – Transglutaminase-Mediated Conjugation*

[00171] The following procedure can be used for transglutaminase mediated conjugation of *seco*-CPI-linker compounds wherein the linker has an amine group that can act as an amine donor. The antibody can be one that has a transglutaminase-reactive glutamine, for example one with an N297A or N297Q substitution. Conjugation is carried out by recombinant bacterial transglutaminase with a molar ratio of antibody:enzyme of 5:1. The conjugation is carried out using standard protocols in 50 mM Tris buffer, pH 8.0, incubated overnight at 37 °C. The resulting conjugate is purified on a Protein A column, pre-equilibrated with 50 mM Tris, pH 8.0. The conjugate is eluted with 0.1 M sodium citrate buffer, pH 3.5. The eluted fractions are neutralized with 1M Tris pH 9.0. The conjugated can be formulated in 20 mg/mL Sorbitol, 10 mg/mL Glycine, pH 5.0.

[00172] Those skilled in the art will understand that the conditions and methodologies in these two examples are illustrative and non-limiting and that variations thereof or other approaches for conjugation are known in the art and usable in the present invention.

*Example 3 – Properties of *seco*-CPI Compounds*

25 **[00173]** Table IV shows properties of compounds of this invention, including proliferation inhibition efficacy against various human cancer cell lines and CLogP. Proliferation inhibition was measured using a 72 hr ATP luminescence assay (Cheng *et al.*, US 8,394,922 B2 (2013)).

[00174] Proliferation inhibition was measured against : (a) H226 (human mesothelioma (lung) cancer cell line); (b) N87 (human gastric (stomach) cancer cell line); (c) OVCAR3 (human ovarian cancer cell line); (d) HCT116 (human colon cancer cell line); and (e) HCT116/VM46 (multidrug and paclitaxel resistant subline of HCT116).

Table IV – Properties of *seco*-CPI Compounds

Cpd. No.	Proliferation Inhibition of Cancer Cells (Cell Line & EC ₅₀ , nM)					CLogP
	H226	N87	OVCAR3	HCT116	HCT116/ VM46	
Ia-01	0.014	0.007	—	—	—	1.81
Ia-02	0.042	0.097	0.048	0.045	0.045	2.45
Ia-03	0.038	0.048	0.026	0.034	0.034	2.97
Ia-04	0.11	0.15	0.11	0.12	0.12	2.89
Ia-05	0.18	0.21	0.076	0.17	0.17	3.37
Ia-06	0.47	0.56	0.32	0.36	0.36	2.83
Ia-07	0.72	0.81	0.41	0.67	0.67	3.41
Ia-08	3.4	1.3	2.1	—	—	1.04
Ia-09	0.055	0.016	0.025	—	—	2.19
Ia-10	0.25	0.15	—	—	—	4.57
Ia-11	1.0	1.2	0.52	0.58	0.92	1.92
Ia-12	0.53	1.3	0.54	0.40	0.29	3.68
Ia-13	0.32	0.48	0.12	0.18	0.24	2.75
Ia-14	0.43	0.36	0.10	0.26	0.77	1.55
Ia-15	0.28	0.32	0.21	0.22	0.26	3.39
Ia-16	0.40	0.50	—	—	—	2.01
Ia-17	1.6	36	18	19	25	1.42
Ia-18	32	48	12	25	19	1.12
Ia-19	4.2	3.1	1.1	3.2	3.5	0.78
Ia-20	6.5	3.5	1.5	1.9	2.6	2.36
Ia-21	0.99	2.2	1.2	0.85	0.50	1.81
Ia-22	5.1	1.9	1.1	1.2	1.2	1.65
Ia-23	7.5	2.6	1.1	0.9	1.9	2.42
Ia-24	250	—	250	250	250	1.60
Ia-25	23	38	22	13	24	0.78

Table IV – Properties of *seco*-CPI Compounds

Cpd. No.	Proliferation Inhibition of Cancer Cells (Cell Line & EC ₅₀ , nM)					CLogP
	H226	N87	OVCAR3	HCT116	HCT116/ VM46	
Ia-26	9.5	—	2.7	4.1	12	1.34
Ia-27	1.4	3.5	—	—	—	3.37
Ia-28	0.30	0.066	0.058	0.079	0.090	2.85
Ia-29	0.63	0.26	—	—	—	2.60
Ia-30	0.018	0.045	0.032	0.033	0.097	1.11
Ia-31	0.24	0.43	0.53	0.62	0.86	3.90
Ib-01	2.39	3.6	2.8	3.2	3.5	1.92
Ib-02	1.0	0.96	0.53	0.64	1.9	1.83
Ic-01	0.91	1.5	0.89	0.43	0.75	1.93
Id-01	—	2.3	1.1	0.93	—	2.18
Id-02	1.2	1.4	—	—	—	1.60

Example 4 – Compound 10

[00175] This example and **FIG. 1A** relate to the synthesis of compound **10**.

[00176] A solution of 3,5-dinitrobenzyl alcohol **1** (5 g, 25.2 mmol) in EtOAc (160 mL) was added to 2-iodoxybenzoic acid (“SIBX,” available from Sigma-Aldrich, 17.67 g, 63.1 mmol). The reaction mixture was refluxed at 90 °C for 20 h, cooled to RT and filtered. The solid was discarded and the filtrate was concentrated by under vacuum to yield crude product **2** as a yellow solid, which was dissolved in 100 mL of EtOAc and washed with saturated NaHCO₃ solution and then water. Then it was dried over anhydrous Na₂SO₄. The solution was concentrated under vacuum to provide 4.2 g (81%) of 3,5-dinitrobenzaldehyde **2** as a yellow solid. ¹H NMR (500MHz, DMSO-d₆) δ 10.24 (s, 1H), 9.08 - 9.06 (m, 1H), 9.04 (d, *J*=2.0 Hz, 2H); Anal. Calcd. for C₇H₄N₂O₅: 196.01; found 194.99 [M-H]⁺.

[00177] A stirred solution of *syn*-benzaldehyde oxime (6.18 g, 51.0 mmol) in *N,N*-dimethyl-formamide (“DMF,” 50 mL) was treated with K₂CO₃ (14.38 g, 102 mmol) and stirred for 10 min. A solution of 3,5-dinitrobenzaldehyde **2** (5 g, 25.5 mmol) in DMF (50 mL) was added. The reaction mixture was stirred for 2 h at 90 °C and cooled to RT. (Bromomethyl)benzene (7.12

mL, 58.6 mmol) was added to the reaction mixture, which was then stirred for 18 h. The reaction mixture was diluted with Et₂O (200 mL). Aqueous HCl (1 N, 100 mL) was added slowly with stirring. The ether layer was collected and the aqueous layer was extracted with Et₂O (80 mL x 2) and EtOAc (80 mL). The combined organic layers were washed with saturated NaHCO₃ solution and then brine, and dried over by MgSO₄, filtered, and concentrated under vacuum to yield 16 g of a brown oil which was purified on a 330 g column from Biotage (45 to 55% dichloromethane/hexane) to provide 3.41 g (52.1%) of 3-(benzyloxy)-5-nitrobenzaldehyde **3** as a white solid. ¹H NMR (500MHz, chloroform-d) δ 10.07 (s, 1H), 8.37 - 8.29 (m, 1H), 8.10 (t, J=2.2 Hz, 1H), 7.82 (dd, J=2.4, 1.2 Hz, 1H), 7.53 - 7.36 (m, 5H), 5.24 (s, 2H); Anal. Calcd. for C₁₄H₁₁N₁O₄: 257.0; found 279.3 [M+Na]⁺.

[00178] Sodium methanolate (25%) (1.313 mL, 5.91 mmol) was added dropwise to a stirred solution of 3-(benzyloxy)-5-nitrobenzaldehyde **3** (400 mg, 1.555 mmol) and methyl 2-azido-acetate **4** (available from Sigma-Aldrich, 591 mg, 5.13 mmol) in a mixture of tetrahydrofuran (“THF,” 3 mL) and MeOH (3 mL) at -25 °C over 20 min. The reaction mixture was stirred for 64 h at -20 °C and then 0 °C for 1 h. The reaction mixture was poured into a mixture of ice and water, extracted with dichloromethane (“DCM,” 15 mL x 3), washed with water, and then brine. The organic layer was dried over MgSO₄ to yield a crude product which was purified on a 330 g column from Biotage (5% to 9 % EtOAc/Hex.) to provide 323 mg (58.6 %) of compound **5**: ¹H NMR (500MHz, chloroform-d) δ 8.22 (s, 1H), 7.84 (s, 1H), 7.80 (t, J=2.1 Hz, 1H), 7.53 - 7.33 (m, 5H), 6.86 (s, 1H), 5.20 (s, 2H), 3.96 (s, 3H); ¹³C NMR (126MHz, chloroform-d) δ 163.4, 159.1, 149.2, 135.6, 135.3, 128.8, 128.5, 128.2, 127.6, 122.8, 122.0, 118.0, 109.8, 70.9, 53.3; Anal. Calcd. for C₁₇H₁₄N₄O₅: 354.09; found 377.30 [M+Na]⁺.

[00179] Compound **5** (313 mg, 0.883 mmol) was added to xylene (80 mL) and sonicated for 5 min. A homogenous solution formed, which was degassed twice. The reaction mixture was refluxed for 20 h at 160 °C and cooled to RT. The xylene was evaporated under vacuum to yield a yellow solid which was purified on a 24 g column from Biotage (5% to 15 % EtOAc/Hexane) to provide 47.3 mg (16.41%) of compound **6a** and 160.3 mg (55.6%) of compound **6b**.

Compound **6a**: ¹H NMR (500MHz, chloroform-d) δ 10.19 (br. s., 1H), 8.06 (d, J=2.2 Hz, 1H), 7.63 (d, J=1.9 Hz, 1H), 7.52 - 7.47 (m, 2H), 7.44 (t, J=7.4 Hz, 2H), 7.41 - 7.35 (m, 1H), 5.20 (s, 2H), 4.01 (s, 3H); ¹³C NMR (126MHz, chloroform-d) δ 161.2, 152.7, 136.2, 133.2, 131.2, 130.5, 128.8, 128.3, 127.6, 125.6, 115.2, 112.0, 108.9, 71.5, 52.4; Anal. Calcd. for C₁₇H₁₄N₂O₅: 327.1; found 327.3 [M+H]⁺.

Compound **6b**: ¹H NMR (500MHz, chloroform-d) δ 9.38 (br. s., 1H), 8.38 (s, 1H), 7.75

(d, $J=1.3$ Hz, 1H), 7.57 - 7.51 (m, 2H), 7.51 - 7.42 (m, 3H), 7.38 (d, $J=2.0$ Hz, 1H), 5.32 (s, 2H), 3.99 (s, 3H); ^{13}C NMR (126MHz, chloroform-d) δ 161.4, 145.2, 143.3, 135.3, 130.9, 129.7, 128.9, 128.8, 128.2, 126.7, 113.2, 111.0, 100.5, 71.1, 52.4; Anal. Calcd. for $\text{C}_{17}\text{H}_{14}\text{N}_2\text{O}_5$: 327.1; found 327.3 $[\text{M}+\text{H}]^+$.

5 [00180] To a stirred solution of compound **6b** (150 mg, 0.460 mmol) in THF (4 mL) was added Boc-anhydride (0.192 mL, 0.827 mmol) and 4-dimethylaminopyridine (“DMAP,” 11.23 mg, 0.092 mmol). The reaction mixture was stirred for 18 h at RT. Brine (5 mL) and EtOAc (5 mL) were added and stirring was continued for 5 min. The organic layer was collected. The aqueous layer was extracted with 10 mL of EtOAc. The combined organic layers were dried over 10 MgSO_4 , filtered, and concentrated under vacuum to yield a yellow solid which was purified on a 24 g column from Biotage (5% to 40% EtOAc/Hexane) to provide 184.2 mg (94%) of compound **7**: ^1H NMR (500MHz, chloroform-d) δ 8.29 (d, $J=1.7$ Hz, 1H), 7.70 (d, $J=1.6$ Hz, 1H), 7.50 (d, $J=7.3$ Hz, 2H), 7.45 - 7.39 (m, 2H), 7.39 - 7.33 (m, 2H), 5.35 (s, 2H), 3.97 (s, 3H), 1.50 (s, 9H); ^{13}C NMR (126MHz, chloroform-d) δ 160.4, 149.2, 145.5, 143.5, 135.1, 130.1, 130.0, 128.8, 128.6, 128.1, 126.3, 112.6, 112.4, 102.1, 86.4, 71.2, 52.4, 27.2; Anal. Calcd. for $\text{C}_{22}\text{H}_{22}\text{N}_2\text{O}_7$: 426.1; found 449.4 $[\text{M}+\text{Na}]^+$.

15 [00181] Zinc powder (130 mg, 1.982 mmol) and NH_4Cl (212 mg, 3.96 mmol) were added to a stirred solution of compound **7** (169 mg, 0.396 mmol) in acetone (4 mL)/water (0.800 mL). The reaction mixture was stirred for 18 hour at RT. The reaction mixture was concentrated under 20 vacuum to yield 180 mg crude compound **8**, which was re-dissolved in EtOAc (8 mL), washed with water (8 mL) and then brine (8 mL), and dried over MgSO_4 . It was concentrated under vacuum to yield 160 mg crude compound **8** (brown) which was purified on a 12 g column from Biotage (15% to 35% EtOAc/Hexane) to provide 138.1 mg (88 %) of compound **8**: ^1H NMR (500MHz, chloroform-d) δ 7.46 (d, $J=7.3$ Hz, 2H), 7.39 (t, $J=7.4$ Hz, 2H), 7.35 - 7.30 (m, 1H), 7.03 (s, 1H), 6.50 (d, $J=1.4$ Hz, 1H), 6.29 (d, $J=1.4$ Hz, 1H), 5.22 (s, 2H), 3.91 (s, 3H), 3.53 (br. s., 2H), 1.49 (s, 9H); Anal. Calcd. for $\text{C}_{22}\text{H}_{24}\text{N}_2\text{O}_5$: 397.17; found 397.13 $[\text{M}+\text{H}]^+$.

25 [00182] Boc-anhydride (0.112 mL, 0.484 mmol) was added to a stirred solution of compound **8** (128 mg, 0.323 mmol) in THF (3.5 mL). The reaction mixture was stirred for 20 h at RT and concentrated under vacuum to yield 178 mg of brown, waxy crude compound **9**, which was 30 purified on a 12 g column from Biotage (5% to 15% EtOAc/Hexane) to provide 156 mg (97%) compound **9**: ^1H NMR (500MHz, chloroform-d) δ 7.48 (d, $J=7.3$ Hz, 2H), 7.39 (t, $J=7.4$ Hz, 2H), 7.36 - 7.30 (m, 2H), 7.13 (s, 1H), 6.86 (s, 1H), 6.41 (br. s., 1H), 5.24 (s, 2H), 3.92 (s, 3H), 1.54 (s, 9H), 1.47 (s, 9H). Anal. Calcd. for $\text{C}_{27}\text{H}_{32}\text{N}_2\text{O}_7$: 496.2; found 497.5 $[\text{M}+\text{H}]^+$.

[00183] N-Iodosuccinimide (127 mg, 0.564 mmol) and acetic acid (0.065 mL, 1.128 mmol) were added to a stirred solution of compound **9** (140 mg, 0.282 mmol) in toluene (8 mL). The reaction flask was covered with aluminum foil and stirred for 18 h at RT. The reaction mixture was poured into water (20 mL). EtOAc (10 mL) was added and the mixture was stirred for 2 min. The organic layer was collected. The aqueous layer was extracted with EtOAc (10 mL). The combined organic layers were dried with MgSO₄, filtered, and concentrated under vacuum to yield a dark purple wax which was purified on a 12 g column from Biotage (5% to 12% EtOAc/Hexane) to provide 110.6 mg (63.0%) compound **10** as yellow wax: ¹H NMR (500MHz, chloroform-d) δ 7.81 (br. s., 1H), 7.50 (d, J=7.1 Hz, 2H), 7.43 - 7.36 (m, 2H), 7.35 - 7.30 (m, 1H), 7.12 (s, 1H), 6.79 (br. s., 1H), 5.27 (s, 2H), 3.94 (s, 3H), 1.56 (s, 9H), 1.43 (s, 9H); Anal. Calcd. for C₂₇H₃₁IN₂O₇: 622.1; found 623.4 [M+H]⁺.

Example 5 – Ester 12

[00184] This example and **FIG. 3B** relate to the preparation of ester **12**.

[00185] NaH (7.71 mg, 0.193 mmol) and 1,3-dichloropropene (23.50 μl, 0.241 mmol) were added to a stirred solution of compound **10** (100 mg, 0.161 mmol) in DMF at 0 °C. The reaction mixture was stirred for 3 h. The reaction mixture was poured into a saturated NH₄Cl 100 mL solution and extracted with Et₂O (2 X 80 mL). The extract was dried over MgSO₄, filtered, and concentrated under vacuum to yield a brown wax which was purified on a 12 g column from Biotage (5% to 12 % EtOAc/Hexane) to provide 88.7 mg (79%) of compound **11**: ¹H NMR (500MHz, chloroform-d) δ 7.47 - 7.31 (m, 5H), 7.21 (s, 1H), 6.53 (d, J=18.3 Hz, 1H), 6.03 - 5.83 (m, 3H), 5.34 - 5.21 (m, 2H), 3.96 (s, 2H), 4.57 - 3.70 (m, 2H), 1.58 (s, 9H), 1.30 (d, J=9.8 Hz, 9H). Anal. Calcd. for C₃₀H₃₄ClIN₂O₇: 696.1; found 719.5 [M+Na]⁺.

[00186] Tri-n-butyltin hydride (1.793 mL, 6.59 mmol) and 2,2'-azobis(2-methylpropionitrile) (“AIBN,” 0.085 g, 0.507 mmol) were added to a stirred solution of compound **11** (3.5305 g, 5.07 mmol) in benzene (100 mL). The reaction mixture was degassed once and stirred for 4 h at 80 °C. The reaction mixture was cooled to RT under vacuum to yield a brown wax that was purified on a 120 g column from Biotage (5% to 10 % EtOAc/Hexane) to provide 2.7552 g mixture of compounds **12** and **12a**. The mixture was separated by SFC chiral separation: Chiralpak: AD-H preparative column, 30 X 250 mm, 5 μm; Mobile Phase: 35% MeOH in CO₂ at 130 bar; temperature: 35 °C; Flow rate: 70.0 mL/min for 13 min; UV monitored at 220 nm. This procedure provided 0.9562 g (33.1%) of compound **12**. ¹H NMR (500MHz, chloroform-d) δ 7.52 - 7.30 (m, 6H), 7.11 (s, 1H), 5.27 (s, 2H), 4.20 - 4.13 (m, 1H), 4.01 - 3.82 (m, 6H), 3.53 (t,

J=9.9 Hz, 1H), 1.57 (s, 9H), 1.43 (br. s., 9H); Anal. Calcd. for: C₃₀H₃₅ClN₂O₇ 570.2; found 571.5 [M+H]⁺; [α]²⁰D -17.06 (c 2.45, CHCl₃) and 0.9252 g (32.0%) of compound **12b**. ¹H NMR (500MHz, chloroform-d) δ 7.53 - 7.30 (m, 6H), 7.11 (s, 1H), 5.27 (s, 2H), 4.22 - 4.13 (m, 1H), 4.12 - 3.83 (m, 6H), 3.53 (t, J=10.1 Hz, 1H), 1.57 (s, 9H), 1.43 (br. s., 9H). Anal. Calcd. for 5 C₃₀H₃₅ClN₂O₇: 570.2; found 571.5 [M+H]⁺; [α]²⁰D +17.06 (c 2.45, CHCl₃).

Example 6 – Compound 16

[00187] This example and **FIG. 2** relate to the preparation of compound **16**.

[00188] A solution of ester **12** (1g, 1.751 mmol) in ACN (Aldrich, 40 mL) was prepared.

LiOH (0.125 g, 5.22 mmol) in distilled water (10 mL) was added. The reaction mixture was 10 stirred at 50 °C for 2 h. LCMS analysis showed the reaction to be complete. The reaction mixture was acidified with 6 N HCl to pH 4 and concentrated under vacuum to remove the ACN. The reaction mixture was extracted with EtOAc (2 x5 0 mL). The organic phases were washed with water and brine, dried with anhydrous Na₂SO₄, and concentrated and dried under high vacuum overnight to yield crude acid **13** (0.98g). ESI-LCMS (M+H): 557.0.

[00189] *N,N*-Diisopropylethylamine (“DIPEA,” Aldrich, 0.612 mL, 3.50 mmol) was added to a solution of acid **13** (0.98 g), morpholine **13a** (Aldrich, 0.458 g, 5.25 mmol) and *N,N,N',N'*-tetramethyl-*O*-(7-azabenzotriazol-1-yl)uronium hexafluorophosphate (“HATU”, CAS Reg. No. 148893-10-1, Oakwood Chemical, 0.999 g, 2.63 mmol) in anhydrous DMF (Acros, 5 mL). The reaction mixture was stirred at RT for 45 min. LCMS analysis showed the reaction to be complete. The reaction mixture was worked up with EtOAc, water and brine. The organic

phases were combined and concentrated with 2 g of silica gel to a slurry. The slurry was purified on a 24 g COMBIFLASH™ column, eluting with 0-35% EtOAc in hexane gradient. The fractions expected to contain product were combined, concentrated and dried under high vacuum to yield compound **14** as a solid (0.872 g, 80% yield over two steps). ESI-LCMS (M+H): 626.1.

[00190] A solution of compound **14** (396 mg, 0.633 mmol) in anhydrous THF (Acros, 5 mL) was prepared. Ammonium formate (Aldrich, 1.104 g, 8.76 mmol) in distilled water (1 mL) was added. Nitrogen gas was bubbled through the reaction mixture for 5 min and then Pd on carbon (Aldrich-Sigma, 10%, 60 mg) was added. The reaction mixture was stirred at RT for 0.5 h.

LCMS analysis showed the reaction to be complete. The reaction mixture was diluted with

EtOAc (50 mL) and filtered. The filtrate was washed with water and brine, dried over anhydrous Na₂SO₄, and concentrated *in vacuo* to leave a residue, which was freeze-dried with ACN and water to yield compound **15** (white solid, 225 mg, 66% yield). ESI-LCMS (M+H): 536.2.

[00191] Trifluoroacetic acid (“TFA,” Aldrich, 1 mL) was added to a solution of compound **15** (78 mg, 0.146 mmol) in anhydrous DCM (Acros, 1 mL). The reaction mixture was stirred at RT for 30 min. LCMS analysis showed the reaction to be complete. The reaction mixture was concentrated under vacuum and freeze-dried with ACN and water to yield compound **16** as green 5 solid (81 mg, double TFA salt, 98%). ESI-LCMS (M+H): 336.0.

Example 7 – Compound 18

[00192] This example relates to the preparation of compound **18**, as shown in **FIG. 2**.

[00193] A solution of ethyl ester **17a** (Alfa Aesar, 300 mg, 1.46 mmol) and triphenylphosphine (“TPP,” Aldrich-Sigma, 767 mg, 2.92 mmol) in anhydrous THF (Acros Organics, 10 mL) was prepared. Di-*tert*-butyl azodicarboxylate (“DBAD,” Aldrich, 673 mg, 2.92 mmol) in anhydrous THF (Acros, anhydrous, 2 mL) was added dropwise over 10 min, followed by 2-methoxyethanol **17b** (Aldrich, 445 mg, 5.85 mmol). The reaction mixture was stirred at RT overnight. LCMS analysis showed the reaction was almost complete. The reaction mixture was worked up with EtOAc, water, and brine. The organic phases were combined and concentrated 15 with silica gel (1 g) under vacuum to yield a slurry. The slurry was purified on 24 g COMBIFLASH™ column, eluting with 0-30% EtOAc in hexane gradient. The fractions expected to contain product were combined and concentrated and dried under high vacuum to yield compound **17** as a solid (314 mg, 1.19 mmol, 82%). ESI-LCMS (M+H): 264.1.

[00194] LiOH (Aldrich, 50 mg) in distilled water (1 mL) was added to a solution of 20 compound **17** (314 mg, 1.19 mmol) in ACN (Acros, 10 mL). The reaction mixture was stirred at 40 °C for 3 h. LCMS analysis showed the reaction to be complete. The reaction mixture was neutralized with acetic acid (0.5 mL) and concentrated under vacuum to 2 mL. The resultant mixture was loaded on a COMBIFLASH™ AQ Gold 50 g column and eluted with 0-60% ACN in water gradient (both with 0.05% formic acid, 50 mL/min). The fractions expected to contain 25 product were combined and freeze-dried to yield compound **18** as white powder (226 g, 0.961 mmol, 81%). ESI-LCMS (M+H): 236.0.

Example 8 – Seco-CPI Compound Ia-01

[00195] This example and **FIG. 2** relate to the preparation of *sec*o-CPI compound **Ia-01**.

[00196] A solution of compounds **16** (5 mg, 0.015 mmol) and **18** (7.0 mg, 0.030 mmol) in 30 anhydrous DMF (Acros, 1 mL) was prepared. *N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (“EDC,” Aldrich, 5.71 mg, 0.030 mmol) was added. The reaction mixture was stirred at RT for 2 h. LCMS analysis showed the reaction to be complete.

The reaction mixture was purified by preparative HPLC, eluting with 45-60% ACN in water (with 0.1% TFA, 20 mL/min) on a KINETEX™ 5 µm EVO C18 150 x 21.2 mm column. The fractions expected to contain product were combined and freeze-dried to yield **Ia-01** as an off-white solid (4.1 mg, 7.4 µmol, 50% yield). ESI-LCMS (M+H): 553.1. ¹H NMR (DMSO-d₆, 400 MHz) 11.54 (1H, s), 11.29 (1H, s), 9.75 (1H, s), 7.75 (1H, s), 7.39 (1H, d), 7.15(1H, d), 7.02(1H, d), 6.91(2H, d), 4.74 (1H, t), 4.45 (1H, d), 4.14(4H, m), 3.89(2H, m), 3.40-3.80(12H, m).

[00197] Generally following the procedures of this example and **FIG. 2**, but using alternative precursor materials, additional *seco*-CPI compounds were prepared as listed in Table V below.

Table V – <i>Seco</i> -CPI Compounds Made Following FIG. 2		
Compound No.	Mass spectrum	Remarks
Ia-02	[M-H] ⁻ = 521.2	Ethanol was used instead of compound 17b .
Ia-08	[M+H] ⁺ = 541.1	Ethane-1,2-diol was used instead of compound 17b .
Ia-09	[M+H] ⁺ = 567.1	3-Methoxy-1-propanol was used instead of compound 17b .
Ia-10	[M+H] ⁺ = 591.1	Cyclohexylmethanol was used instead of compound 17b .
Ia-16	[M+H] ⁺ = 566.1	2-(Dimethylamino)ethanol was used instead of compound 17b .
Ia-30	[M+H] ⁺ = 569	5,6,7-Trimethoxy-1 <i>H</i> -indole-2-carboxylic acid (CAS Reg. No. 128781-0707) was used instead of compound 18 .
Ia-31	[M+H] ⁺ = 565.0	3-Methylbutan-1-ol was used instead of compound 17b .

10

Example 9 – Compound 26

[00198] This example and **FIG. 3A** relate to the synthesis of compound **26**.

[00199] A solution of compound **19a** (Bachem, 7.5 g, 24.09 mmol) and (4-aminophenyl)-methanol **19b** (Aldrich, 3.86 g, 31.3 mmol) in anhydrous DCM (Acros, 50 mL) and anhydrous methanol (Acros, 50 mL) was prepared. 2-Ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (“EEDQ,” Bachem, 8.94 g, 36.1 mmol) was added. The reaction mixture was stirred at RT overnight. LCMS analysis showed the reaction was almost complete. The reaction mixture was diluted with EtOAc (400 mL) and the resultant solution was washed with water and brine. The

organic phases were dried over anhydrous Na_2SO_4 and concentrated under vacuum to 100 mL volume. The precipitated white solid was collected by filtration and air dried over a weekend to yield compound **20** (white solid, 8.9 g, 21.37 mmol, 89%). ESI-LCMS (M+H): 417.1.

[00200] Piperidine (Aldrich, 1 mL) was added to a solution of compound **20** (2.2 g, 5.28

5 mmol) in DMF (Acros, anhydrous, 10 mL). The reaction mixture was stirred at RT for 1 h. LCMS analysis showed the reaction to be complete. The reaction mixture was freeze-dried with ACN and water to yield crude compound **21** with some Fmoc residue as white solid (2.12 g). ESI-LCMS (M+H): 195.0.

[00201] A solution of compound **21** (2.12 g, crude) and compound **22a** (Aldrich, 1.610 g, 5.28

10 mmol) in DMF (Acros, anhydrous, 10 mL) was prepared. DIPEA (Aldrich) was added to adjust the pH of reaction mixture to above 8. The reaction mixture was stirred at RT for 2 h. LCMS analysis showed the reaction to be complete. The reaction mixture was worked up with EtOAc, water and brine. The organic phases were combined and concentrated to leave a residue. The residue was re-dissolved with ACN (Aldrich, 5 mL) and distilled water (10 mL). The suspension 15 was filtered. The filtrate was loaded onto a 130 g COMBIFLASH™ AQ Gold column and eluted at 75 mL/min with 25-40% ACN (with 0.05% formic acid) and water (also with 0.05% formic acid). The fractions expected to contain product were combined and freeze-dried to yield compound **22** as a white solid (0.89 g, 2.262 mmol, 42.8%). ESI-LCMS (M+H): 394.1.

[00202] A solution of compound **22** (147 mg, 0.374 mmol) and perbromomethane (Aldrich,

20 217 mg, 0.653 mmol) in anhydrous DCM (Acros, 4 mL) was prepared. TPP (Aldrich, 171 mg, 0.653 mmol) was added. The reaction mixture was stirred at RT for 5 min, after which a clear solution had formed. LCMS analysis showed the reaction to be complete. The reaction mixture was loaded onto a 12 g COMBIFLASH™ Gold column and eluted with 50-100% EtOAc in hexane (25 mL/min). The fractions expected to contain product were combined, concentrated 25 and dried under high vacuum for 15 min to yield compound **23** as a solid (92 mg, 54%). ESI-LCMS (M+H): 456.1.

[00203] Methyl lithium in diethyl ether (Aldrich, 1.6 M, 0.233 mL, 0.373 mmol) was added to a solution of compound **15** (FIG. 2, 100 mg, 0.187 mmol) in anhydrous THF (Acros, 1 mL).

30 The reaction mixture was stirred at RT for 5 min. Compound **23** (92 mg, 0.202 mmol) was added. The reaction mixture was stirred at RT for 10 min. LCMS showed the reaction to be complete. The reaction mixture was worked up with EtOAc, water and brine. The organic phases were dried over anhydrous Na_2SO_4 , concentrated and dried under high vacuum to yield compound **24** (153 mg, crude). ESI-LCMS (M+H): 911.2.

[00204] TFA (Aldrich, 2 mL) was added to a solution of crude compound **24** (153 mg) in anhydrous DCM (Acros, 0.5 mL). The reaction mixture was stirred at RT for 30 min. LCMS analysis showed the reaction to be complete. The reaction mixture was concentrated and purified on a COMBIFLASH™ AQ Gold 30 g column, eluting with 15-30% ACN (with 0.05% formic acid) in deionized water (with 0.05% formic acid) at 35 mL/min. The fractions expected to contain product were combined and freeze-dried to yield compound **25** as a white solid (61 mg, 53.5%). ESI-LCMS (M+H): 611.2.

[00205] A solution of compound **25** (61 mg, 0.100 mmol) and BOC-anhydride (Oakwood, 0.030 mL, 0.130 mmol) in anhydrous DMF (Acros, 1 mL) was prepared. DIPEA (Aldrich, 0.060 mL, 0.348 mmol) was added. The reaction mixture was stirred at RT for 30 min. LCMS analysis showed the reaction to be complete. The reaction mixture was purified by preparative HPLC with 30-50% ACN in water (with 0.1% TFA, 20 mL/min) on a KINETEX™ C18 EVO 5 μ 150x22.1 mm column. The fractions expected to contain product were combined and freeze-dried to yield compound **26** as a white solid (60 mg, 84.4%). ESI-LCMS (M+H): 711.2.

15 *Example 10 – Seco-CPI-Linker Compound IIIa-03*

[00206] This example and **FIG. 3B** relate to the preparation of compound **IIIa-03**.

[00207] DIPEA (Aldrich, 0.074 mL, 0.422 mmol) was added to a solution of compound **18** (**FIG. 2**, 29.8 mg, 0.127 mmol) and HATU (Oakwood, 48.1 mg, 0.127 mmol) in anhydrous DMF (Acros, 0.7 mL). The reaction mixture was stirred at RT for 5 min. Compound **26** (**FIG. 3B**, 30 mg, 0.042 mmol) was added. The reaction mixture was stirred at RT overnight. LCMS analysis showed the reaction was 70% complete. The reaction was purified by preparative HPLC, eluting with 50-65% ACN in water (with 0.1% TFA, 20 mL/min) on a KINETEX™ C18 EVO 5 μ 150x22.1 mm column. The fractions expected to contain product were combined and freeze-dried to yield compound **27** as a powder (27 mg, 68.8%). ESI-LCMS (M+H): 928.3.

25 [00208] TFA (Sigma, 1 mL) was added to a solution of compound **27** (27 mg, 0.029 mmol) in anhydrous DCM (Acros, 1 mL). The reaction mixture was stirred at RT for 15 min. LCMS analysis showed the reaction to be complete. The reaction was purified by preparative HPLC, eluting with 40-50% ACN in water (with 0.1% TFA, 20 mL/min) on a KINETEX™ C18 EVO 5 μ 150x22.1 mm column. The fractions expected to contain product were combined and freeze-dried to yield compound **28** as a powder (20 mg, 83.0%). ESI-LCMS (M+H): 828.2.

[00209] DIPEA (Sigma, 20 μ L, 0.114 mmol) was added to a solution of compound **28** (20 mg, 0.024 mmol) and 6-maleimidohexanoic acid *N*-hydroxysuccinimide ester **29** (available from

SIGMA-Aldrich, 11.17 mg, 0.036 mmol) in anhydrous DMF (Acros, 0.7 mL). The reaction mixture was stirred at RT for 30 min. LCMS analysis showed the reaction to be complete. The reaction mixture was purified by preparative HPLC, eluting with 50-65% acetonitrile in water (with 0.1% TFA, 20 mL/min) on a KINETEX™ C18 EVO 5 μ 150x22.1 mm column. The

5 fractions expected to contain product were combined and freeze-dried to yield *seco*-CPI-linker compound **IIIa-03** as a powder (12.8 mg, 51.9%). ESI-LCMS (M+H): 1021.5. 1 H NMR (DMSO-d6): δ 11.98(s, 1H), 11.77(s, 1H), 9.79(s, 1H), 8.11(d, 1H, J = 6.9 Hz), 7.79(d, 1H, J = 8.6 Hz), 7.42(d, 1H, J = 8.8 Hz), 7.29(d, 3H, J = 8.3 Hz), 7.19(s, 1H), 6.98-7.03(m, 4H), 6.61(d, 1H, J = 7.19 Hz), 6.53(d, 2H, J = 8.5 Hz), 5.05(t, 2H, J = 4.7 Hz), 4.45(d, 1H, J = 10.7 Hz),
10 4.34(d, 1H, J = 7.1 Hz), 4.11-4.17(m, 3H), 3.52-3.72(m, 10H), 3.09-3.38(m, 9H), 2.15(m, 2H), 1.95(m, 1H), 1.48(m, 4H), 1.27(m, 3H), 1.17(m, 2H), 0.84(m, 6H).

[00210] Additional *seco*-CPI-linker compounds as listed in Table VI below were prepared generally following the procedures of this example and the scheme of FIGs. 3A-3B, but using alternative precursor materials.

15

Table VI – *Seco*-CPI-Linker Compounds Made Following FIGs. 3A-3B

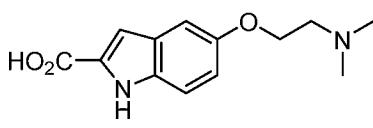
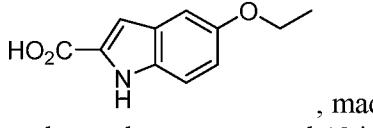
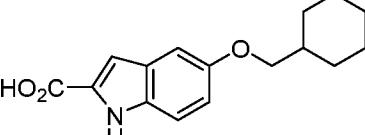
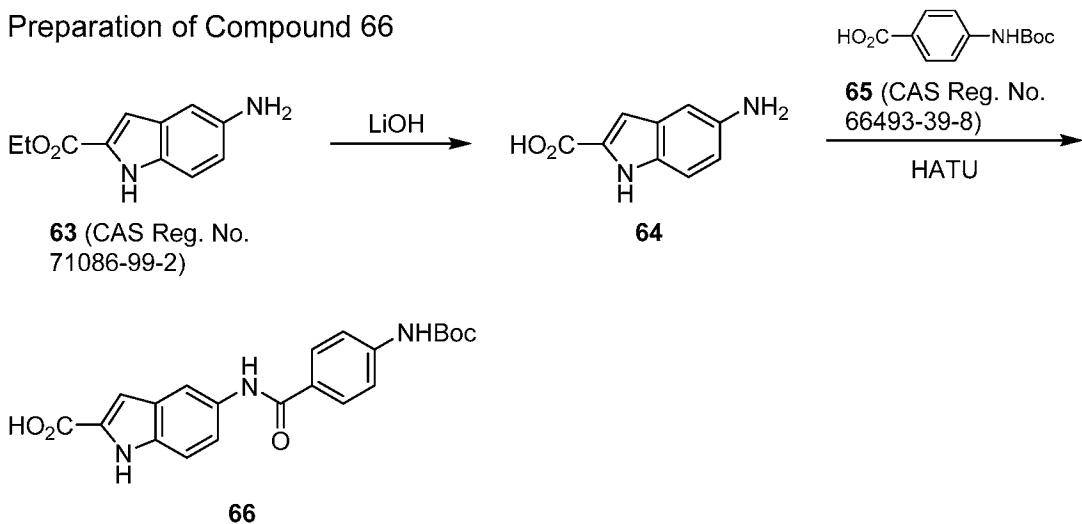
Compound No.	Mass spectrum	Remarks
IIIa-01	$[M+H]^+ = 1034.4$	 , made analogously to compound 18 in FIG. 2, was used instead.
IIIa-02	$[M+H]^+ = 1081.4$	Compound 66 , with later removal of Boc protecting group, was used instead of compound 18 . Scheme for synthesis of compound 66 is shown below.
IIIa-04	$[M+H]^+ = 991.4$	 , made analogously to compound 18 in FIG. 2, was used instead.

Table VI – Seco-CPI-Linker Compounds Made Following FIGs. 3A-3B

Compound No.	Mass spectrum	Remarks
IIIa-13	$[\text{M}+\text{H}]^+ = 1059.4$	 , made analogously to compound 18 in FIG. 2, was used instead.

Preparation of Compound 66



5 Example 11 – Seco-CPI Compound Ia-21

[00211] This example and FIG. 4 relate to the preparation of *sec*o-CPI compound **Ia-21**.

[00212] HCl in dioxane (4 N, 15 mL) was added to ester **12** (0.25 g, 0.4388 mmol). The reaction mixture was stirred at 50 °C for 5h. LCMS analysis showed the reaction to be complete. The reaction mixture was blow dried under nitrogen to yield a dark blue residue as crude acid **30**, used in next step without further purification. ESI-LCMS (M+H): 371.1.

[00213] N,N-Dimethylacetamide (“DMA,” 5 mL) was added to crude acid **30** (hydrochloride salt, 178 mg, 0.437 mmol), 5-methoxy-1H-indole-2-carboxylic acid **30a** (CAS Reg. No. 4382-54-1, available from Sigma-Aldrich, 125 mg, 0.656 mmol), EDC (251 mg, 1.311 mmol), 1-hydroxybenzotriazole (“HOBT,” 201 mg, 1.311 mmol), and NaHCO₃ (184 mg, 2.185 mmol). The black solution was stirred at RT for 4.5 h. LCMS analysis showed appearance of the product peak. The reaction mixture was diluted with water (15 mL), stirred at RT for 15 min and filtered. The crude product was collected as a brown solid and re-dissolved in DCM/methanol. The resultant solution was concentrated to dryness with silica gel (1.3 g) to form a slurry. The

slurry was purified on a 15 g silica gel column with 0-3% ethyl acetate in DCM eluent gradient. The fractions containing expected product were combined, concentrated and dried under vacuum to yield crude product (206 mg). The crude product was re-dissolved in 20% methanol in chloroform and concentrated with 1.3 g silica gel to a slurry. The slurry was purified on silica 5 gel column with 100% DCM and 1-5% ethyl acetate in hexane eluent. The fractions containing expected product were combined, concentrated and dried under high vacuum to yield slightly impure compound **31**(0.108 g, 0.199 mmol, 45.4%). ESI-LCMS (M+H): 544.4.

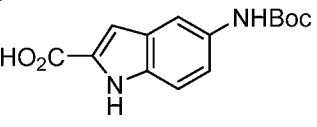
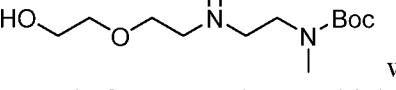
[00214] THF (3 mL), water (1.000 mL), and MeOH (2.000 mL) were added to compound **31** (104 mg, 0.191 mmol) and lithium hydroxide hydrate (138 mg, 3.29 mmol) was added. The 10 yellow heterogeneous mixture stirred overnight. LC/MS showed the reaction to be complete. The reaction mixture was acidified with 1 N HCl and then filtered. The solid was collected and dried to yield crude compound **32** (0.168 g, 88%). ESI-LCMS (M+H): 529.9.

[00215] DMA (0.5 mL) was added to compound **32** (15 mg, 0.028 mmol), N,N'-dimethyl-ethane-1,2-diamine **32a** (CAS Reg. No. 110-70-3, available from Sigma-Aldrich, 1.247 mg, 15 0.014 mmol), EDC (10.85mg, 0.057 mmol), HOBT (8.67 mg, 0.057 mmol), and NaHCO₃ (9.51 mg, 0.113mmol). LC/MS shows reaction to be 50% complete. The reaction mixture was diluted with MeOH and purified by preparative LC with 15-100% ACN in water (both with 0.1% TFA, 40 mL/min) gradient. The fractions containing expected product were combined and freeze-dried to yield compound **33** (5.8 mg, 7.98 μ mol, 28.2%). ESI-LCMS (M+H): 600.10.

[00216] MeOH (0.5mL) was added to compound **33** (TFA salt, 5.7 mg, 7.98 μ mol), ammonium formate (15.10 mg, 0.239 mmol), and Pd-C (1.699 mg, 1.596 μ mol). The reaction mixture was heated to reflux for 30 sec. After 10 min, LC/MS showed the reaction to be complete. The reaction mixture was filtered and the filtrate was purified by preparative HPLC with 0-75% ACN in water (both with 0.1% TFA, 25 ml/min) on Waters-YMC-OBD S5 20x100 25 mm column. The fractions containing expected product were combined and freeze-dried to yield *seco*-CPI compound **Ia-21** as an amber solid (1.5 mg, 2.12 μ mol, 26.5%). ESI-LCMS (M+H): 510.15. ¹H NMR (MeOD, 500 MHz) 7.92 (1H, s), 7.41(1H, d, *J* = 5 Hz), 7.18(1H, d, *J* = 1 Hz), 7.14(1H, s), 7.07(1H, s), 6.96(1H, dd, *J* = 10 Hz), 4.76 (1H, t), 4.61 (1H, dd), 4.09 (2H, m), 3.86 (3H, s), 3.80(3H, m), 3.40(2H, t, *J* = 5 Hz), 3.03(6H, s).

[00217] Generally following the procedures of this example and **FIG. 4**, but using alternative precursor materials, additional *seco*-CPI compounds were prepared as listed in Table VII below.

Table VII – *Seco-CPI* Compounds Made Following FIG. 4

Compound No.	Mass spectrum	Remarks
Ia-11	$[M+H]^+ = 509.3$	Morpholine was used instead of compound 32a .
Ia-19	$[M+H]^+ = 494.1$	Morpholine was used instead of compound 32a and  was used instead of compound 30a , with later removal of Boc protecting group.
Ia-20	$[M+H]^+ = 522.2$	1-Methylpiperazine was used instead of compound 32a .
Ia-22	$[M+H]^+ = 527.2$	2-(2-Aminoethoxy)ethanol was used instead of compound 32a .
Ia-23	$[M+H]^+ = 511.2$	(<i>S</i>)-2-Aminobutan-1-ol was used instead of compound 32a .
Ia-24	$[M+H]^+ = 584.4$	 was used instead of compound 32a , with later removal of Boc protecting group.

Example 12 – Seco-CPI-Linker Compound IIIa-05

[00218] This example and **FIG. 5** relate to the preparation of *seco*-CPI-linker compound **IIIa-05**.

5 **[00219]** DIPEA (Sigma, 0.052 mL, 0.299 mmol) was added to a solution of compound **25** (61 mg, 0.100 mmol) and hydroxysuccinimide ester **25a** (QuantaBiodesign, 76 mg, 0.130 mmol) in DMF (Acros, anhydrous, 1 mL). The reaction mixture was stirred at RT for 60 min. LCMS analysis showed the reaction to be complete. The reaction mixture was neutralized with acetic acid (40 uL), loaded onto 30 g COMBIFLASH™ AQ Gold column and purified with 50-75% ACN in water (both with 0.05% formic acid, 35mL/min). The fractions containing the expected product were combined and freeze-dried to yield compound **34** (52 mg, 0.048 mmol, 48.2 % yield). ESI-LCMS ($M+H$): 1080.3.

10 **[00220]** DIPEA (Sigma, 0.042 mL, 0.241 mmol) was added to a solution of acid **18** (34.0 mg, 0.144 mmol), compound **34** (52 mg, 0.048 mmol) and HATU (Oakwood, 54.9 mg, 0.144 mmol) 15 in DMF (Acros, anhydrous, 0.7 mL). The reaction mixture was stirred at RT overnight. LCMS

analysis showed the reaction to be 70% complete. The reaction mixture was purified by preparative HPLC with 45-65% ACN in water (with 0.1% TFA, 20 mL/min) on a KINETEX™ 5 μ C18 EVO 150x21.2 mm column. The fractions containing product were combined and freeze-dried to yield compound **35** (31 mg, 49.8%). ESI-LCMS (M+H): 1297.3.

5 [00221] Piperidine (Aldrich, 50 μ L) was added to a solution of compound **35** (31 mg) in DMF (Acros, anhydrous, 0.5 mL). The reaction mixture was stirred at RT for 30 min. LCMS analysis showed the reaction to be complete. The reaction mixture was neutralized with AcOH (50 μ L) and purified by preparative HPLC with 30-40% ACN in water gradient (with 0.1% TFA, 20 mL/min) on a KINETEX™ C18 5 μ 150x21.2 mm column. The fractions containing the expected product were combined and freeze-dried to yield *seco*-CPI-linker compound **IIIa-05** (22 mg, 64.2%), as a TFA salt. ESI-LCMS (M+H): 1075.6. 1 H NMR(DMSO-d6): δ 11.97(s, 1H), 11.77(s, 1H), 9.81(s, 1H), 8.15(d, 1H, J = 3.1 Hz), 7.88(d, 1H, J = 8.8 Hz), 7.79(s, 3H), 7.42(d, 1H, J = 9.1 Hz), 7.30(d, 3H, J = 8.6 Hz), 7.19(s, 1H), 6.96-7.02(m, 2H), 6.63(d, 1H, J = 1.9 Hz), 6.53(d, 2H, J = 8.6 Hz), 5.06(t, 2H, J = 4.9 Hz), 4.45(d, 1H, J = 10.7 Hz), 4.34(d, 1H, J = 7.1 Hz), 4.21(m, 1H), 4.12(s, 2H), 2.96-3.72(m, 32H), 2.36-2.48(m, 4H), 1.97(m, 2H), 1.28(d, 3H, J = 7.1 Hz), 0.85(m, 6H).

10 [00222] Additional *seco*-CPI-linker compounds, as listed in Table VIII below, were prepared generally following the procedures of this example and the scheme of FIG. 5, but using alternative precursor materials.

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Table VIII – *Seco*-CPI-Linker Compounds Made Following FIG. 5

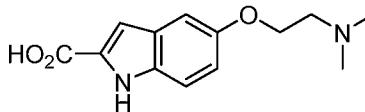
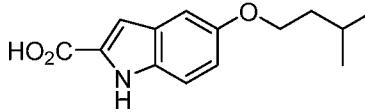
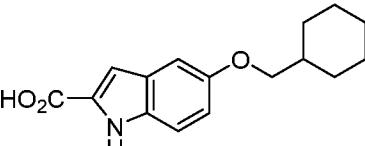
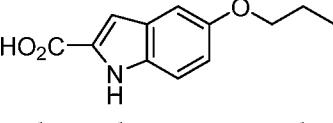
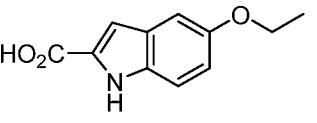
Compound No.	Mass spectrum	Remarks
IIIa-06	$[M+H]^+ = 1088.4$	 , made analogously to compound 18 in FIG. 2, was used instead.
IIIa-07	$[M+H]^+ = 1087.3$	 , made analogously to compound 18 in FIG. 2, was used instead.

Table VIII – *Seco-CPI-Linker Compounds Made Following FIG. 5*

Compound No.	Mass spectrum	Remarks
IIIa-08	$[M+H]^+ = 1113.4$	 , made analogously to compound 18 in FIG. 2 , was used instead.
IIIa-09	$[M+H]^+ = 1091.3$	5,6,7-Trimethoxy-1 <i>H</i> -indole-2-carboxylic acid (CAS Reg. No. 128781-0707) was used instead of compound 18 .
IIIa-10	$[M+H]^+ = 1134.5$	Compound 48a (FIG. 9) was used instead of compound 18 , followed by removal of the Boc protecting group and HATU mediated coupling with 4-hydroxybenzoic acid.
IIIa-11	$[M+H]^+ = 1059.5$	 , made analogously to compound 18 in FIG. 2 , was used instead.
IIIa-12	$[M+H]^+ = 1045.4$	 , made analogously to compound 18 in FIG. 2 , was used instead.

Example 13 – Seco-CPI Compound Ia-15

[00223] **FIG. 6** shows the scheme for the synthesis of *seco*-CPI compound **Ia-15**. The reagents used, and the conditions for their use, are known in the art and/or have been exemplified in the preceding examples. Table IX shows analytical data for compound **Ia-15** and other compounds similarly prepared.

Table IX – Seco-CPI Compounds Made Following FIG. 6

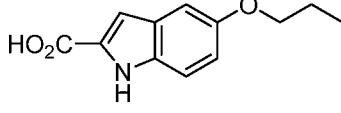
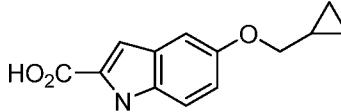
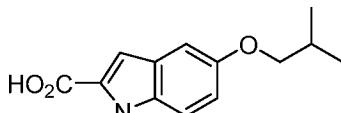
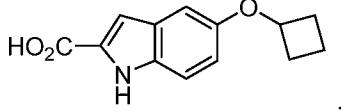
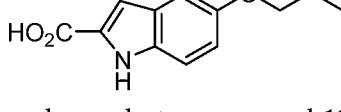
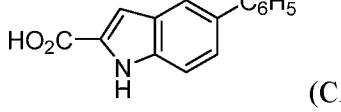
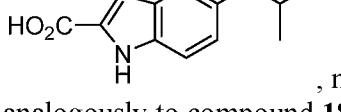
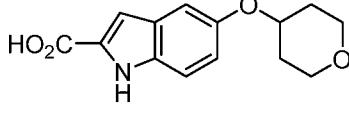
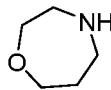
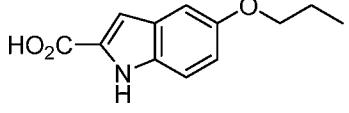
Compound No.	Mass spectrum	Remarks
Ia-03	$[M+H]^+ = 537.3$	 , made analogously to compound 18 in FIG. 2 , was used instead of compound 38 .
Ia-04	$[M+H]^+ = 549.1$	 , made analogously to compound 18 in FIG. 2 , was used instead of compound 38 .
Ia-05	$[M+H]^+ = 551.3$	 , made analogously to compound 18 in FIG. 2 , was used instead of compound 38 .
Ia-06	$[M+H]^+ = 549.2$	 , made analogously to compound 18 in FIG. 2 , was used instead of compound 38 .
Ia-07	$[M+H]^+ = 563.5$	 , made analogously to compound 18 in FIG. 2 , was used instead of compound 38 .
Ia-12	$[M+H]^+ = 555.3$	 (CAS Reg. No. 66616-71-5) was used instead of compound 38 .
Ia-13	$[M+H]^+ = 537.3$	 , made analogously to compound 18 in FIG. 2 , was used instead of compound 38 .

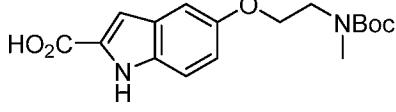
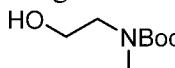
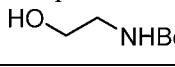
Table IX – Seco-CPI Compounds Made Following FIG. 6

Compound No.	Mass spectrum	Remarks
Ia-14	$[M+H]^+ = 579.2$	 , made analogously to compound 18 in FIG. 2, was used instead of compound 38 .
Ia-15	$[M+H]^+ = 563.4$	As depicted
Ia-28	$[M+H]^+ = 551.1$	 was used instead of morpholine to make counterpart of compound 14 per the procedure of FIG. 2 and  was used instead of compound 38 .

Example 14 – Seco-CPI Compound Ia-18

[00224] FIG. 7 shows the scheme for the synthesis of *secoc*-CPI compound **Ia-18**. The reagents used, and the conditions for their use, are known in the art and/or have been exemplified in the preceding examples. Table X shows analytical data for compound **Ia-18** and another compound similarly prepared.

Table X – Seco-CPI Compounds Made Following FIG. 7

Compound No.	Mass spectrum	Remarks
Ia-17	$[M+H]^+ = 552.2$	 , made analogously to compound 18 (FIG. 2) using  , was used instead of compound 40 .
Ia-18	$[M+H]^+ = 538.2$	Compound 40 was made analogously to compound 18 (FIG. 2), using 

Example 15 – Seco-CPI Compounds Ib-01 and Ib-02

[00225] **FIG. 8** shows a scheme for the synthesis of *sec*-CPI compounds **Ib-01** and **Ib-02**.

The reagents used, and the conditions for their use, are known in the art and/or have been exemplified in the preceding examples. **Ib-01** $[M+H]^+ = 615.0$; **Ib-02** $[M+H]^+ = 659.4$.

5 *Example 16 – Seco-CPI Compound Id-02*

[00226] **FIG. 9** shows the scheme for the synthesis of *sec*-CPI compound **Id-02**. The reagents used, and the conditions for their use, are known in the art and/or have been exemplified in the preceding examples. Table XI shows analytical data for compound **Id-02** and other compounds similarly prepared.

10

Table XI – *Seco-CPI Compounds Made Following FIG. 9*

Compound No.	Mass spectrum	Remarks
Ic-01	$[M+H]^+ = 652.3$	Compound 48a was used instead of compound 50 .
Id-01	$[M+H]^+ = 614.1$	4-Hydroxybenzoic acid was used instead of compound 50 .
Id-02	$[M+H]^+ = 613.1$	As depicted.

Example 17 – Seco-CPI Compound Ia-25

[00227] **FIG. 10** shows the scheme for the synthesis of *sec*-CPI compound **Ia-25**. The reagents used, and the conditions for their use, are known in the art and/or have been exemplified in the preceding examples.

[00228] Compound **Ia-26** was analogously prepared, using 1-methylpiperazine instead of 1-Cbz-piperazine.

[00229] The mass spectral data for the two compounds were: **Ia-25** $[M+H]^+ = 493.2$; **Ia-26** $[M+H]^+ = 507.3$.

20 *Example 18 – Seco-CPI Compound Ia-27*

[00230] **FIG. 11** shows the scheme for the synthesis of *sec*-CPI compound **Ia-27**. The reagents used, and the conditions for their use, are known in the art and/or have been exemplified in the preceding examples. Mass spectral data for compound **Ia-27**: $[M+H]^+ = 588.1$.

Example 19 – Seco-CPI Compound Ia-29

[00231] FIG. 12 shows the scheme for the synthesis of *seco*-CPI compound **Ia-29**. The reagents used, and the conditions for their use, are known in the art and/or have been exemplified in the preceding examples. Mass spectral data for compound **Ia-29**: $[M+H]^+ = 574.1$.

5 *Example 20 – Biological Activity of Conjugates*

[00232] *Seco*-CPI-linker compounds **IIIa-01**, **IIIa-02**, **IIIa-03**, and **IIIa-04** were conjugated to the anti-mesothelin antibody 6A4 (Terrett *et al.*, US 8,268,970 B2 (2012)) generally following the Michael addition procedure of Example 1 above. The resulting antibody-drug conjugates were designated **6A4/IIIa-01 ADC**, **6A4/IIIa-02 ADC**, **6A4/IIIa-03 ADC**, and **6A4/IIIa-04 ADC**, respectively.

[00233] Additionally, *seco*-CPI-linker compounds **IIIa-06** and **IIIa-12** were conjugated to anti-mesothelin antibody 6A4 engineered to have an N297A (EU as in Kabat) substitution in the heavy chain, generally following the transglutaminase mediated conjugation procedure of Example 2. The N297A substitution abrogates glycosylation of the antibody and makes nearby glutamine 295 (Q295) available as an amine acceptor for a transglutamine-catalyzed transamidation reaction (Jeger *et al.*, *Angew. Chem. Int. Ed.* **2010**, *49*, 9995). The resulting antibody-drug conjugates were designated **6A4(N297A)/IIIa-06 ADC** and **6A4(N297A)/IIIa-12 ADC**, respectively.

[00234] The activities of these ADCs against H226 and N87 cancer cells, both of which express mesothelin, was measured using a ^3H thymidine incorporation assay (Cheng *et al.*, US 8,394,922 B2 (2013)). Results are presented in Table XII.

Table XII – Biological Activity of Conjugates

Conjugate	EC ₅₀ (nM)	
	H226 Cells	N87 Cells
6A4/IIIa-01 ADC	1.2, 0.45	256
6A4/IIIa-02 ADC	5.8	—
6A4/IIIa-03 ADC	0.3	3.5
6A4/IIIa-04 ADC	0.10	0.12
6A4(N297A)/IIIa-06 ADC	0.18	—
6A4(N297A)/IIIa-12 ADC	0.20	—

[00235] 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.

[00236] 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

[00237] Full citations for the following references cited in abbreviated fashion by first author (or inventor) and date earlier in this specification are provided below. Each of these references is incorporated herein by reference for all purposes.

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5 [00257] Ng *et al.*, US 7,507,420 B2 (2009).

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10 [00262] Tietze *et al.*, *ChemBioChem* **2001**, 2, 758.

[00263] Tietze *et al.*, *Bioorg. Med. Chem.* **2008**, 16, 6312.

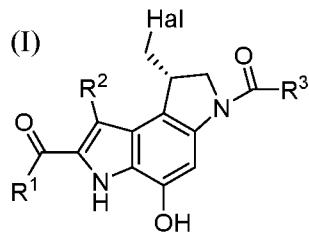
[00264] Zhang *et al.*, US 8,852,599 B2 (2014).

[00265] Zhao *et al.*, US 7,655,660 B2 (2010).

CLAIMS

What is claimed is:

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



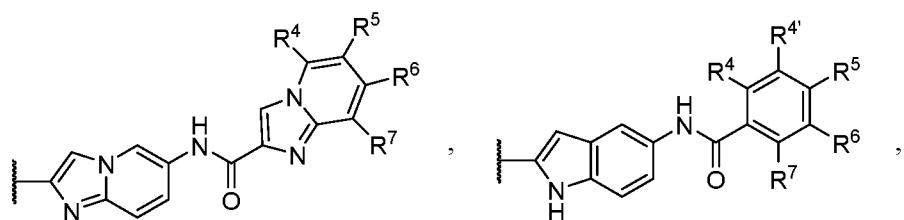
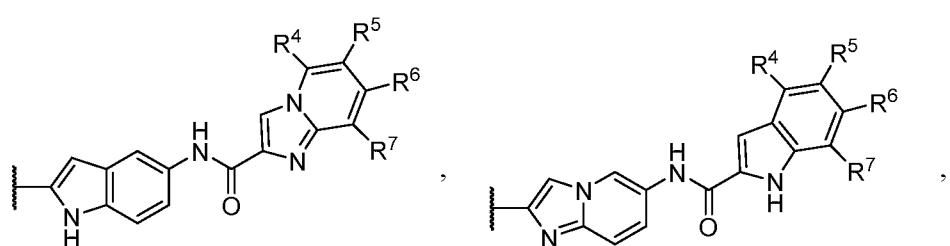
5 wherein

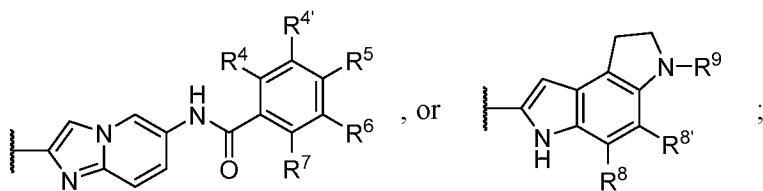
Hal is Cl or Br;

R¹ is $\begin{array}{c} \text{---N} \\ | \\ \text{H} \end{array} \text{---} \text{CH}_2 \text{---} \text{X}^1$, $\begin{array}{c} \text{---N} \\ | \\ \text{X}^2 \end{array}$, or $\begin{array}{c} \text{---N} \\ | \\ \text{---CH}_2 \text{---} \text{X}^3 \end{array}$;

R² is H, C₁-C₃ alkyl, CO₂H, CO₂(C₁-C₃ alkyl), C(=O)NH₂, C(=O)NH(C₁-C₃ alkyl), or C(=O)N(C₁-C₃ alkyl)₂;

10 R³ is , , ,





R^4 , $R^{4'}$, R^5 , R^6 , or R^7 are independently H, OMe, OH, a 6-membered aryl group, a 5- or 6-membered heteroaryl group, NH₂, NHMe, NMe₂, NH(C₂-C₄ alkyl), N(C₂-C₄ alkyl)₂, NHC(=O)X¹, O(C₂-C₄ alkyl), O(CH₂)₀₋₂(C₃-C₆ cycloalkyl), O(CH₂)₀₋₂X¹, or

5 $\text{---O(CH}_2\text{)}_{0-2}\text{---}\begin{array}{c} (\text{CH}_2)_{1-2} \\ | \\ \text{---X}^3 \\ | \\ (\text{CH}_2)_{1-2} \end{array}$;

wherein a C₂-C₄ alkyl group may unsubstituted or substituted with OCH₂CH₂OH, OCH₂CH₂NH₂, NHCH₂CH₂OH, NHCH₂CH₂NH₂, OH, or NH₂ and an aryl or heteroaryl group may be substituted with C₁-C₂ alkyl, OH, NH₂, NH(C₁-C₂ alkyl), N(C₁-C₂ alkyl)₂, F, Cl, Br, NO₂, or CN;

10 with the proviso that at least one of R^4 , $R^{4'}$, R^5 , R^6 , and R^7 is other than H;

R^8 and $R^{8'}$ are independently H, OH, O(C₁-C₃ alkyl), Cl, Br, F, O(CH₂)₂₋₄NH₂, or O(CH₂)₂₋₄OH;

R^9 is H, C(=O)(C₁-C₃ alkyl), C(=O)NH₂, C(=O)NH(C₁-C₃ alkyl), C(=O)(C₁-C₃ alkyl)₂, (CH₂)₂₋₄OH, (CH₂)₂₋₄O(C₁-C₃ alkyl), (CH₂)₂₋₄NH₂, (CH₂)₂₋₄NH(C₁-C₃ alkyl), or (CH₂)₂₋₄N(C₁-C₃ alkyl)₂;

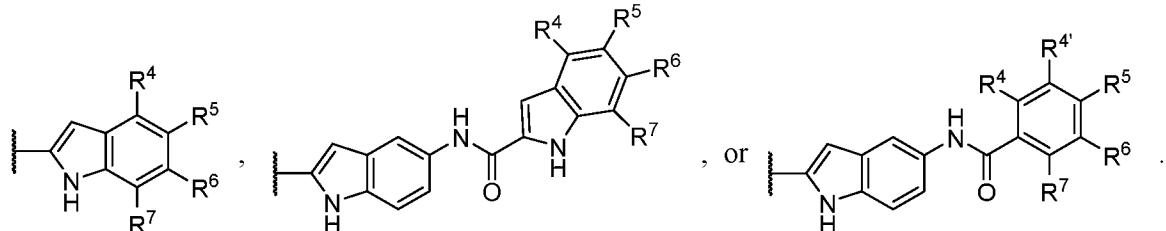
15 each X^1 is independently a 6-membered aryl or 5- to 6-membered heteroaryl group that is unsubstituted or substituted with C₁-C₃ alkyl, OH, O(C₁-C₃ alkyl), NH₂, NH(C₁-C₃ alkyl), N(C₁-C₃ alkyl)₂, F, Cl, Br, NO₂, or CN;

 each X^2 is independently H, Me, or a C₂-C₄ alkyl group that may be unsubstituted or substituted with OCH₂CH₂OH, OCH₂CH₂NH₂, NHCH₂CH₂OH, NHCH₂CH₂NH₂, OH, or NH₂; and

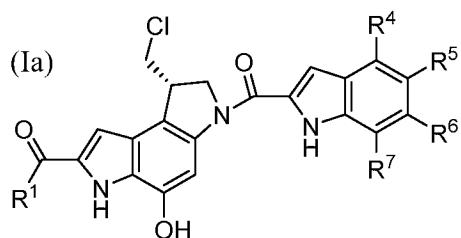
20 each X^3 is independently O, NH, N(C₁-C₃ alkyl), or S; or a pharmaceutically acceptable salt thereof.

2. A compound according to claim 1, wherein R^2 is H and Hal is Cl.

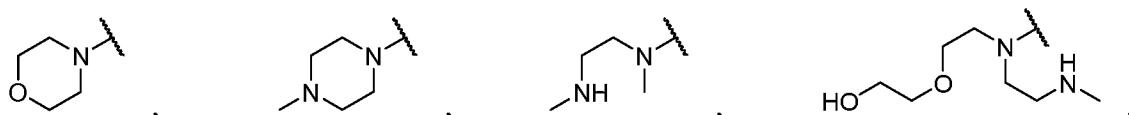
3. A compound according to claim 1, wherein R^3 is



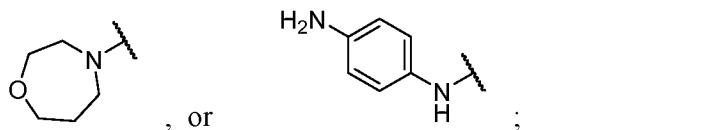
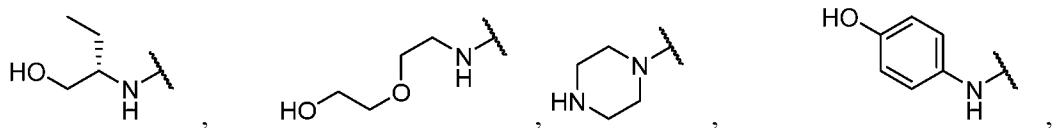
4. A compound according to claim 1, having a structure represented by formula (Ia):



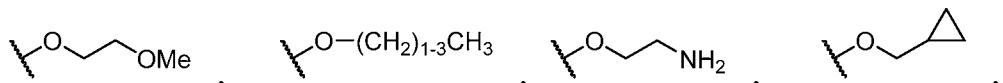
5. A compound according to claim 4, wherein R^4 , R^6 , and R^7 are each H, R^1 is



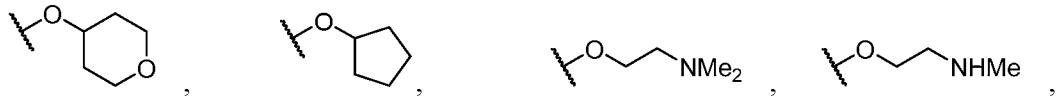
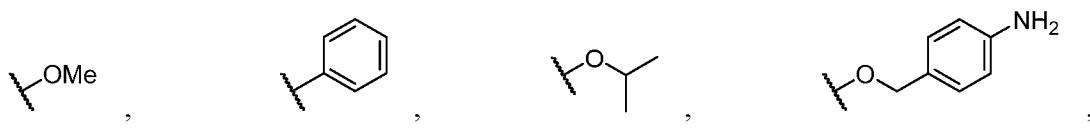
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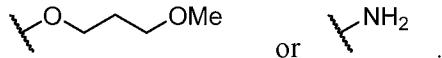
10 and R^5 is



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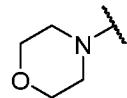


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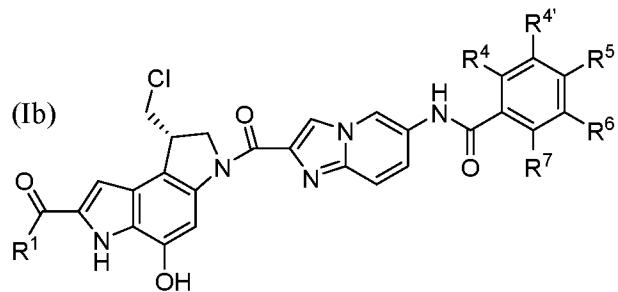


6. A compound according to claim 1, wherein R¹ is a group whose corresponding compound R¹H has a CLogP value of less than 0.300.

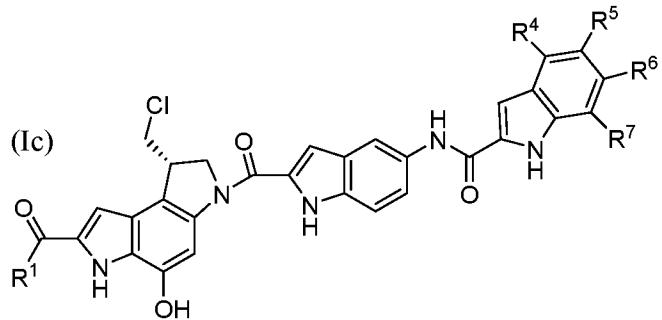
7. A compound according to claim 4, wherein R¹ is



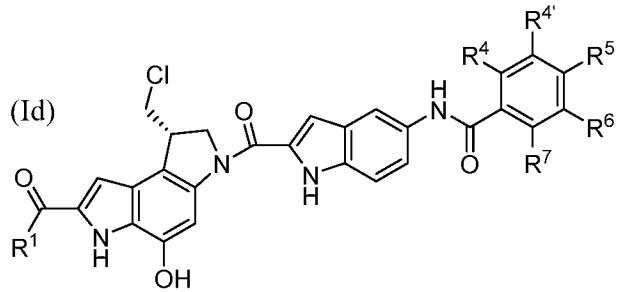
8. A compound according to claim 1, having a structure represented by formula (Ib):



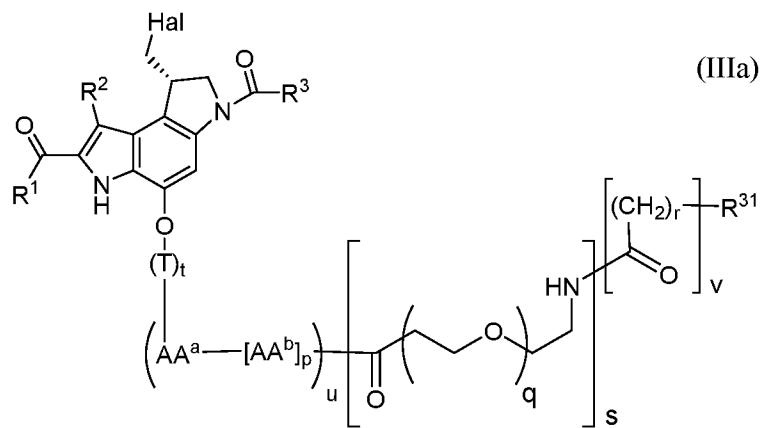
5 9. A compound according to claim 1, having a structure represented by formula (Ic):



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



11. A *seco*-CPI-linker compound, having a structure according to formula (IIIa):



wherein

T is a self-immolating group;

t is 0 or 1;

5 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;

10 u is 0 or 1;

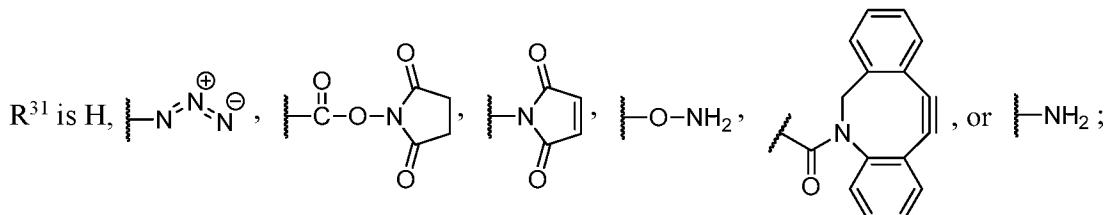
p is 1, 2, 3, or 4;

q is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 (preferably 2, 3, 4, or 8);

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

s is 0 or 1;

15 v is 0 or 1;

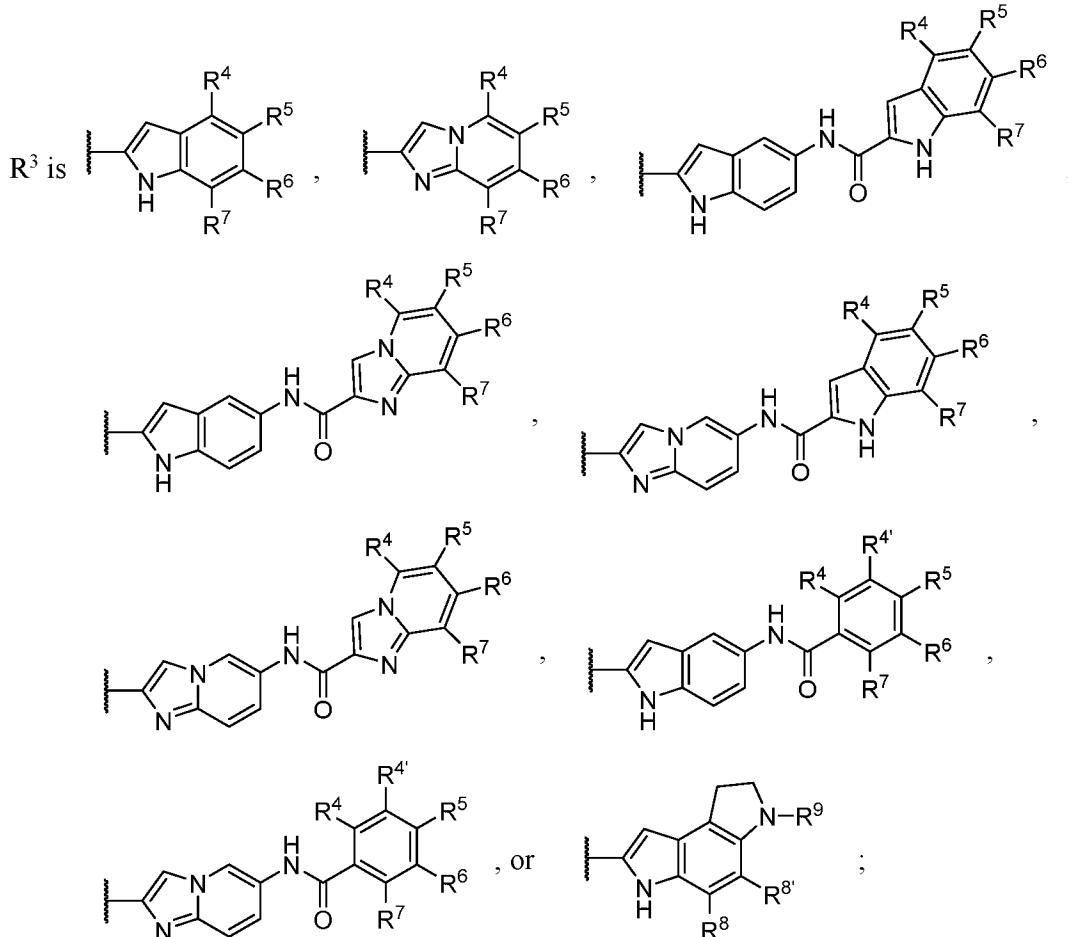


with the provisos that R³¹ can be H only if s is 1 and v is 0 and that v can be 0 only if s is 1 and R³¹ is H;

Hal is Cl or Br;

20 R¹ is $\text{--N}^{(\text{CH}_2)_{0-2}-\text{X}^1}$, $\text{--N}^{\text{X}^2}_{\text{X}^2}$, or $\text{--N}^{(\text{CH}_2)_{2-3}}_{(\text{CH}_2)_{2-3}\text{X}^3}$;

R² is H, C₁-C₃ alkyl, CO₂H, CO₂(C₁-C₃ alkyl), C(=O)NH₂, C(=O)NH(C₁-C₃ alkyl), or C(=O)N(C₁-C₃ alkyl)₂;



R⁹ is H, C(=O)(C₁-C₃ alkyl), C(=O)NH₂, C(=O)NH(C₁-C₃ alkyl), C(=O)(C₁-C₃ alkyl)₂,

(CH₂)₂₋₄OH, (CH₂)₂₋₄O(C₁-C₃ alkyl), (CH₂)₂₋₄NH₂, (CH₂)₂₋₄NH(C₁-C₃ alkyl), or

(CH₂)₂₋₄N(C₁-C₃ alkyl)₂;

each X¹ is independently a 6-membered aryl or 5- to 6-membered heteroaryl group that is

5 unsubstituted or substituted with C₁-C₃ alkyl, OH, O(C₁-C₃ alkyl), NH₂, NH(C₁-C₃ alkyl), N(C₁-C₃ alkyl)₂, F, Cl, Br, NO₂, or CN;

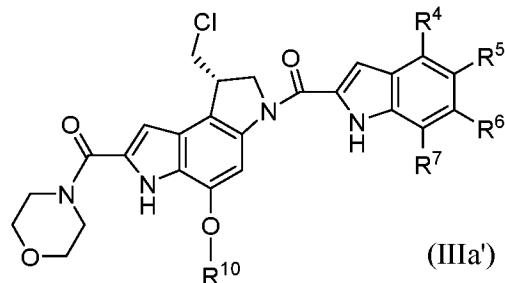
each X² is independently H, Me, or a C₂-C₄ alkyl group that may be unsubstituted or substituted

with OCH₂CH₂OH, OCH₂CH₂NH₂, NHCH₂CH₂OH, NHCH₂CH₂NH₂, OH, or NH₂; and

each X³ is independently O, NH, N(C₁-C₃ alkyl), or S;

10 or a pharmaceutically acceptable salt thereof.

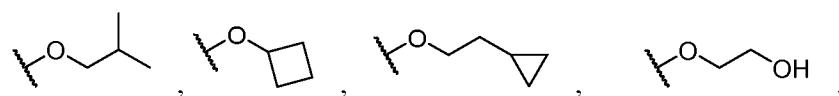
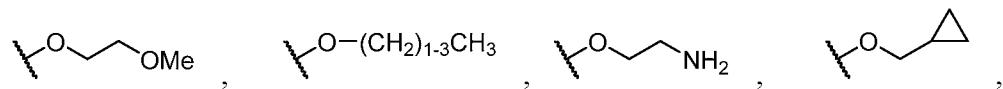
12. A *seco*-CPI-linker compound according to claim 11, having a structure according to formula (IIIa'):



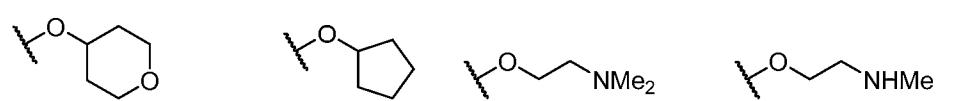
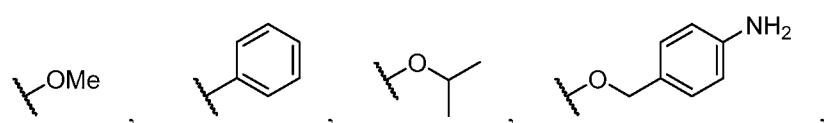
wherein

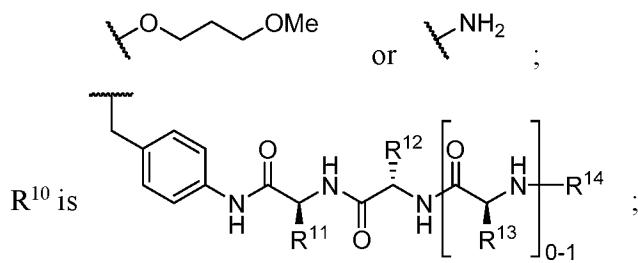
15 R⁴, R⁶, and R⁷ are each H; and

R⁵ is

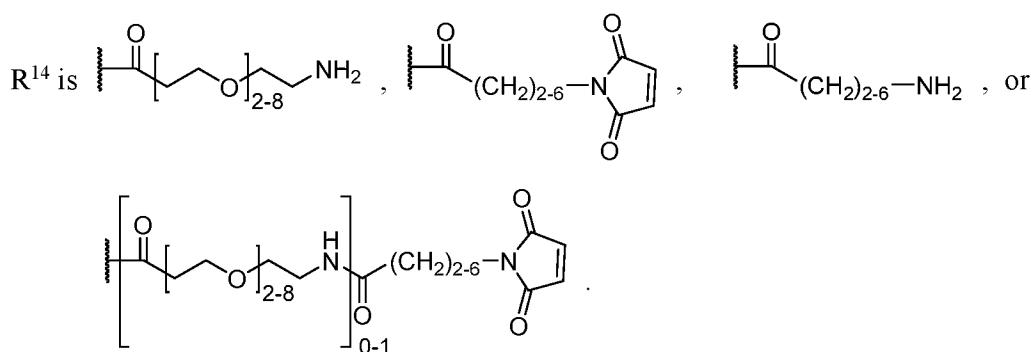


20

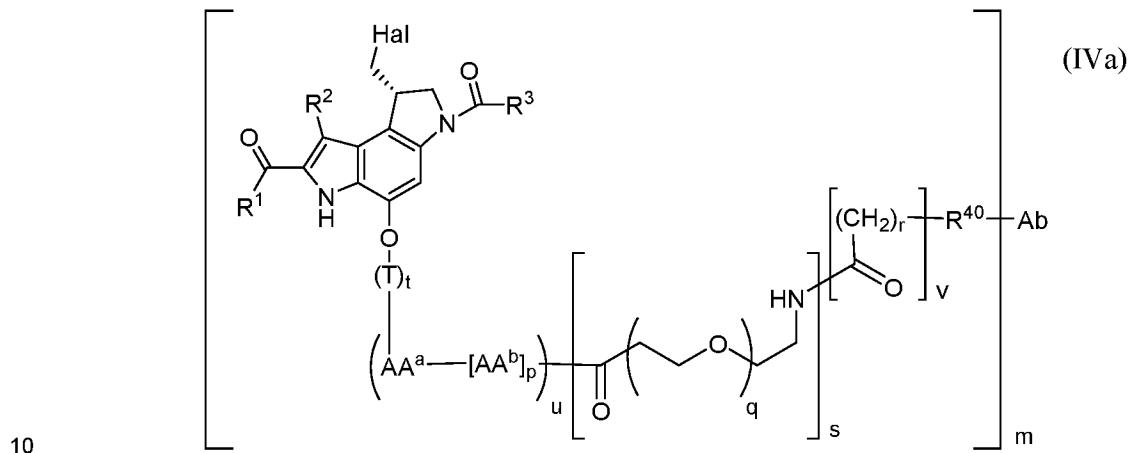




5 R¹¹, R¹² and R¹³ are independently H, CH₃, CH(CH₃)₂, CH₂CO₂H, CH₂CH₂CO₂H,
CH₂C(=O)NH₂, CH₂CH₂C(=O)NH₂, (CH₂)₄NH₂, (CH₂)₃NHC(=NH)NH₂ or
(CH₂)₃NHC(=O)NH₂; and

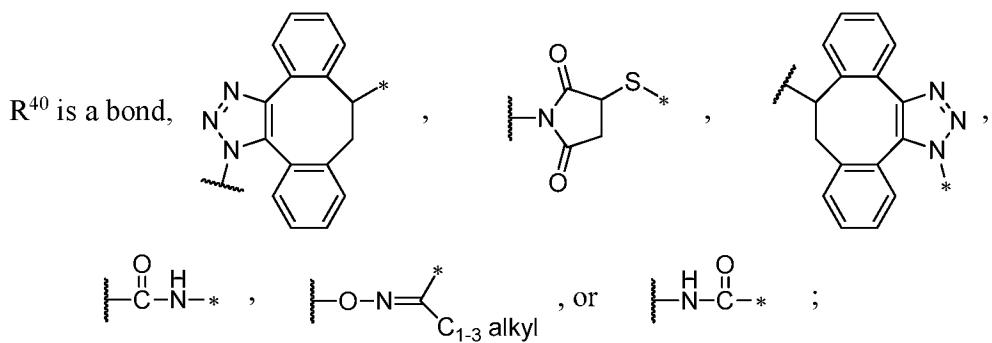


13. An antibody-drug conjugate, having a structure according to formula (IVa):



wherein

Ab is an antibody;



where the open valence of R^{40} that is bonded to Ab is denoted by an asterisk (*) and the open valence of R^{40} that is bonded to $(CH_2)_r$ is denoted by a wavy line (~~~~~);

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

v is 0 or 1, with the provisos that v can be 0 only if s is 1 and R^{40} is a bond and that R^{40} can be a bond only if v is 0 and s is 1;

T is a self-immolating group;

t is 0 or 1;

10 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;

15 u is 0 or 1;

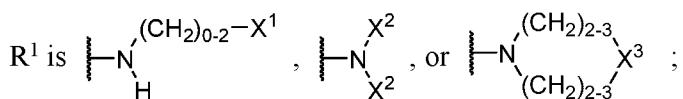
p is 1, 2, 3, or 4;

q is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 (preferably 2, 3, 4, or 8);

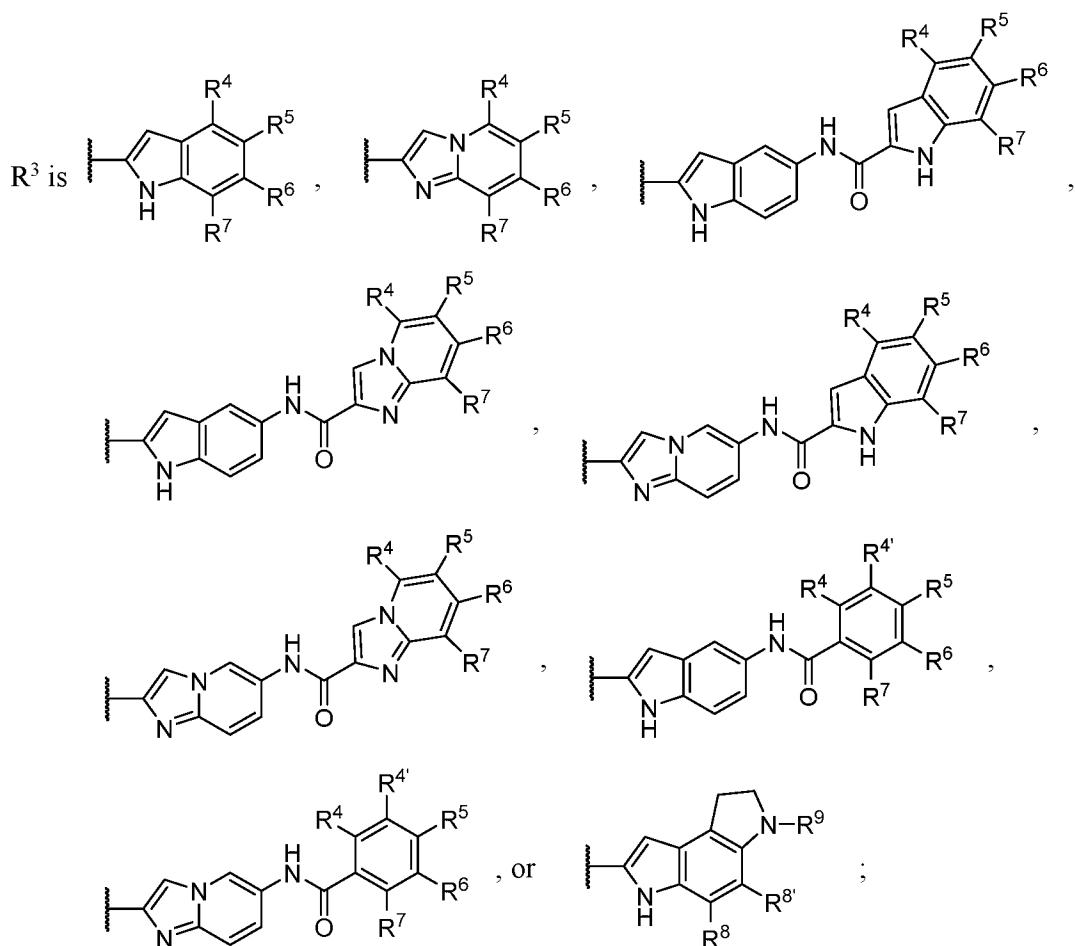
r is 1, 2, 3, 4, or 5;

s is 0 or 1;

20 Hal is Cl or Br;



R^2 is H, C_1 - C_3 alkyl, CO_2H , $CO_2(C_1$ - C_3 alkyl), $C(=O)NH_2$, $C(=O)NH(C_1$ - C_3 alkyl), or $C(=O)N(C_1$ - C_3 alkyl) $_2$;



5 R^4 , $R^{4'}$, R^5 , R^6 , or R^7 are independently H, OMe, OH, a 6-membered aryl group, a 5- or 6-membered heteroaryl group, NH₂, NHMe, NMe₂, NH(C₂-C₄ alkyl), N(C₂-C₄ alkyl)₂, NHC(=O)X¹, O(C₂-C₄ alkyl), O(CH₂)₀₋₂(C₃-C₆ cycloalkyl), O(CH₂)₀₋₂X¹, or

wherein a C₂-C₄ alkyl group may unsubstituted or substituted with OCH₂CH₂OH, OCH₂CH₂NH₂, NHCH₂CH₂OH, NHCH₂CH₂NH₂, OH, or NH₂ and an aryl or heteroaryl group may be substituted with C₁-C₂ alkyl, OH, NH₂, NH(C₁-C₂ alkyl), N(C₁-C₂ alkyl)₂, F, Cl, Br, NO₂, or CN;

with the proviso that at least one of R^4 , R^4' , R^5 , R^6 , and R^7 is other than H;

15 R⁸ and R^{8'} are independently H, OH, O(C₁-C₃ alkyl), Cl, Br, F, O(CH₂)₂₋₄NH₂, or O(CH₂)₂₋₄OH;
R⁹ is H, C(=O)(C₁-C₃ alkyl), C(=O)NH₂, C(=O)NH(C₁-C₃ alkyl), C(=O)(C₁-C₃ alkyl)₂,
(CH₂)₂₋₄OH, (CH₂)₂₋₄O(C₁-C₃ alkyl), (CH₂)₂₋₄NH₂, (CH₂)₂₋₄NH(C₁-C₃ alkyl), or
(CH₂)₂₋₄N(C₁-C₃ alkyl)₂;

each X¹ is independently a 6-membered aryl or 5- to 6-membered heteroaryl group that is unsubstituted or substituted with C₁-C₃ alkyl, OH, O(C₁-C₃ alkyl), NH₂, NH(C₁-C₃ alkyl), N(C₁-C₃ alkyl)₂, F, Cl, Br, NO₂, or CN;

each X² is independently H, Me, or a C₂-C₄ alkyl group that may be unsubstituted or substituted with OCH₂CH₂OH, OCH₂CH₂NH₂, NHCH₂CH₂OH, NHCH₂CH₂NH₂, OH, or NH₂; and

5 each X³ is independently O, NH, N(C₁-C₃ alkyl), or S;

each X⁴ is independently a pharmaceutically acceptable salt thereof.

14. A compound according to claim 1 or a conjugate thereof with an antibody, for use in treating a cancer in a subject suffering from such cancer.

10 **15.** A compound according to claim 14, wherein the cancer is lung cancer, gastric cancer, ovarian cancer, or colon cancer.

16. A compound according to claim 14, wherein the compound is conjugated to an antibody.

17. A compound according to claim 16, wherein the cancer is lung cancer, gastric cancer, ovarian cancer, or colon cancer.

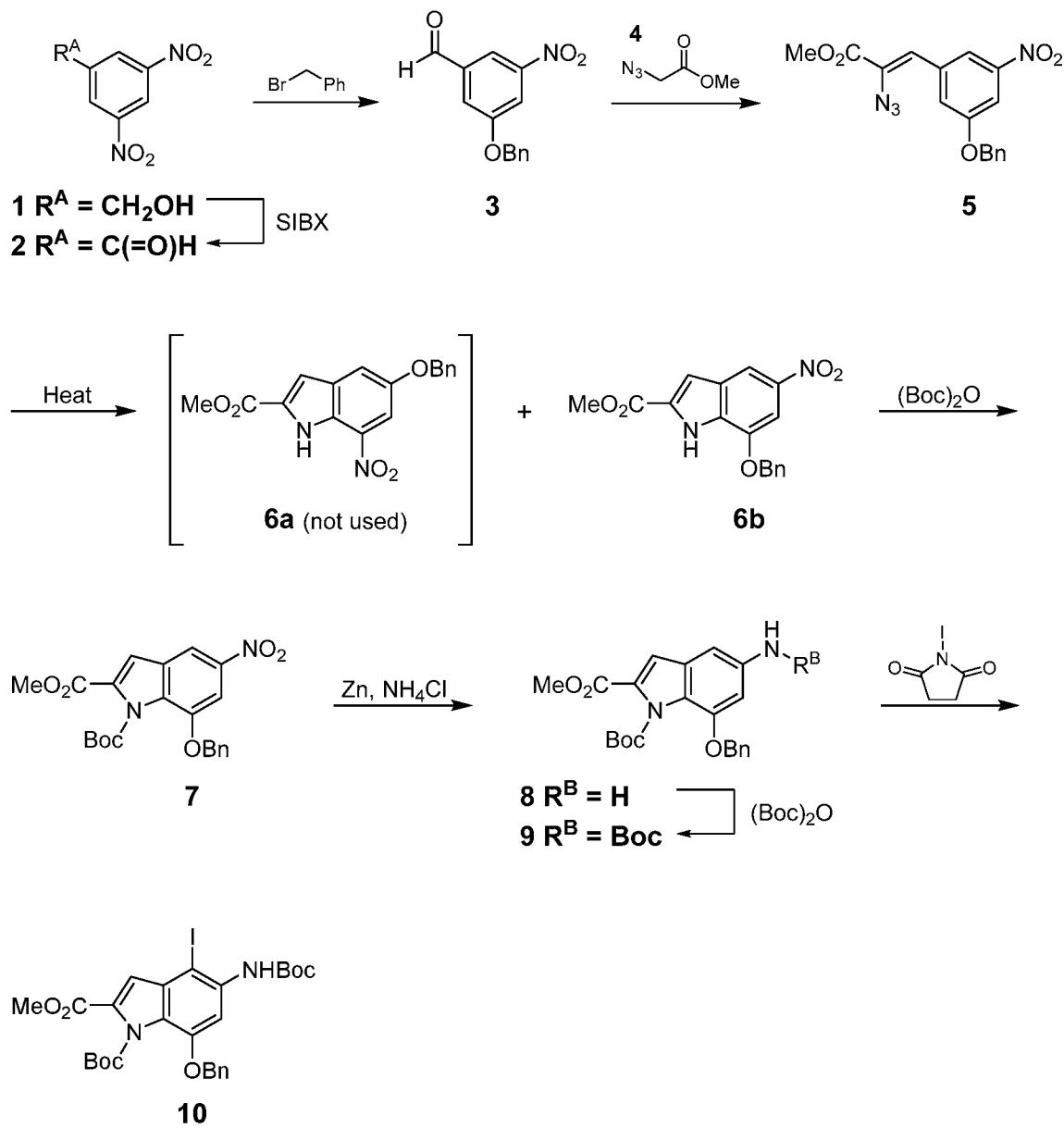
FIG. 1A

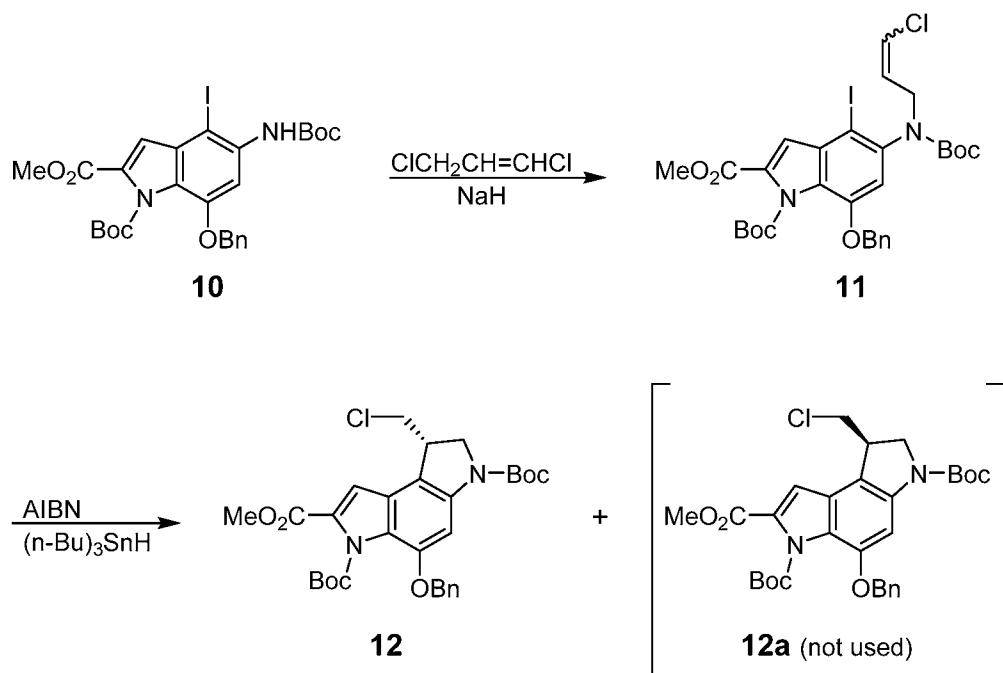
FIG. 1B

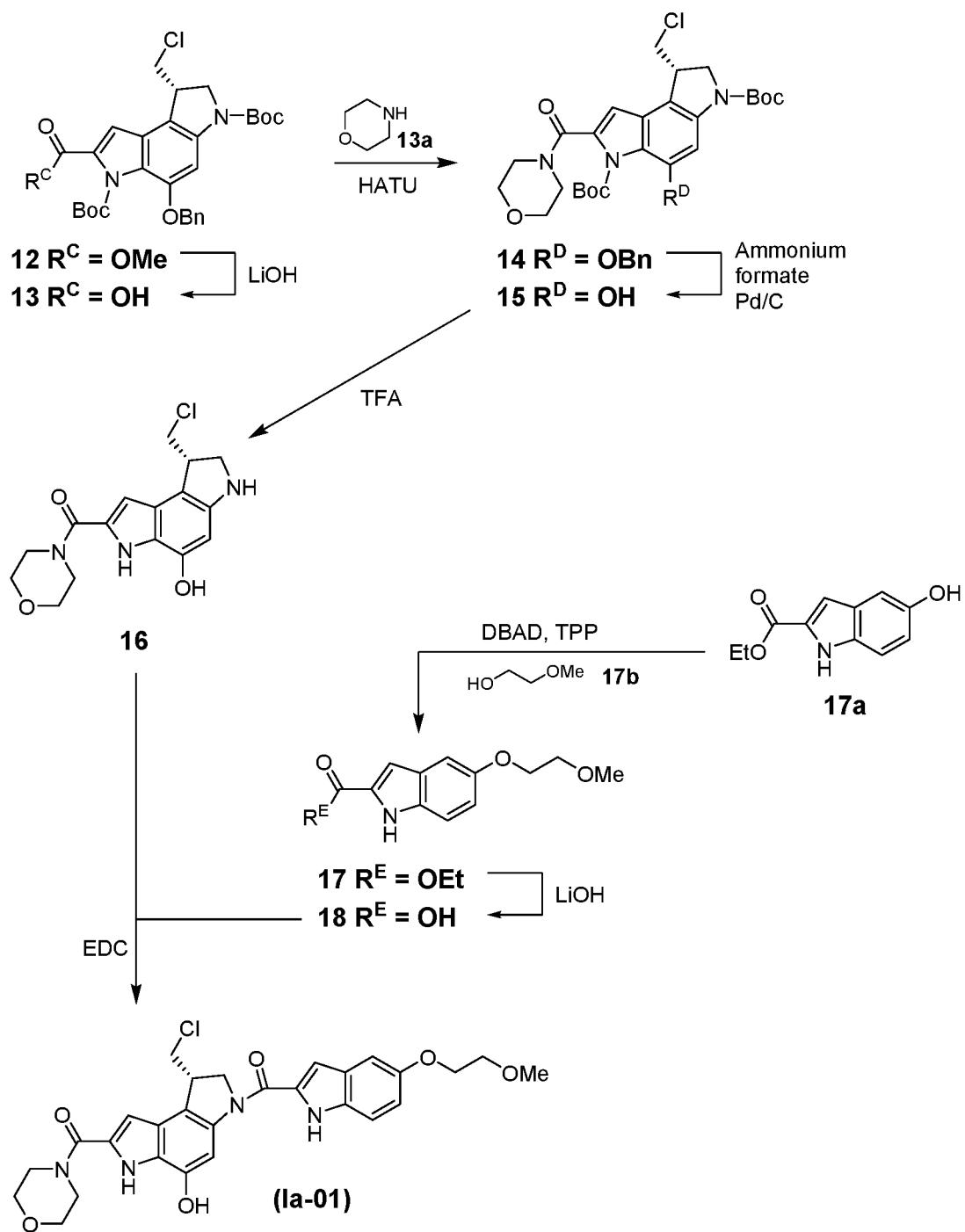
FIG. 2

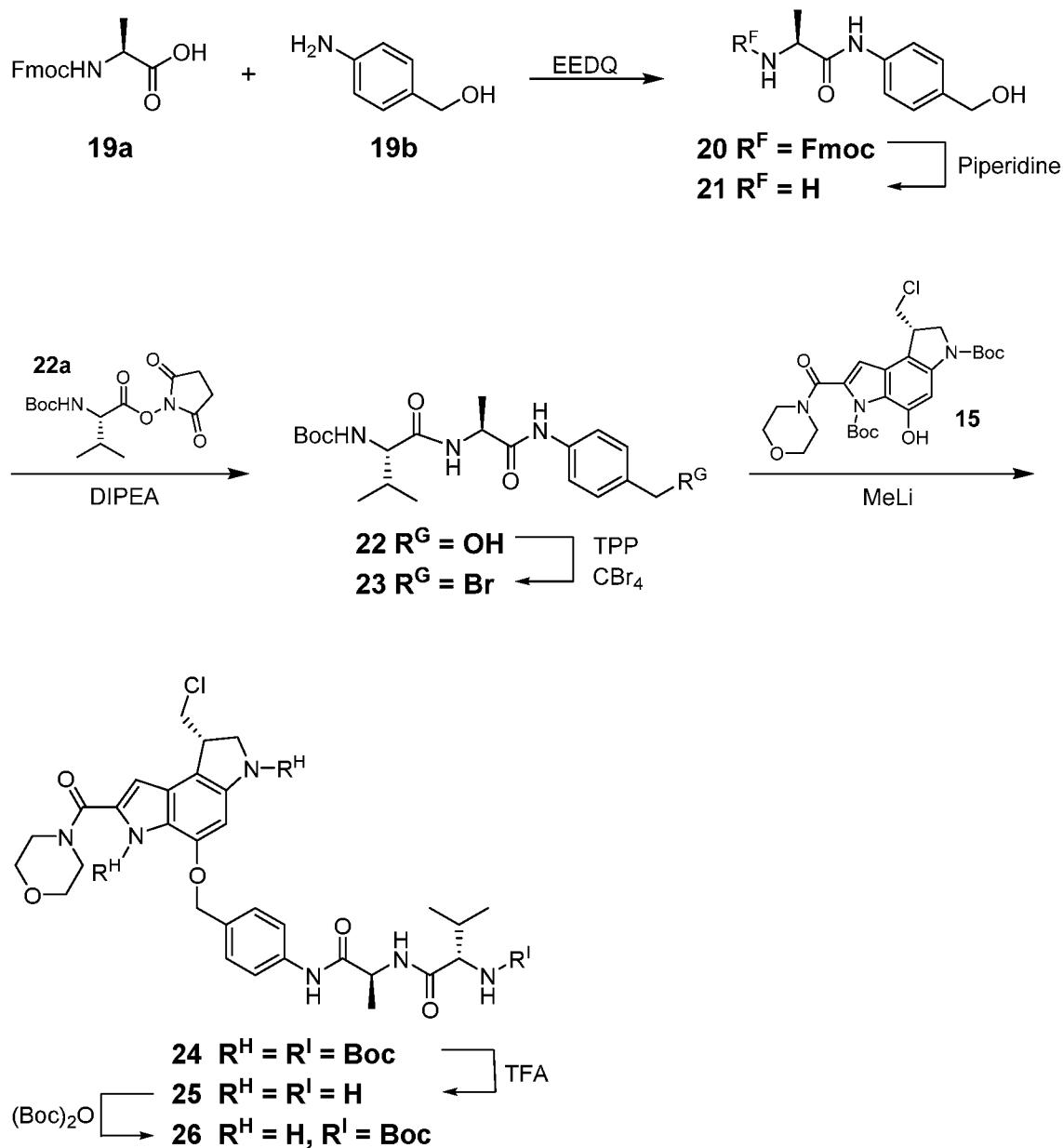
FIG. 3A

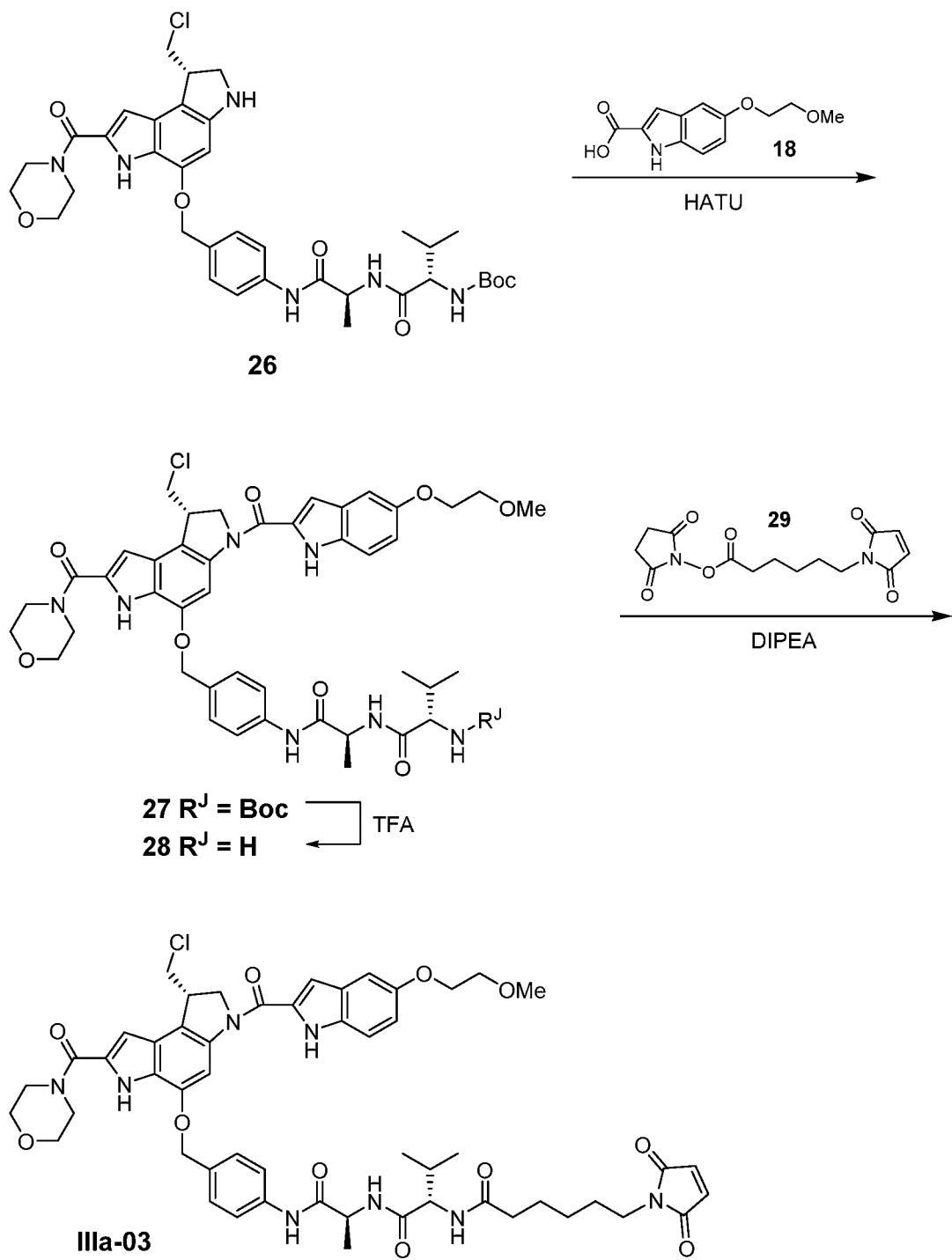
FIG. 3B

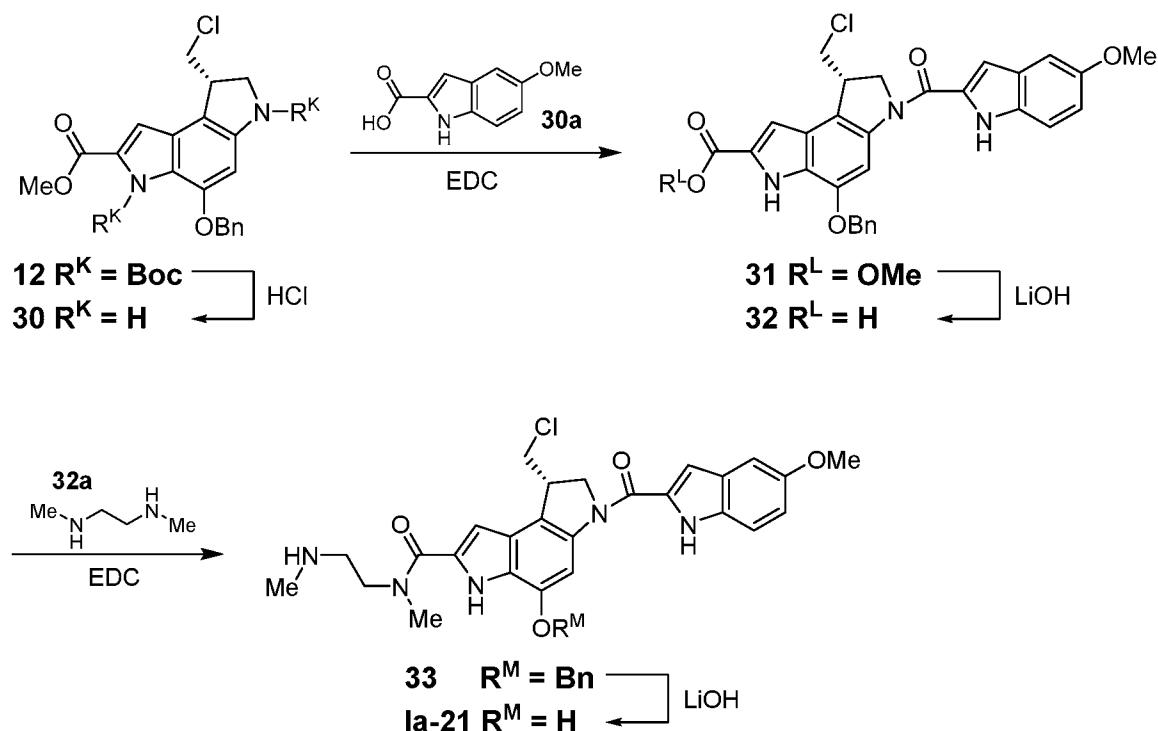
FIG. 4

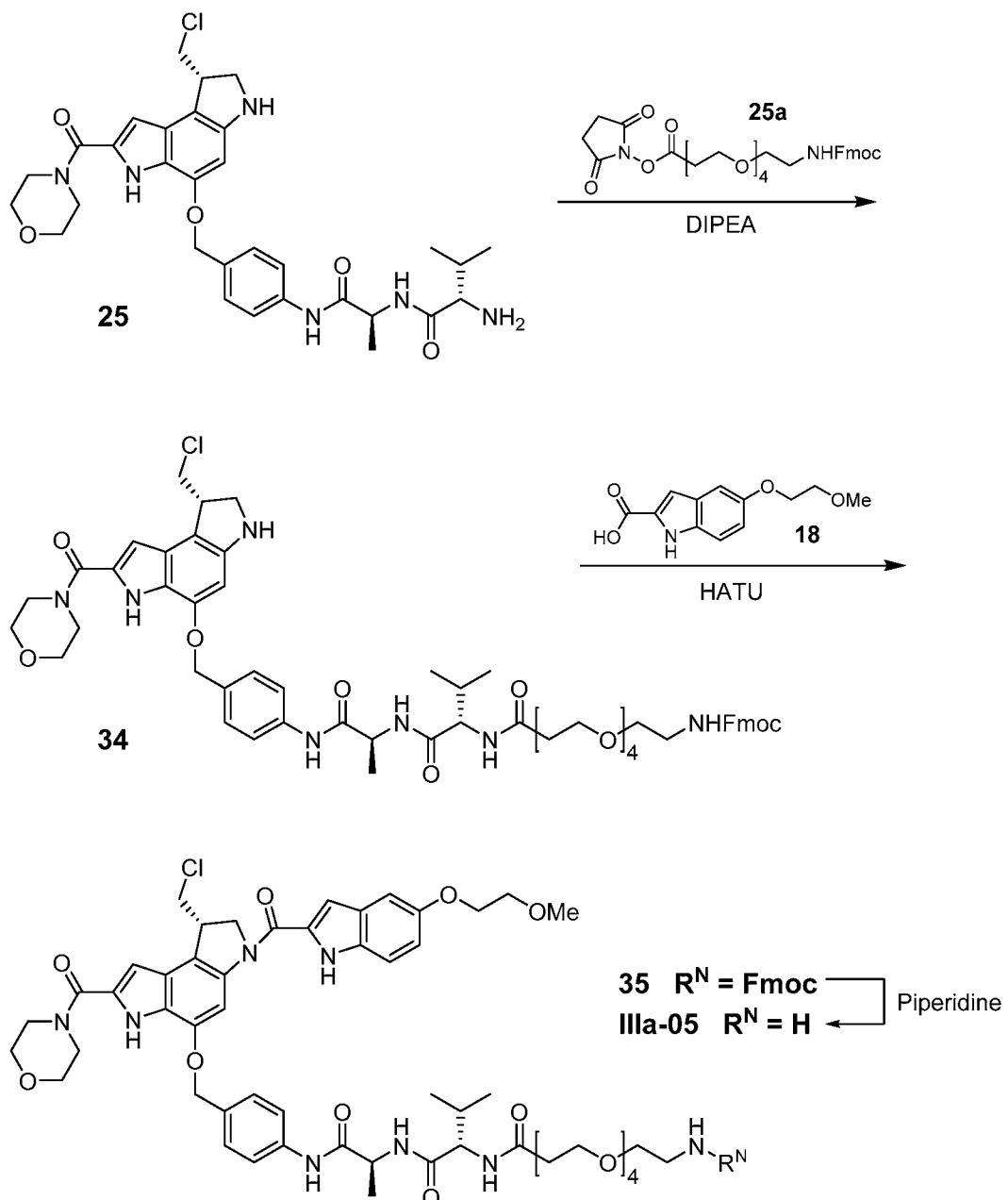
FIG. 5

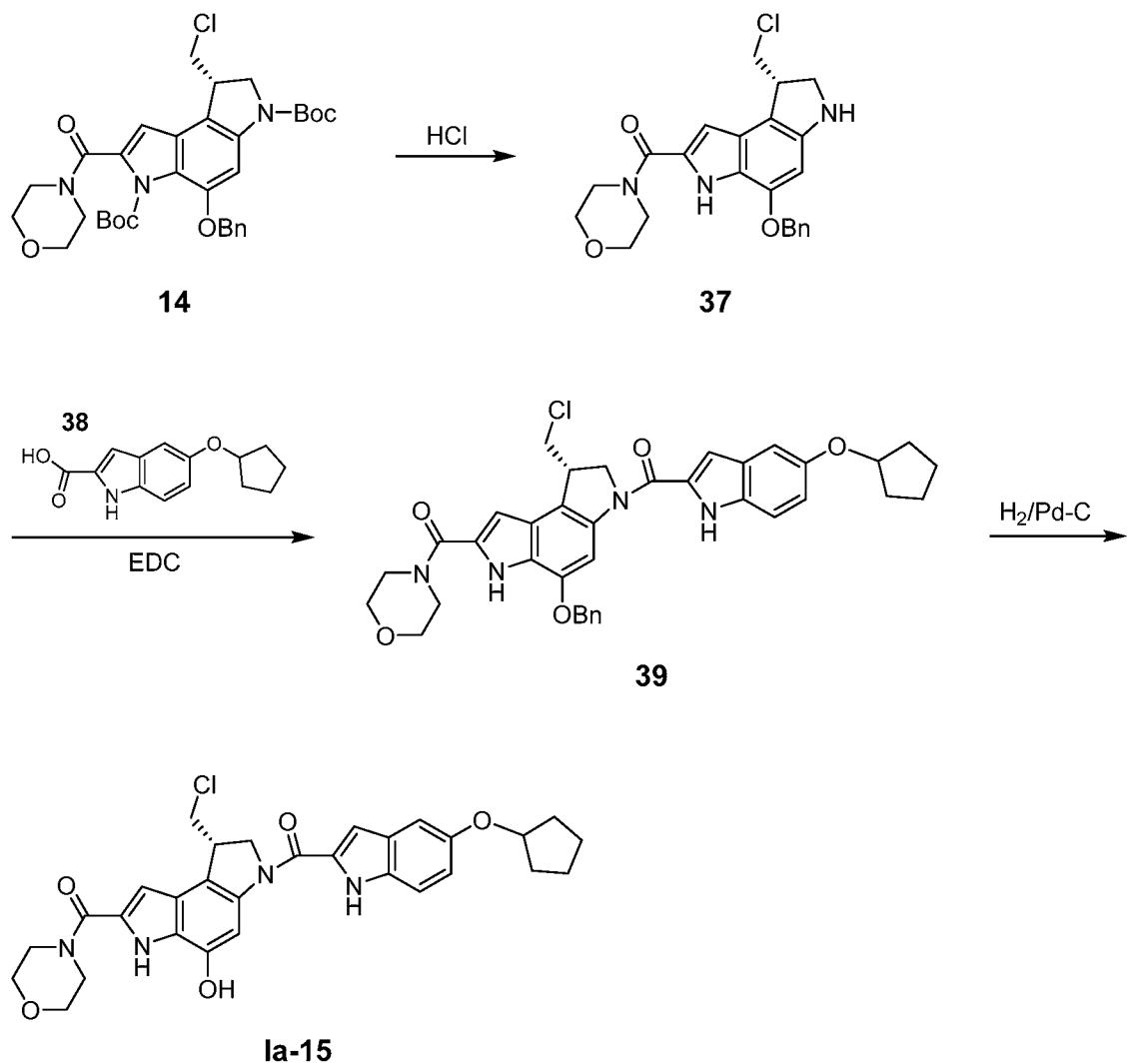
FIG. 6

FIG. 7

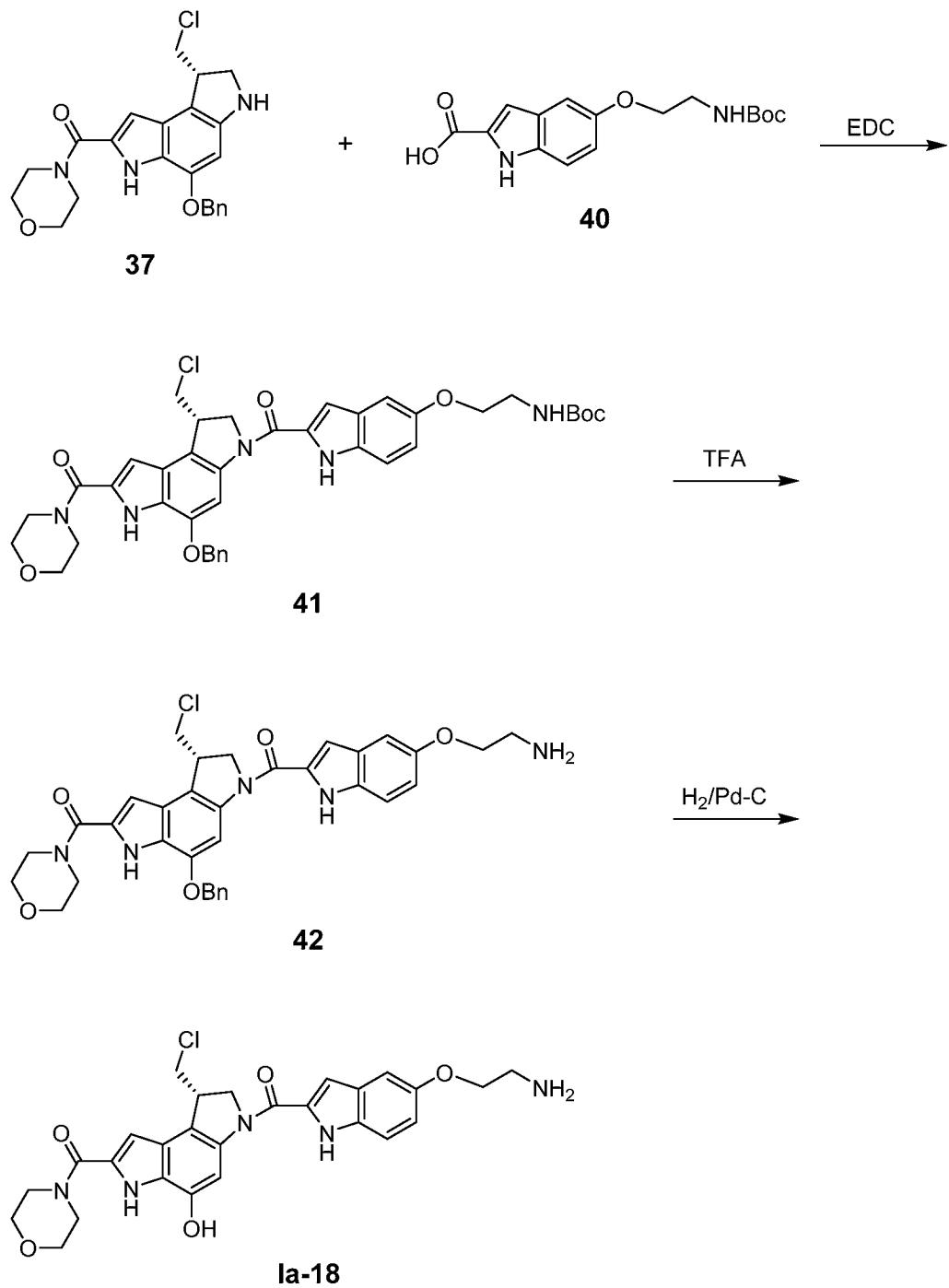


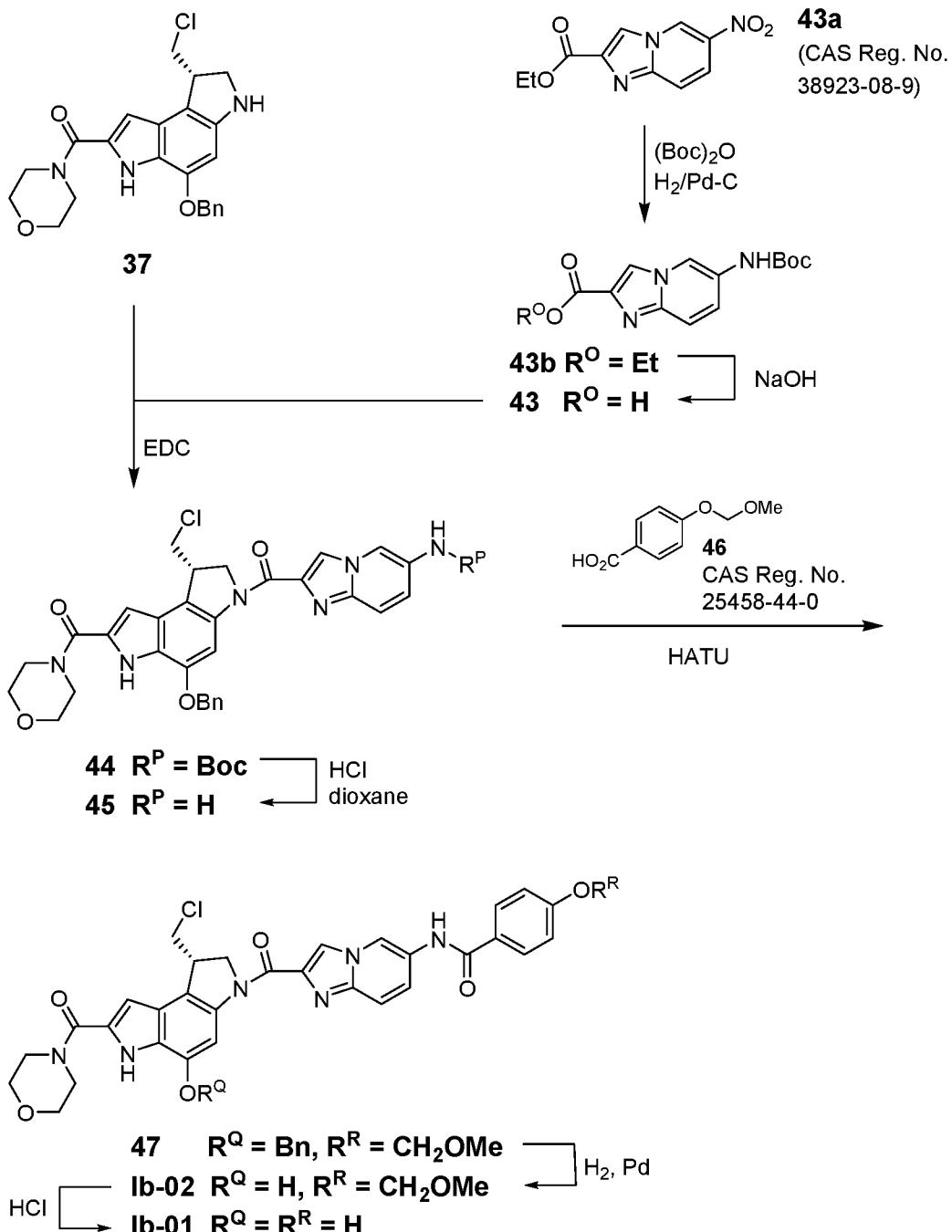
FIG. 8

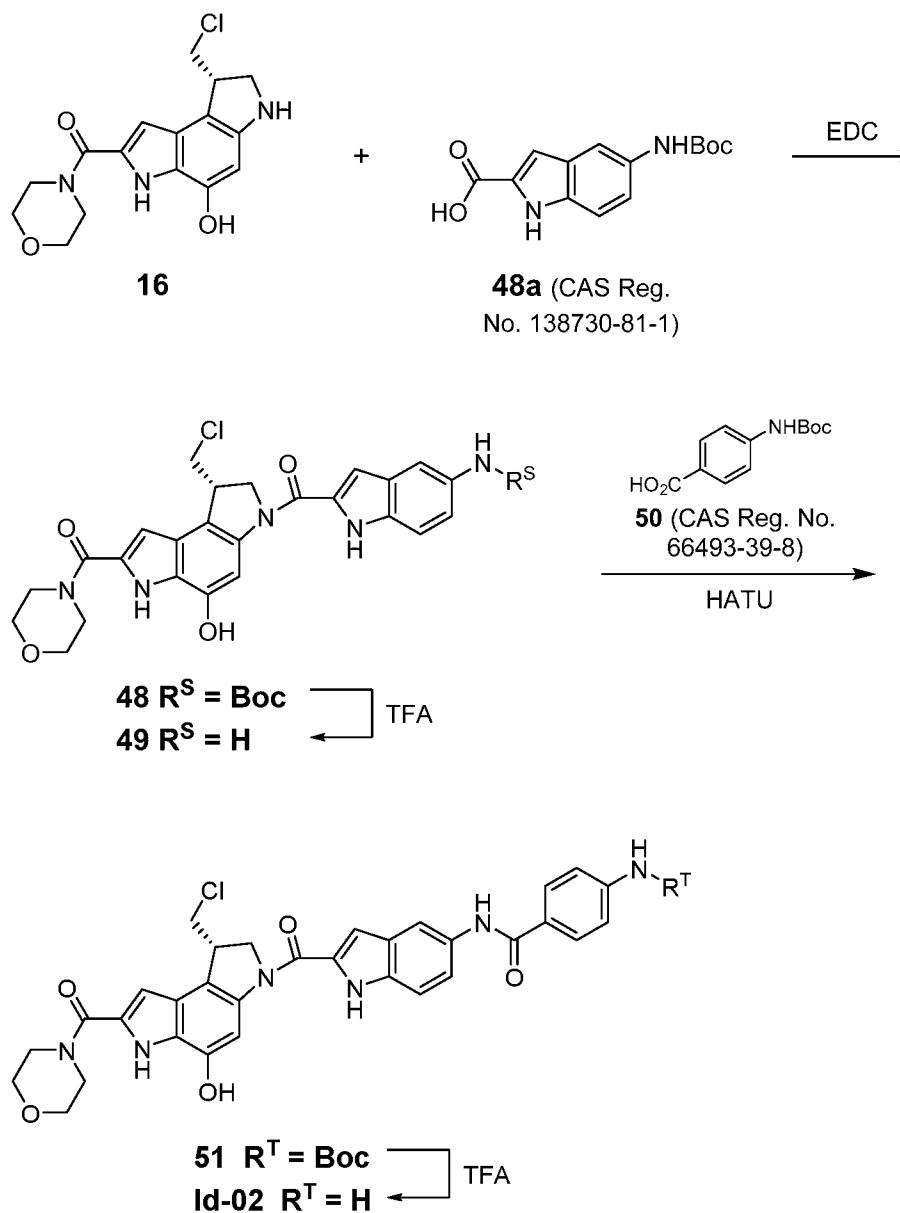
FIG. 9

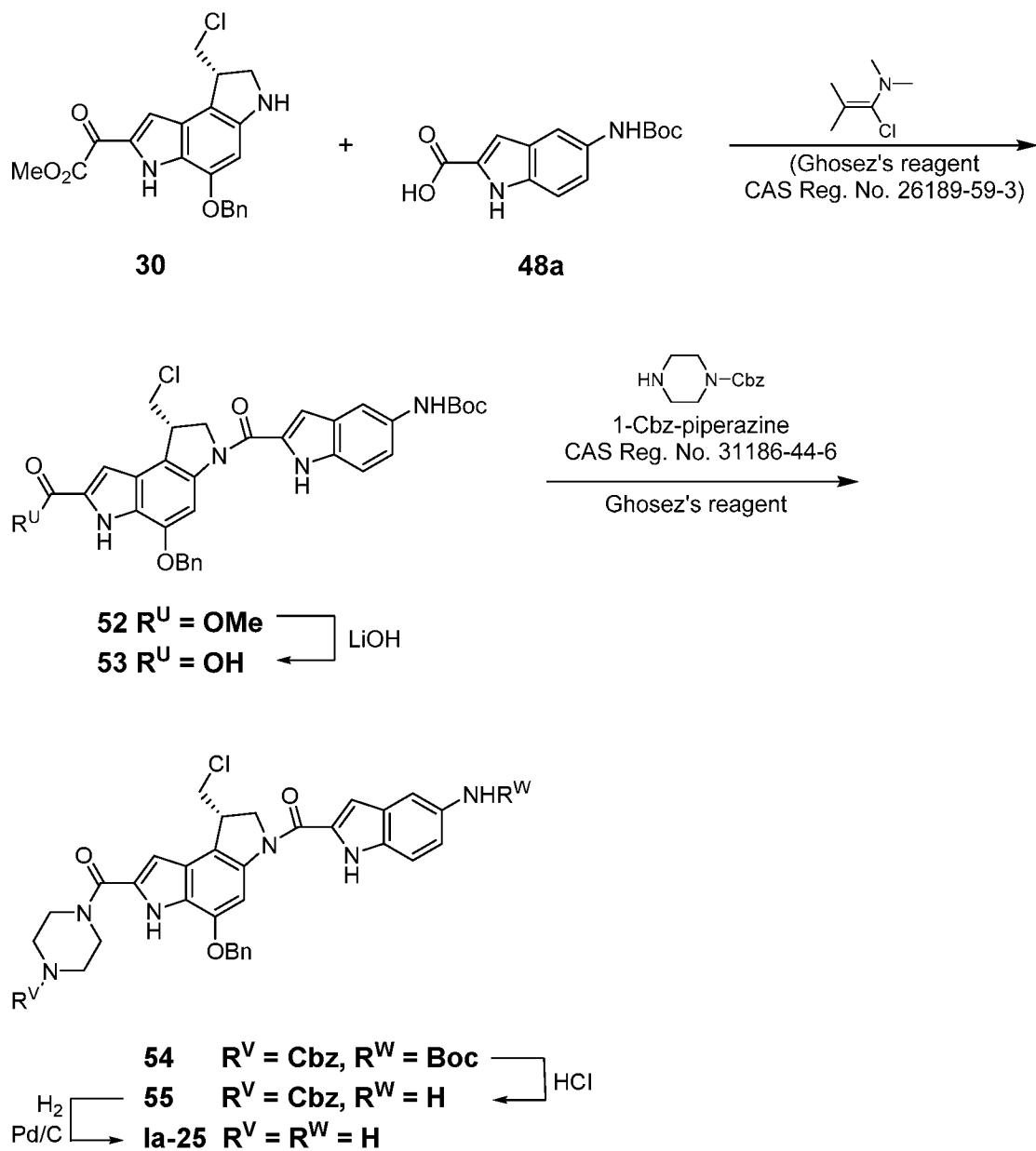
FIG. 10

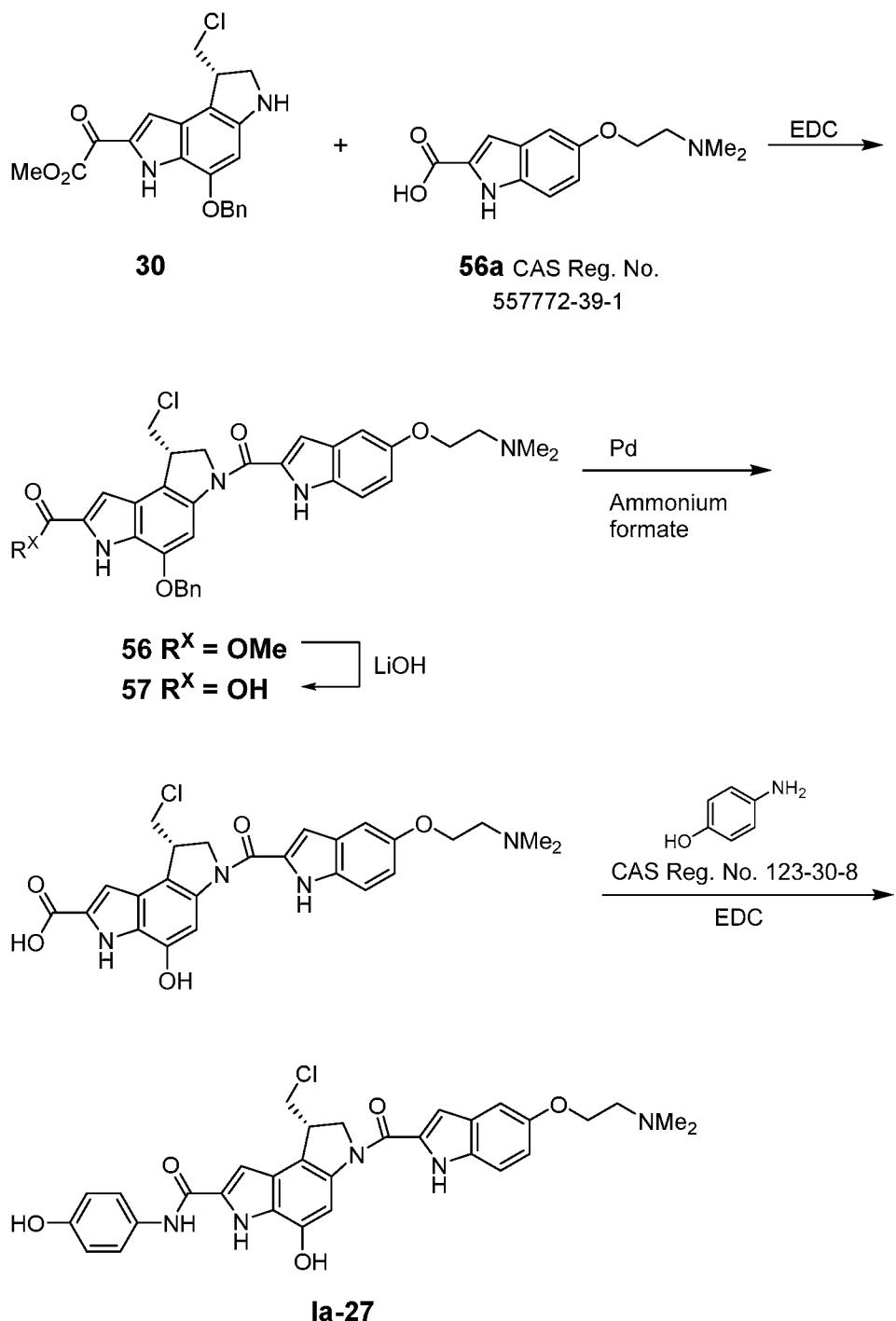
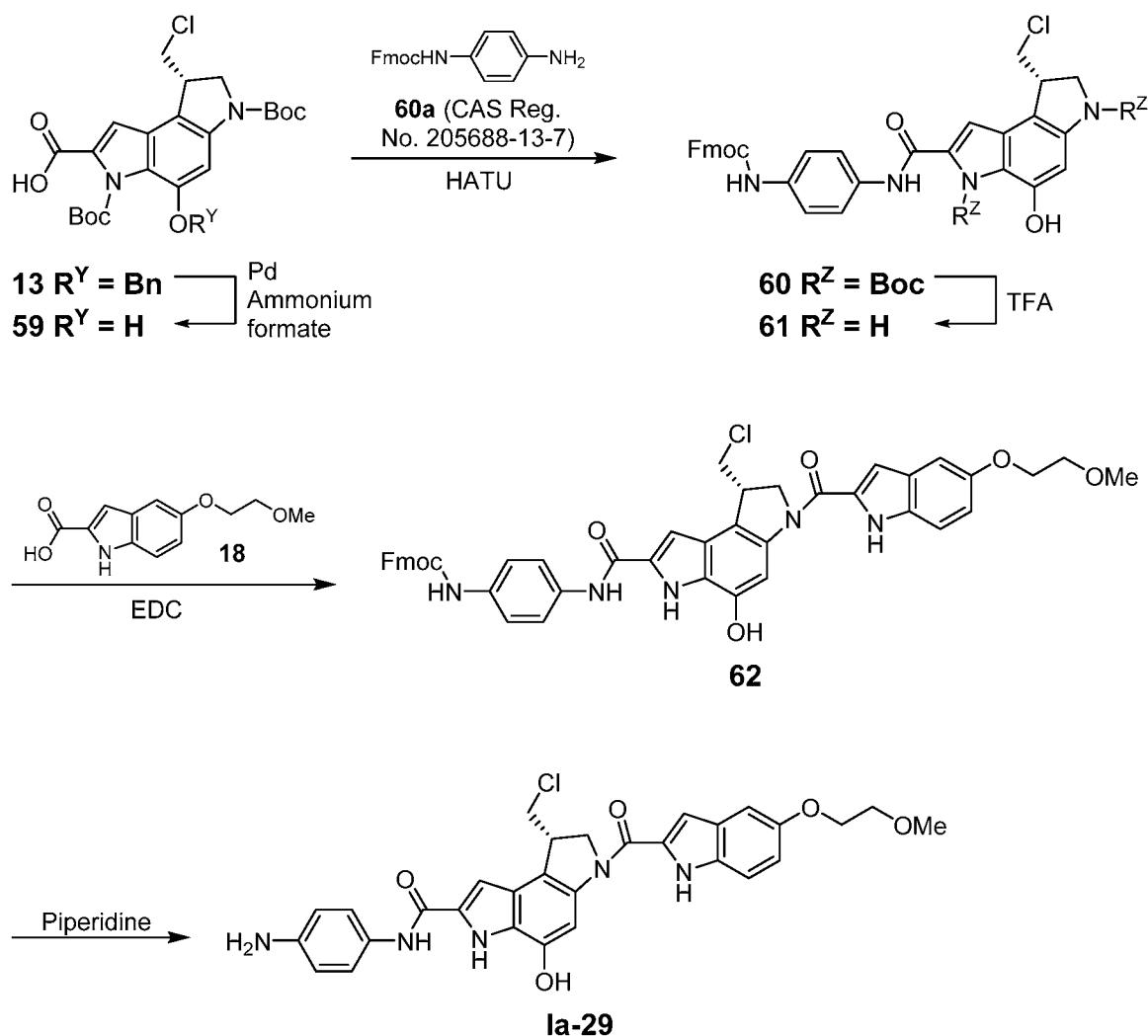
FIG. 11

FIG. 12

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2017/047465

A. CLASSIFICATION OF SUBJECT MATTER		
INV. C07D487/04 C07D519/00 A61K47/68 A61K31/407 A61K31/437 A61P35/00		
ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) C07D A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>MARK S. TICHENOR ET AL: "Systematic Exploration of the Structural Features of Yatakemycin Impacting DNA Alkylation and Biological Activity", JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, vol. 129, no. 35, 1 September 2007 (2007-09-01), pages 10858-10869, XP055042780, ISSN: 0002-7863, DOI: 10.1021/ja072777z Whole document, specially compounds 15a, 15b in p. 10860, or 12f in p. 10861</p> <p style="text-align: center;">-----</p> <p style="text-align: center;">-/-</p>	1-17
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C.		<input checked="" type="checkbox"/> See patent family annex.
<p>* Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier application or patent but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>		
Date of the actual completion of the international search	Date of mailing of the international search report	
18 October 2017	25/10/2017	
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Sahagún Krause, H	

INTERNATIONAL SEARCH REPORT

International application No PCT/US2017/047465

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>MICHAEL J. STEPHENSON ET AL: "Solid-Phase Synthesis of Duocarmycin Analogues and the Effect of C-Terminal Substitution on Biological Activity", THE JOURNAL OF ORGANIC CHEMISTRY, vol. 80, no. 19, 2 October 2015 (2015-10-02), pages 9454-9467, XP055292162, US ISSN: 0022-3263, DOI: 10.1021/acs.joc.5b01373 Whole document, specially compounds 14-18 in p. 9455</p> <p>-----</p>	1-17
A, P	<p>WO 2016/193709 A1 (UNIV OF EAST ANGLIA [GB]) 8 December 2016 (2016-12-08) Claims, compounds 14-18 in p. 62</p> <p>-----</p>	1-17

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2017/047465

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 2016193709	A1 08-12-2016	US 2016347753	A1	01-12-2016
		WO 2016193709	A1	08-12-2016