CHEMICAL LINKERS AND CLEAVABLE SUBSTRATES AND CONJUGATES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority of US Provisional Patent Application No. 60/882,461 filed on December 28, 2006, and US Provisional Patent Application No. 60/991,300, filed November 30, 2007, the benefits of which are hereby claimed under 35 U.S.C. § 119 and the disclosures of which are herein incorporated by reference in their entirety.

FIELD OF THE INVENTION

The present invention provides linkers and cleavable substrates that attach to a drug and a ligand and are cleaved in vivo. The linkers and cleavable substrates are of use in forming prodrugs and conjugates of the cytotoxins of the invention as well as other diagnostic and therapeutic moieties.

BACKGROUND OF THE INVENTION

Many therapeutic agents, particularly those that are especially effective in cancer chemotherapy, often exhibit acute toxicity *in vivo*, especially bone marrow and mucosal toxicity, as well as chronic cardiac and neurological toxicity. Such high toxicity can limit their applications. Development of more and safer specific therapeutic agents, particularly antitumor agents, is desirable for greater effectiveness against tumor cells and a decrease in the number and severity of the side effects of these products (toxicity, destruction of non-tumor cells, etc.). Another difficulty with some existing therapeutic agents is their less than optimal stability in plasma. Addition of functional groups to stabilize these compounds resulted in a significant lowering of the activity. Accordingly, it is desirable to identify ways to stabilize compounds while maintaining acceptable therapeutic activity levels.

The search for more selective cytotoxic agents has been extremely active for many decades, the dose limiting toxicity (i.e. the undesirable activity of the cytotoxins on normal tissues) being one of the major causes of failures in cancer therapy. For example, CC-1065 and the duocarmycins are known to be extremely potent cytotoxins.

CC-1065 was first isolated from *Streptomyces zelensis* in 1981 by the Upjohn Company (Hanka *et al., J. Antibiot.* **31**: 1211 (1978); Martin *et al., J. Antibiot.* **33**: 902 (1980); Martin *et al., J. Antibiot.* **34**: 1119 (1981)) and was found to have potent antitumor and antimicrobial activity both *in vitro* and in experimental animals (Li *et al., Cancer Res.* **42**: 999 (1982)). CC-1065 binds to double-stranded B-DNA within the minor groove (Swenson *et al., Cancer Res.* **42**: 2821 (1982)) with the sequence preference of 5'-d(A/GNTTA)-3' and 5'-d(AAAAA)-3' and alkylates the N3 position of the 3'-adenine by its CPI left-hand unit present in the molecule (Hurley *et al., Science* **226**: 843 (1984)). Despite its potent and broad antitumor activity, CC-1065 cannot be used in humans because it causes delayed death in experimental animals.

Many analogues and derivatives of CC-1065 and the duocarmycins are known in the art. The research into the structure, synthesis and properties of many of the compounds has been reviewed. *See*, for example, Boger *et al., Angew. Chem. Int. Ed. Engl.* **35**: 1438 (1996); and Boger *et al., Chem. Rev.* **97**: 787 (1997).

A group at Kyowa Hakko Kogya Co., Ltd. has prepared a number of CC-1065 derivatives. See, for example, U.S. Pat. No. 5,101, 038; 5,641,780; 5,187,186; 5,070,092; 5,703,080; 5,070,092; 5,641,780; 5,101,038; and 5,084,468; and published PCT application, WO 96/10405 and published European application 0 537 575 A1.

The Upjohn Company (Pharmacia Upjohn) has also been active in preparing derivatives of CC-1065. See, for example, U.S. Patent No. 5,739,350; 4,978,757, 5,332, 837 and 4,912,227.

Research has also focused on the development of new therapeutic agents which are in the form of prodrugs, compounds that are capable of being converted to drugs (active therapeutic compounds) *in vivo* by certain chemical or enzymatic modifications of their structure. For purposes of reducing toxicity, this conversion is preferably confined to the site of action or target tissue rather than the circulatory system or non-target tissue. However, even prodrugs are problematic as many are characterized by a low stability in blood and serum, due to the presence of enzymes that degrade or activate the prodrugs before the prodrugs reach the desired sites within the patient's body.

Bristol-Myers Squibb has described particular lysosomal enzyme-cleavable antitumor drug conjugates. See, for example, U.S. Patent No. 6,214,345. This patent provides an aminobenzyl oxycarbonyl.

Seattle Genetics has published applications U.S. Pat. Appl. 2003/0096743 and U.S. Pat. Appl. 2003/0130189, which describe p-aminobenzylethers in drug delivery agents. The linkers described in these applications are limited to aminobenzyl ether compositions.

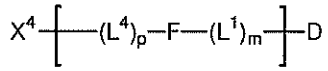
Other groups have also described linkers. See for example de Groot *et al., J. Med. Chem.* 42, 5277 (1999); de Groot *et al. J. Org. Chem.* 43, 3093 (2000); de Groot *et al., J. Med. Chem.* 66, 8815, (2001); WO 02/083180; Carl *et al., J. Med. Chem. Lett.* 24, 479, (1981); Dubowchik *et al., Bioorg & Med. Chem. Lett.* 8, 3347 (1998). These linkers include aminobenzyl ether spacer, elongated electronic cascade and cyclization spacer systems, cyclisation eliminations spacers, such as w-amino aminocarbonyls, and a *p* aminobenzy oxycarbonyl linker.

Stability of cytotoxin drugs, including *in vivo* stability, is still an important issue that needs to be addressed. In addition, the toxicity of many compounds makes them less useful, so compositions that will reduce drug toxicity, such as the formation of a cleaveable prodrug, are needed. Therefore, in spite of the advances in the art, there continues to be a need for the development of improved therapeutic agents for the treatment of mammals, and humans in particular, more specifically cytotoxins that exhibit high specificity of action, reduced toxicity, and improved stability in blood relative to known compounds of similar structure. The instant invention addresses those needs.

SUMMARY OF THE INVENTION

The present invention relates to drug-ligand conjugates, where the drug and ligand are linked through a peptidyl or other linker, and to drug-enzyme cleavable substrate conjugates. These conjugates are potent cytotoxins that can be selectively delivered to a site of action of interest in an active form and then cleaved to release the active drug.

One embodiment is a compound of the formula

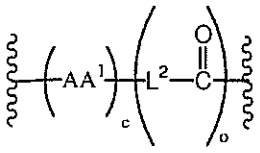


wherein

L^1 is a self-immolative linker;

m is an integer 0, 1, 2, 3, 4, 5, or 6;

F is a linker comprising the structure:

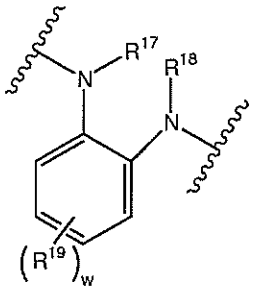


wherein

AA^1 is one or more members independently selected from the group consisting of natural amino acids and unnatural α-amino acids;

c is an integer from 1 to 20;

L^2 is a self-immolative linker and comprises



wherein each R^{17}, R^{18}, and R^{19} is independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl,

and w is an integer from 0 to 4;

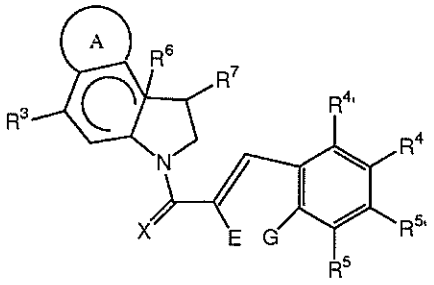
o is 1;

L^4 is a linker member;

p is 0 or 1;

X^4 is a member selected from the group consisting of protected reactive functional groups, unprotected reactive functional groups, detectable labels, and targeting agents; and

D comprises a structure:



wherein the ring system A is a member selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl groups;

E and G are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, a heteroatom, a single bond, or E and G are joined to form a ring system selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl;

X is a member selected from O, S and NR^{23};

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^3 is OR^{11},

wherein R^{11} is a member selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, monophosphates, diphosphates, triphosphates, sulfonates, acyl, $C(O)R^{12}R^{13}$, $C(O)OR^{12}$, $C(O)NR^{12}R^{13}$, $P(O)(OR^{12})_2$, , $C(O)CHR^{12}R^{13}$, SR^{12} and $SiR^{12}R^{13}R^{14}$,

in which R^{12}, R^{13}, and R^{14} are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl, wherein R^{12} and R^{13} together with the nitrogen or carbon atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

R^4 , $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, $CR^{15}{=}NR^{16}$, and $O(CH_2)_nN(CH_3)_2$, or any adjacent pair of R^4, $R^{4'}$, R^5 and $R^{5'}$, together with the carbon atoms to which they are attached, are joined to form a substituted or unsubstituted cycloalkyl or heterocycloalkyl ring system having from 4 to 6 members;

wherein

n is an integer from 1 to 20;

R^{15} and R^{16} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, and substituted or unsubstituted peptidyl, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

R^6 is a single bond which is either present or absent and when present R^6 and R^7 are joined to form a cyclopropyl ring; and

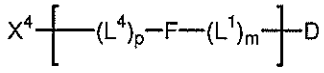
R^7 is CH_2-X^1 or –CH_2- joined in said cyclopropyl ring with R^6, wherein

X^1 is a leaving group,

wherein R^{11} links said drug to L^1, if present, or to F;

or a pharmaceutically acceptable salt thereof.

Another embodiment is a compound of the formula



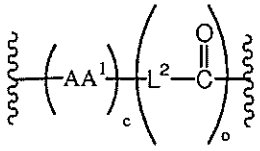
wherein

L^1 is a self-immolative linker;

m is an integer 0, 1, 2, 3, 4, 5, or 6;

F is a linker comprising the structure:

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wherein

AA^1 is one or more members independently selected from the group consisting of natural amino acids and unnatural α-amino acids;

c is an integer from 1 to 20;

L^2 is a self-immolative linker;

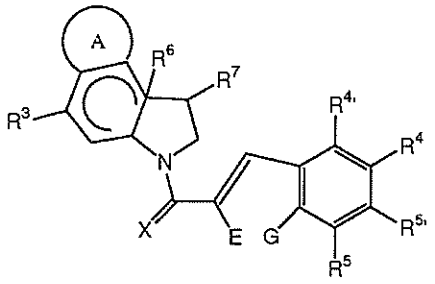
o is 0 or 1;

L^4 is a linker member;

p is 0 or 1;

X^4 is a member selected from the group consisting of protected reactive functional groups, unprotected reactive functional groups, detectable labels, and targeting agents; and

D comprises a structure:



wherein the ring system A is a member selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl groups;

E and G are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, a heteroatom, a single bond, or E and G are joined to form a ring system selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl;

X is a member selected from O, S and NR^{23};

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^3 is a member selected from the group consisting of (=O), SR^{11}, NHR^{11} and OR^{11},

wherein R^{11} is a member selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, monophosphates, diphosphates, triphosphates, sulfonates, acyl, $C(O)R^{12}R^{13}$, $C(O)OR^{12}$, $C(O)NR^{12}R^{13}$, $P(O)(OR^{12})_2$, , $C(O)CHR^{12}R^{13}$, SR^{12} and $SiR^{12}R^{13}R^{14}$,

in which R^{12}, R^{13}, and R^{14} are members independently selected from H, substituted or unsubstituted alkyl, substituted or

unsubstituted heteroalkyl and substituted or unsubstituted aryl, wherein R^{12} and R^{13} together with the nitrogen or carbon atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

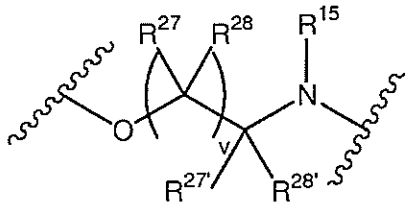
R^4, $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, $CR^{15}{=}NR^{16}$, and $O(CH_2)_nN(CH_3)_2$, or any adjacent pair of R^4, $R^{4'}$, R^5 and $R^{5'}$, together with the carbon atoms to which they are attached, are joined to form a substituted or unsubstituted cycloalkyl or heterocycloalkyl ring system having from 4 to 6 members,

wherein

n is an integer from 1 to 20;

R^{15} and R^{16} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, and substituted or unsubstituted peptidyl, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

wherein at least one of R^4, $R^{4'}$, R^5 and $R^{5'}$ links said drug to L^1, if present, or to F, and comprises



wherein v is an integer from 1 to 6; and

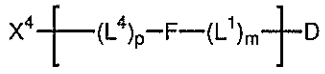
each R^{27}, $R^{27'}$, R^{28}, and $R^{28'}$ is independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, and substituted or unsubstituted heterocycloalkyl;

R^6 is a single bond which is either present or absent and when present R^6 and R^7 are joined to form a cyclopropyl ring; and

R^7 is CH_2-X^1 or –CH_2- joined in said cyclopropyl ring with R^6, wherein X^1 is a leaving group;

or a pharmaceutically acceptable salt thereof.

Yet another embodiment is a compound of the formula

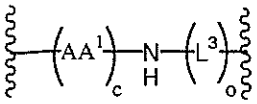


wherein

L^1 is a self-immolative linker;

m is an integer 0, 1, 2, 3, 4, 5, or 6;

F is a linker comprising the structure:



wherein

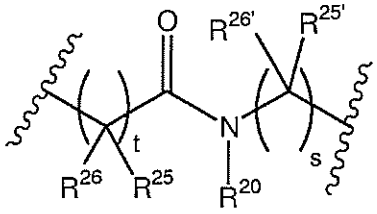
AA^1 is one or more members independently selected from the group consisting of natural amino acids and unnatural α-amino acids;

c is an integer from 1 to 20;

L^3 is a spacer group comprising a primary or secondary amine or a carboxyl functional group; wherein if L^3 is present, m is 0 and either the amine of L^3 forms an amide bond with a pendant carboxyl functional group of D or the carboxyl of L^3 forms an amide bond with a pendant amine functional group of D;

o is 0 or 1;

L^4 is a linker member, wherein L^4 comprises



directly attached to the N-terminus of $(AA^1)_c$, wherein

R^{20} is is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl,

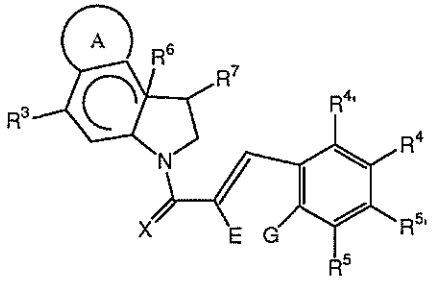
each R^{25}, $R^{25'}$, R^{26}, and $R^{26'}$ is independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, and substituted or unsubstituted heterocycloalkyl;

and s and t are independently integers from 1 to 6;

p is 1;

X^4 is a member selected from the group consisting of protected reactive functional groups, unprotected reactive functional groups, detectable labels, and targeting agents; and

D comprises a structure:



wherein the ring system A is a member selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl groups;

E and G are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, a heteroatom, a single bond, or E and G are joined to form a ring system selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl;

X is a member selected from O, S and NR^{23};

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^3 is a member selected from the group consisting of (=O), SR^{11}, NHR^{11} and OR^{11},

wherein

R^{11} is a member selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, monophosphates, diphosphates, triphosphates, sulfonates, acyl, $C(O)R^{12}R^{13}$, $C(O)OR^{12}$, $C(O)NR^{12}R^{13}$, $P(O)(OR^{12})_2$, , $C(O)CHR^{12}R^{13}$, SR^{12} and $SiR^{12}R^{13}R^{14}$,

in which

R^{12}, R^{13}, and R^{14} are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl, wherein R^{12} and R^{13} together with the nitrogen or carbon atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

R^4 , $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, $CR^{15}{=}NR^{16}$, and $O(CH_2)_nN(CH_3)_2$, or any adjacent pair of R^4, $R^{4'}$, R^5 and $R^{5'}$, together with the carbon atoms to which they are attached, are joined to form a substituted or unsubstituted cycloalkyl or heterocycloalkyl ring system having from 4 to 6 members,

wherein

n is an integer from 1 to 20;

R^{15} and R^{16} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or

unsubstituted heterocycloalkyl, and substituted or unsubstituted peptidyl, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

R^6 is a single bond which is either present or absent and when present R^6 and R^7 are joined to form a cyclopropyl ring; and

R^7 is CH_2-X^1 or –CH_2- joined in said cyclopropyl ring with R^6, wherein

X^1 is a leaving group,

wherein at least one of R^4 , $R^{4'}$, R^5, $R^{5'}$, R^{15} or R^{16} links said drug to L^1, if present, or to F;

or a pharmaceutically acceptable salt thereof.

A further embodiment is a compound having the following structure:

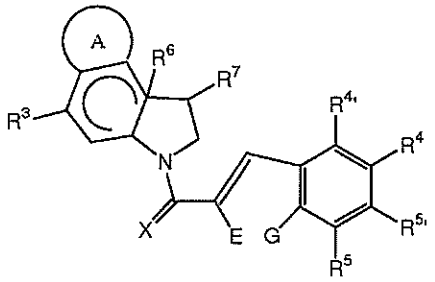
$$X^2\text{—}(L^1)_m\text{—}D$$

wherein L^1 is a self-immolative spacer;

m is an integer of 0, 1, 2, 3, 4, 5, or 6;

X^2 is a cleavable substrate; and

D comprises a structure:



wherein the ring system A is a member selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl groups;

E and G are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, a heteroatom, a single bond, or E and G are joined to form a ring system selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl;

X is a member selected from O, S and NR^{23};

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^3 is a member selected from the group consisting of (=O), SR^{11}, NHR^{11} and OR^{11},

wherein

R^{11} is a member selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, monophosphates, diphosphates, triphosphates, sulfonates, acyl, $C(O)R^{12}R^{13}$, $C(O)OR^{12}$, $C(O)NR^{12}R^{13}$, $P(O)(OR^{12})_2$, , $C(O)CHR^{12}R^{13}$, SR^{12} and $SiR^{12}R^{13}R^{14}$,

in which

R^{12}, R^{13}, and R^{14} are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl, wherein R^{12} and R^{13} together with the nitrogen or carbon atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

R^6 is a single bond which is either present or absent and when present R^6 and R^7 are joined to form a cyclopropyl ring; and

R^7 is CH_2-X^1 or $-CH_2$- joined in said cyclopropyl ring with R^6, wherein

X^1 is a leaving group

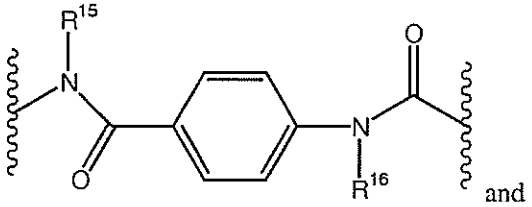
R^4 , $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, CR^{15}=NR^{16}, and $O(CH_2)_nN(CH_3)_2$, or any adjacent pair of R^4, $R^{4'}$, R^5 and $R^{5'}$, together with the carbon atoms to which they are attached, are joined to form a substituted or unsubstituted cycloalkyl or heterocycloalkyl ring system having from 4 to 6 members,

wherein

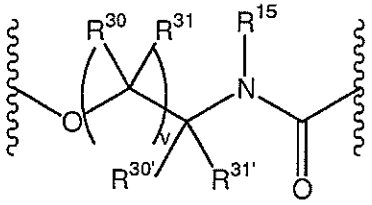
n is an integer from 1 to 20;

R^{15} and R^{16} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, and substituted or unsubstituted peptidyl, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

wherein at least one of members R^4 , $R^{4'}$, R^5 and $R^{5'}$ links said drug to L^1, if present, or to X^2, and is selected from the group consisting of



and



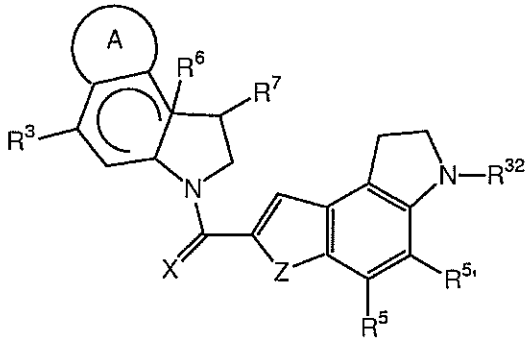
wherein

R^{30}, $R^{30'}$, R^{31}, and $R^{31'}$ are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, and substituted or unsubstituted heterocycloalkyl; and

v is an integer from 1 to 6;

or a pharmaceutically acceptable salt thereof.

Another embodiment is a compound having the following structure:



wherein the ring system A is a member selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl groups;

Z and X are independently members selected from O, S and NR^{23};

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^3 is a member selected from the group consisting of (=O), SR^{11}, NHR^{11} and OR^{11},

R^{11} is a member selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, monophosphates, diphosphates, triphosphates, sulfonates, acyl, $C(O)R^{12}R^{13}$, $C(O)OR^{12}$, $C(O)NR^{12}R^{13}$, $P(O)(OR^{12})_2$, , $C(O)CHR^{12}R^{13}$, SR^{12} and $SiR^{12}R^{13}R^{14}$,

in which

R^{12}, R^{13}, and R^{14} are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl, wherein R^{12} and R^{13} together with the nitrogen or carbon atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

R^6 is a single bond which is either present or absent and when present R^6 and R^7 are joined to form a cyclopropyl ring;

R^7 is CH_2-X^1 or –CH_2- joined in said cyclopropyl ring with R^6, wherein

X^1 is a leaving group; and

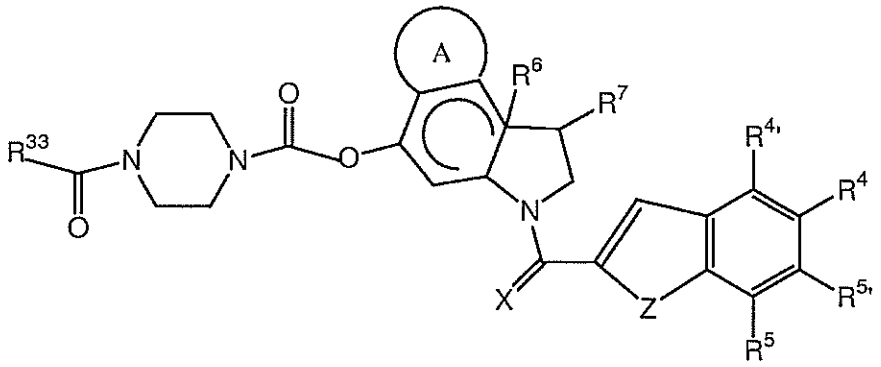
R^5, $R^{5'}$, and R^{32} are members independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, CR^{15}=NR^{16}, and $O(CH_2)_nN(CH_3)_2$

n is an integer from 1 to 20;

R^{15} and R^{16} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, and substituted or unsubstituted peptidyl, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

or a pharmaceutically acceptable salt thereof.

A further embodiment is a compound having the following structure:



wherein the ring system A is a member selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl groups;

Z and X are independently members selected from O, S and NR^{23};

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^4, $R^{4'}$, R^5, $R^{5'}$, and R^{33} are members independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, CR^{15}=NR^{16}, and $O(CH_2)_nN(CH_3)_2$, or any adjacent pair of R^4, $R^{4'}$, R^5 and $R^{5'}$, together with the carbon atoms to which they are attached, are joined to form a substituted or unsubstituted cycloalkyl or heterocycloalkyl ring system having from 4 to 6 members;

wherein

n is an integer from 1 to 20;

R^{15} and R^{16} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, and substituted or unsubstituted peptidyl, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms,

wherein R^{33} comprises one or more cleaveable groups;

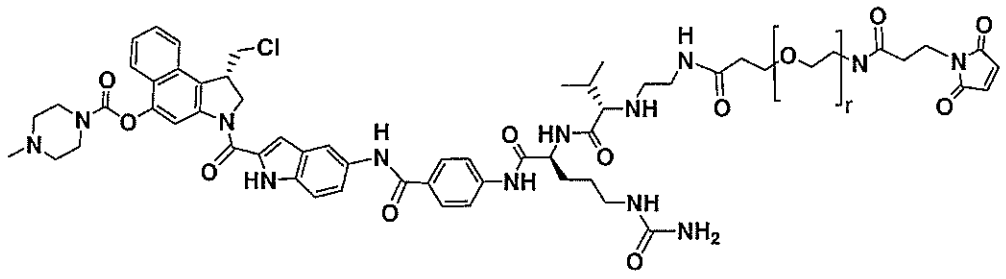
R^6 is a single bond which is either present or absent and when present R^6 and R^7 are joined to form a cyclopropyl ring; and

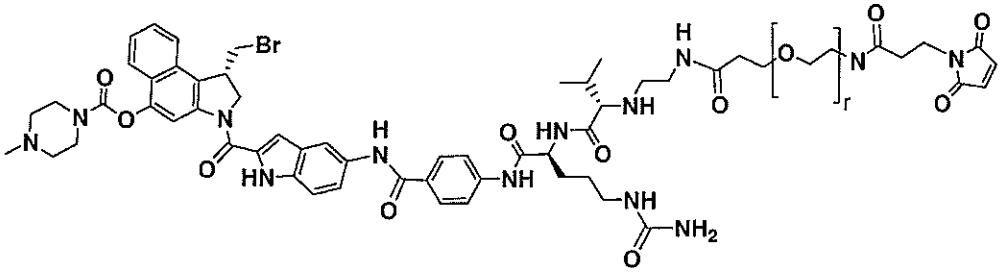
R^7 is CH_2-X^1 or –CH_2- joined in said cyclopropyl ring with R^6, wherein

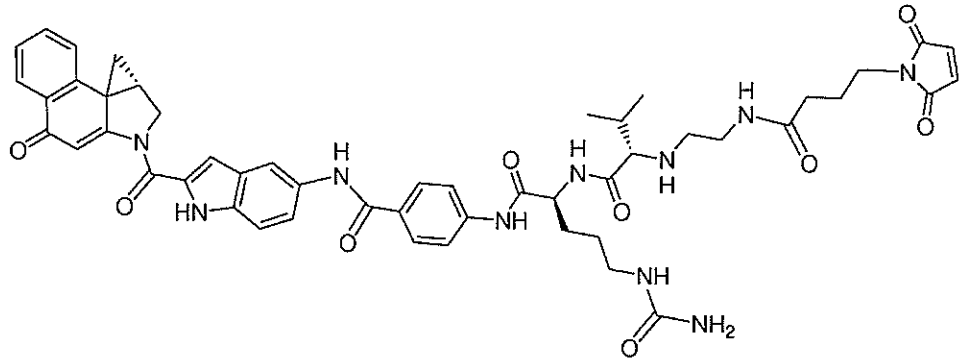
X^1 is a leaving group;

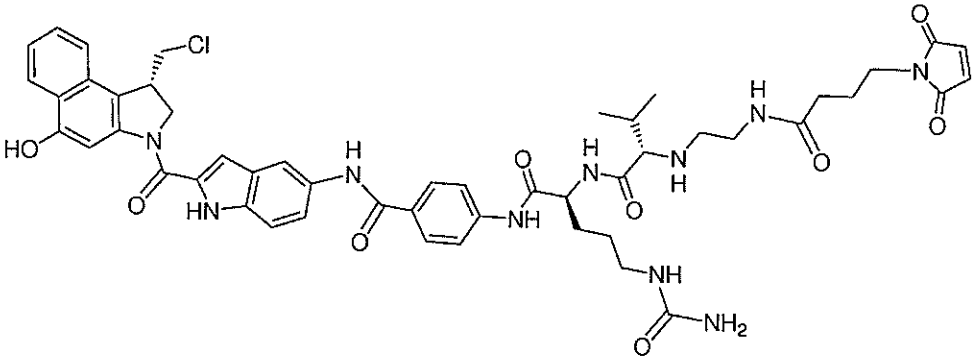
or a pharmaceutically acceptable salt thereof.

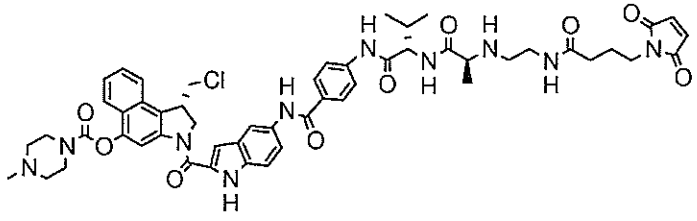
Another embodiment is a compound selected from:

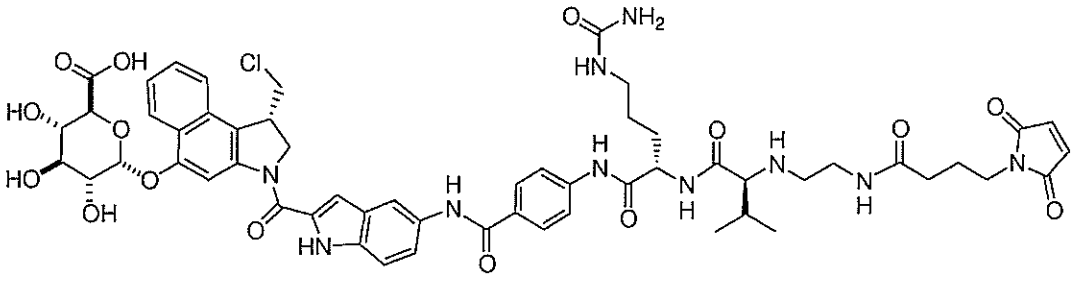




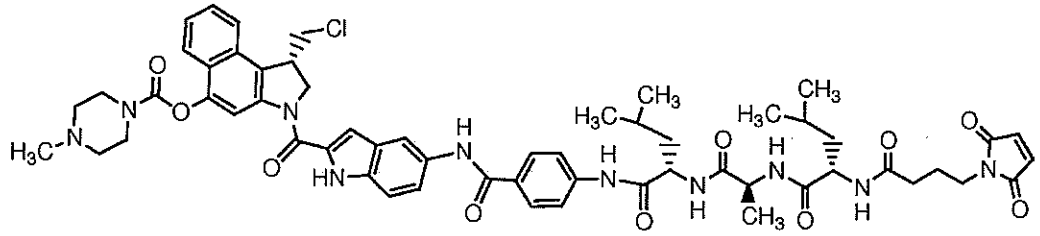


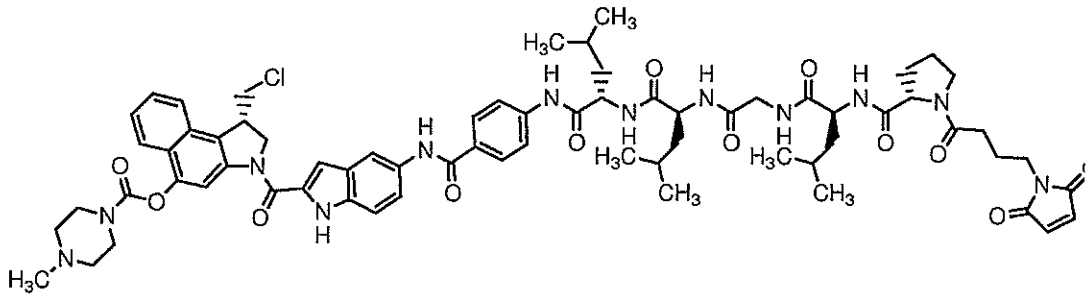


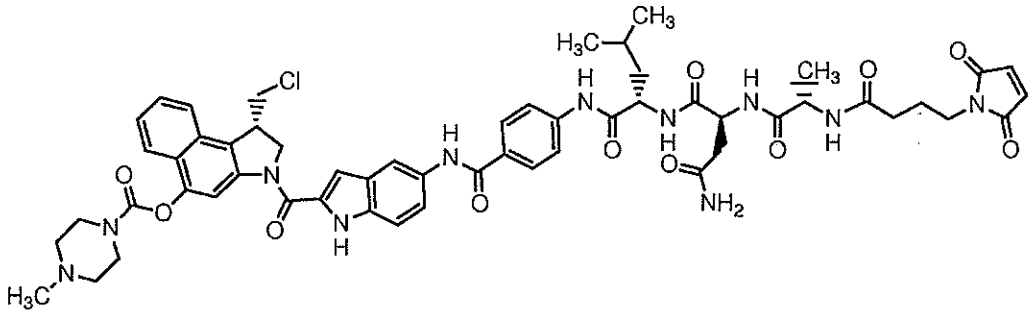




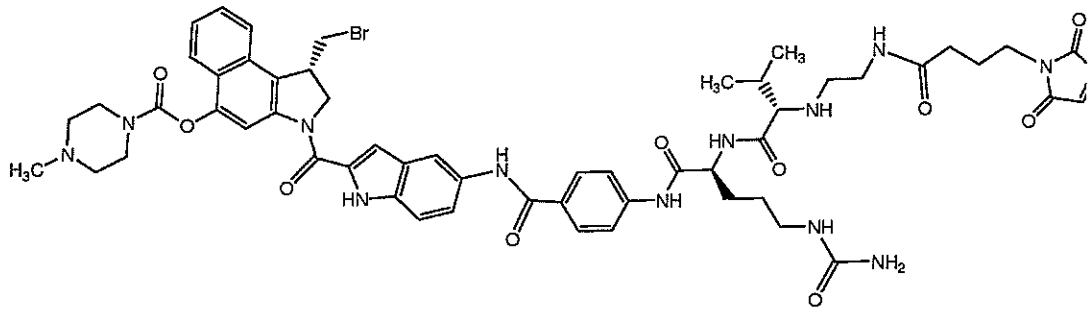
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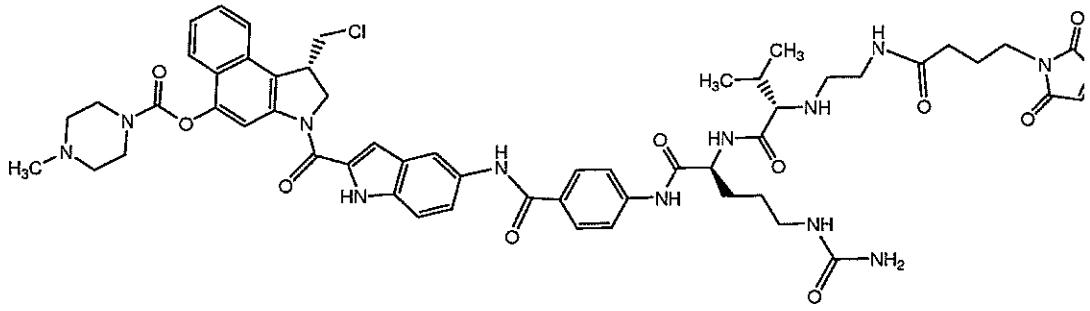


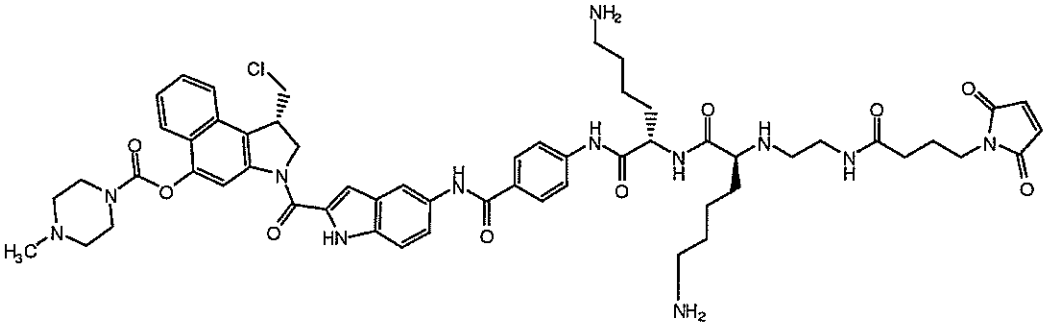


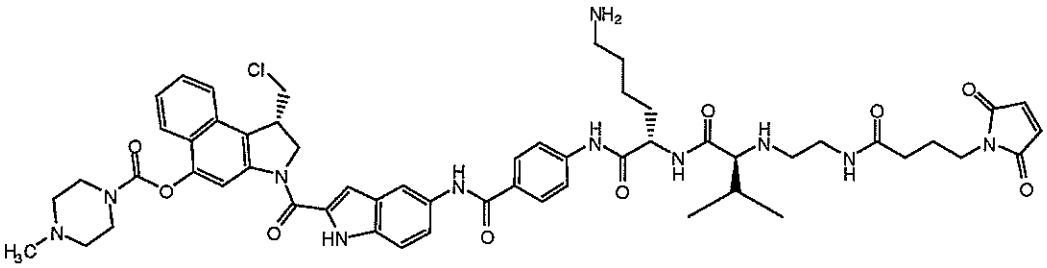
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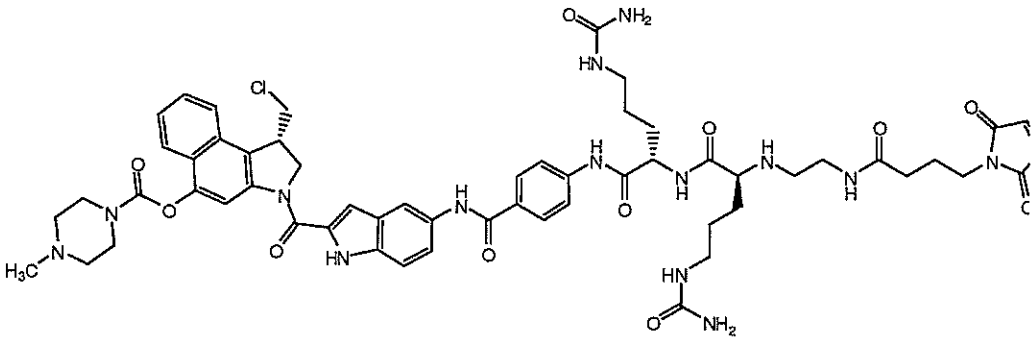


and









wherein r is an integer in the range from 0 to 24.

Any of these compounds can be used as, or used to form, drug-ligand conjugates.

In yet another aspect, the invention pertains to pharmaceutical formulations. Such formulations typically comprise a conjugate compound of the invention and a pharmaceutically acceptable carrier.

In still a further aspect, the invention pertains to methods of using the conjugate compounds of the invention. For example, the invention provides a method of killing a cell, wherein a conjugate compound of the invention is administered to the cell an amount sufficient to kill the cell. In a preferred embodiment, the cell is a tumor cell. In another embodiment, the invention provides a method of retarding or stopping the growth of a tumor in a mammalian subject, wherein a conjugate compound of the invention is administered to the subject an amount sufficient to retard or stop growth of the tumor.

Other aspects, advantages and objects of the invention will be apparent from review of the detailed description below.

BRIEF DESCRIPTION OF THE DRAWINGS

Non-limiting and non-exhaustive embodiments of the present invention are described with reference to the following drawings. In the drawings, like reference numerals refer to like parts throughout the various figures unless otherwise specified.

For a better understanding of the present invention, reference will be made to the following Detailed Description, which is to be read in association with the accompanying drawings, wherein:

FIGS. 1-3 are graphs of mean tumor volume, median tumor volume, and median % body weight change, respectively, versus days past dosing for a first *in vivo* study;

FIGS. 4-6 are graphs of mean tumor volume, median tumor volume, and median % body weight change, respectively, versus days past dosing for a second *in vivo* study;

FIG. 7 contains logistic curves fitted for various antibody-toxin conjugates and the resulting EC_{50} values, in LNCaP and 786-O cells;

FIG. 8 contains graphs of mean tumor volume versus days past dosing for a third *in vivo* study;

FIG. 9 contains graphs of median body weight change versus days past dosing for the third *in vivo* study;

FIG. 10 contains graphs of mean tumor volume versus days past dosing for a fourth *in vivo* study;

FIG. 11 contains graphs of median body weight change versus days past dosing for the fourth *in vivo* study;

FIG. 12 is a graph of mean tumor volume versus days past dosing for a fifth *in vivo* study;

FIG. 13 is a graph of mean tumor volume versus days past dosing for a sixth *in vivo* study;

FIG. 14 is a graph of mean tumor volume versus days past dosing for a seventh *in vivo* study;

FIG. 15 is a graph of mean tumor volume versus days past dosing for a eighth *in vivo* study; and

FIG. 16 is a graph of mean tumor volume versus days past dosing for a ninth *in vivo* study.

DETAILED DESCRIPTION OF THE INVENTION

Abbreviations

"Ala," refers to alanine.

"Boc," refers to *t*-butyloxycarbonyl.

"CPI," refers to cyclopropapyrroloindole.

"Cbz," refers to carbobenzoxy.

"DCM" refers to dichloromethane.

"DDQ" refers to 2,3-dichloro-5,6-dicyano-1,4-benzoquinone.

"DIPEA" refers to diisopropylethalamine

"DMDA" refers to N,N'-dimethylethylene diamine

"RBF" refers to a round bottom flask

"DMF" refers to N,B-dimethylformamide

"HATU" refers to N-[[(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridin-1-yl]methylene]-N-methylmethanaminium hexafluorophosphate N-oxide

The symbol "E," represents an enzymatically cleaveable group.

"EDCI" refers to 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide.

"FMOC" refers to 9-fluorenylmethyloxycarbonyl.

"HOAt" refers to 7-aza-1-hydroxybenzotriazole.

"Leu" refers to leucine.

"PABA" refers to *para*-aminobenzoic acid.

"PEG" refers to polyethylene glycol.

"DBU" refers to 1,8-diazabicyclo(5.4.0)undec-7-ene.

"DIEA" refers to diisopropylethyl amine.

"PMB" refers to *para*-methoxybenzyl.

"TBAF," refers to tetrabutylammonium fluoride.

"TBSO," refers to *t*-butyldimethylsilyl ether.

"TEA" refers to triethylamine.

"TFA" refers to trifluororoacetic acid.

"EDC" refers to (1-Ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride)

"TBTU" refers to (2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate)

"HOBT" refers to N-Hydroxybenzotriazole

The symbol "Q" refers to a therapeutic agent, diagnostic agent or detectable label.

Definitions

Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, organic chemistry and nucleic acid chemistry and hybridization described below are those well known and commonly employed in the art. Standard techniques are used for nucleic acid and peptide synthesis. Generally, enzymatic reactions and purification steps are performed according to the manufacturer's specifications. The techniques and procedures are generally performed according to conventional methods in the art and various general references (*see generally*, Sambrook *et al.* MOLECULAR CLONING: A LABORATORY MANUAL, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., which is incorporated herein by reference), which are provided throughout this document. The nomenclature used herein and the laboratory procedures in analytical chemistry, and organic synthetic described below are those well known and commonly employed in the art. Standard techniques, or modifications thereof, are used for chemical syntheses and chemical analyses.

The term "therapeutic agent" is intended to mean a compound that, when present in a therapeutically effective amount, produces a desired therapeutic effect on a mammal. For treating carcinomas, it is desirable that the therapeutic agent also be capable of entering the target cell.

The term "cytotoxin" is intended to mean a therapeutic agent having the desired effect of being cytotoxic to cancer cells. Cytotoxic means that the agent arrests the growth of, or kills the cells. Exemplary cytotoxins include, by way of example and not limitation, combretastatins, duocarmycins, the CC-1065 anti-tumor antibiotics, anthracyclines, and related compounds. Other cytotoxins include mycotoxins, ricin and its analogues, calicheamycins, doxirubicin and maytansinoids.

The term "prodrug" and the term "drug conjugate" are used herein interchangeably. Both refer to a compound that is relatively innocuous to cells while still in the conjugated form but which is selectively degraded to a pharmacologically active form by conditions, e.g., enzymes, located within or in the proximity of target cells.

The term "marker" is intended to mean a compound useful in the characterization of tumors or other medical condition, for example, diagnosis, progression of a tumor, and assay of the factors secreted by tumor cells. Markers are considered a subset of "diagnostic agents."

The term "selective" as used in connection with enzymatic cleavage means that the rate of rate of cleavage of the linker moiety is greater than the rate of cleavage of a peptide having a random sequence of amino acids.

The terms "targeting group" and "targeting agent" are intended to mean a moiety that is (1) able to direct the entity to which it is attached (e.g., therapeutic agent or marker) to a target cell, for example to a specific type of tumor cell or (2) is preferentially activated at a target tissue, for example a tumor. The targeting group or targeting agent can be a small molecule, which is intended to include both non-peptides and peptides. The targeting group can also be a macromolecule, which includes saccharides, lectins, receptors, ligand for receptors, proteins such as BSA, antibodies, and so forth. In a preferred embodiment of the current invention, the targeting group is an antibody or an antibody fragment, more preferably a monoclonal antibody or monoclonal antibody fragment

The term "self-immolative spacer" refers to a bifunctional chemical moiety that is capable of covalently linking two chemical moieties into a normally stable tripartate

molecule. The self-immolative spacer is capable of spontaneously separating from the second moiety if the bond to the first moiety is cleaved.

The term "detectable label" is intended to mean a moiety having a detectable physical or chemical property.

The term "cleaveable group" is intended to mean a moiety that is unstable *in vivo*. Preferably the "cleaveable group" allows for activation of the marker or therapeutic agent by cleaving the marker or agent from the rest of the conjugate. Operatively defined, the linker is preferably cleaved *in vivo* by the biological environment. The cleavage may come from any process without limitation, e.g., enzymatic, reductive, pH, etc. Preferably, the cleaveable group is selected so that activation occurs at the desired site of action, which can be a site in or near the target cells (e.g., carcinoma cells) or tissues such as at the site of therapeutic action or marker activity. Such cleavage may be enzymatic and exemplary enzymatically cleaveable groups include natural amino acids or peptide sequences that end with a natural amino acid, and are attached at their carboxyl terminus to the linker. While the degree of cleavage rate enhancement is not critical to the invention, preferred examples of cleaveable linkers are those in which at least about 10% of the cleaveable groups are cleaved in the blood stream within 24 hours of administration, most preferably at least about 35%.

The term "ligand" means any molecule that specifically binds or reactively associates or complexes with a receptor, substrate, antigenic determinant, or other binding site on a target cell or tissue. Examples of ligands include antibodies and fragments thereof (e.g., a monoclonal antibody or fragment thereof), enzymes (e.g., fibrinolytic enzymes), biologic response modifiers (e.g., interleukins, interferons, erythropeoitin, or colony stimulating factors), peptide hormones, and antigen-binding fragments thereof.

The term "cyclization reaction," when referring to the cyclization of a peptide, hydrazine, or disulfide linker, indicates the cyclization of that linker into a ring and initiates the separation of the drug-ligand complex. This rate can be measured *ex situ*, and is completed when at least 90%, 95%, or 100% of the product is formed.

The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino

acid polymers and non-naturally occurring amino acid polymer. These terms also encompass the term "antibody."

The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. One amino acid that may be used in particular is citrulline, which is a precursor to arginine and is involved in the formation of urea in the liver. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but functions in a manner similar to a naturally occurring amino acid. The term "unnatural amino acid" is intended to represent the "D" stereochemical form of the twenty naturally occurring amino acids described above. It is further understood that the term unnatural amino acid includes homologues of the natural amino acids, and synthetically modified forms of the natural amino acids. The synthetically modified forms include, but are not limited to, amino acids having alkylene chains shortened or lengthened by up to two carbon atoms, amino acids comprising optionally substituted aryl groups, and amino acids comprised halogenated groups, preferably halogenated alkyl and aryl groups. When attached to a linker or conjugate of the invention, the amino acid is in the form of an "amino acid side chain", where the carboxylic acid group of the amino acid has been replaced with a keto (C(O)) group. Thus, for example, an alanine side chain is -C(O)-CH(NH_2)-CH_3, and so forth.

Amino acids and peptides may be protected by blocking groups. A blocking group is an atom or a chemical moiety that protects the N-terminus of an amino acid or a peptide from undesired reactions and can be used during the synthesis of a drug-ligand conjugate. It should remain attached to the N-terminus throughout the synthesis, and may be removed after completion of synthesis of the drug conjugate by chemical or other conditions that selectively achieve its removal. The blocking groups suitable for N-

terminus protection are well known in the art of peptide chemistry. Exemplary blocking groups include, but are not limited to, hydrogen, D-amino acid, and carbobenzoxy (Cbz) chloride.

"Nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs).

Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.* **19**:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.* **260**: 2605-2608 (1985); Rossolini *et al.*, *Mol. Cell. Probes* **8**: 91-98 (1994)). The term nucleic acid is used interchangeably with gene, cDNA, mRNA, oligonucleotide, and polynucleotide.

The symbol $\sim\!\!\sim\!\!\sim$, whether utilized as a bond or displayed perpendicular to a bond indicates the point at which the displayed moiety is attached to the remainder of the molecule, solid support, etc.

The term "alkyl," by itself or as part of another substituent, means, unless otherwise stated, a straight or branched chain, or cyclic hydrocarbon radical, or combination thereof, which may be fully saturated, mono- or polyunsaturated and can include di- and multivalent radicals, having the number of carbon atoms designated (*i.e.* C_1-C_{10} means one to ten carbons). Examples of saturated hydrocarbon radicals include, but are not limited to, groups such as methyl, ethyl, n-propyl, isopropyl, n-butyl, t-butyl, isobutyl, sec-butyl, cyclohexyl, (cyclohexyl)methyl, cyclopropylmethyl, homologs and isomers of, for example, n-pentyl, n-hexyl, n-heptyl, n-octyl, and the like. An unsaturated alkyl group is one having one or more double bonds or triple bonds. Examples of unsaturated alkyl groups include, but are not limited to, vinyl, 2-propenyl, crotyl, 2-

isopentenyl, 2-(butadienyl), 2,4-pentadienyl, 3-(1,4-pentadienyl), ethynyl, 1- and 3-propynyl, 3-butynyl, and the higher homologs and isomers. The term "alkyl," unless otherwise noted, is also meant to include those derivatives of alkyl defined in more detail below, such as "heteroalkyl." Alkyl groups, which are limited to hydrocarbon groups are termed "homoalkyl".

The term "alkylene" by itself or as part of another substituent means a divalent radical derived from an alkane, as exemplified, but not limited, by $-CH_2CH_2CH_2CH_2-$, and further includes those groups described below as "heteroalkylene." Typically, an alkyl (or alkylene) group will have from 1 to 24 carbon atoms, with those groups having 10 or fewer carbon atoms being preferred in the present invention. A "lower alkyl" or "lower alkylene" is a shorter chain alkyl or alkylene group, generally having eight or fewer carbon atoms.

The term "heteroalkyl," by itself or in combination with another term, means, unless otherwise stated, a stable straight or branched chain, or cyclic hydrocarbon radical, or combinations thereof, consisting of the stated number of carbon atoms and at least one heteroatom selected from the group consisting of O, N, Si, and S, and wherein the nitrogen, carbon and sulfur atoms may optionally be oxidized and the nitrogen heteroatom may optionally be quaternized. The heteroatom(s) O, N, S, and Si may be placed at any interior position of the heteroalkyl group or at the position at which the alkyl group is attached to the remainder of the molecule. Examples include, but are not limited to, $-CH_2-CH_2-O-CH_3$, $-CH_2-CH_2-NH-CH_3$, $-CH_2-CH_2-N(CH_3)-CH_3$, $-CH_2-S-CH_2-CH_3$, $-CH_2-CH_2,-S(O)-CH_3$, $-CH_2-CH_2-S(O)_2-CH_3$, $-CH=CH-O-CH_3$, $-Si(CH_3)_3$, $-CH_2-CH=N-OCH_3$, and $-CH=CH-N(CH_3)-CH_3$. Up to two heteroatoms may be consecutive, such as, for example, $-CH_2-NH-OCH_3$ and $-CH_2-O-Si(CH_3)_3$. Similarly, the term "heteroalkylene" by itself or as part of another substituent means a divalent radical derived from heteroalkyl, as exemplified, but not limited by, $-CH_2-CH_2-S-CH_2-CH_2-$ and $-CH_2-S-CH_2-CH_2-NH-CH_2-$. For heteroalkylene groups, heteroatoms can also occupy either or both of the chain termini (*e.g.*, alkyleneoxy, alkylenedioxy, alkyleneamino, alkylenediamino, and the like). The terms "heteroalkyl" and "heteroalkylene" encompass poly(ethylene glycol) and its derivatives (see, for example, Shearwater Polymers Catalog, 2001). Still further, for alkylene and heteroalkylene linking groups, no orientation of the linking group is implied by the direction in which the formula of the linking group is written. For example, the formula $-C(O)_2R'-$ represents both $-C(O)_2R'-$ and $-R'C(O)_2-$.

The term "lower" in combination with the terms "alkyl" or "heteroalkyl" refers to a moiety having from 1 to 6 carbon atoms.

The terms "alkoxy," "alkylamino," "alkylsulfonyl," and "alkylthio" (or thioalkoxy) are used in their conventional sense, and refer to those alkyl groups attached to the remainder of the molecule via an oxygen atom, an amino group, an SO_2 group or a sulfur atom, respectively. The term "arylsulfonyl" refers to an aryl group attached to the remainder ofhte molecule via an SO_2 group, and the term "sulfhydryl" refers to an SH group.

In general, an "acyl substituent" is also selected from the group set forth above. As used herein, the term "acyl substituent" refers to groups attached to, and fulfilling the valence of a carbonyl carbon that is either directly or indirectly attached to the polycyclic nucleus of the compounds of the present invention.

The terms "cycloalkyl" and "heterocycloalkyl", by themselves or in combination with other terms, represent, unless otherwise stated, cyclic versions of substituted or unsubstituted "alkyl" and substituted or unsubstituted "heteroalkyl", respectively. Additionally, for heterocycloalkyl, a heteroatom can occupy the position at which the heterocycle is attached to the remainder of the molecule. Examples of cycloalkyl include, but are not limited to, cyclopentyl, cyclohexyl, 1-cyclohexenyl, 3-cyclohexenyl, cycloheptyl, and the like. Examples of heterocycloalkyl include, but are not limited to, 1 –(1,2,5,6-tetrahydropyridyl), 1-piperidinyl, 2-piperidinyl, 3-piperidinyl, 4-morpholinyl, 3-morpholinyl, tetrahydrofuran-2-yl, tetrahydrofuran-3-yl, tetrahydrothien-2-yl, tetrahydrothien-3-yl, 1 –piperazinyl, 2-piperazinyl, and the like. The heteroatoms and carbon atoms of the cyclic structures are optionally oxidized.

The terms "halo" or "halogen," by themselves or as part of another substituent, mean, unless otherwise stated, a fluorine, chlorine, bromine, or iodine atom. Additionally, terms such as "haloalkyl," are meant to include monohaloalkyl and polyhaloalkyl. For example, the term "halo(C_1-C_4)alkyl" is mean to include, but not be limited to, trifluoromethyl, 2,2,2-trifluoroethyl, 4-chlorobutyl, 3-bromopropyl, and the like.

The term "aryl" means, unless otherwise stated, a substituted or unsubstituted polyunsaturated, aromatic, hydrocarbon substituent which can be a single ring or multiple rings (preferably from 1 to 3 rings) which are fused together or linked covalently. The term "heteroaryl" refers to aryl groups (or rings) that contain from one to four

heteroatoms selected from N, O, and S, wherein the nitrogen, carbon and sulfur atoms are optionally oxidized, and the nitrogen atom(s) are optionally quaternized. A heteroaryl group can be attached to the remainder of the molecule through a heteroatom. Non-limiting examples of aryl and heteroaryl groups include phenyl, 1-naphthyl, 2-naphthyl, 4-biphenyl, 1-pyrrolyl, 2-pyrrolyl, 3-pyrrolyl, 3-pyrazolyl, 2-imidazolyl, 4-imidazolyl, pyrazinyl, 2-oxazolyl, 4-oxazolyl, 2-phenyl-4-oxazolyl, 5-oxazolyl, 3-isoxazolyl, 4-isoxazolyl, 5-isoxazolyl, 2-thiazolyl, 4-thiazolyl, 5-thiazolyl, 2-furyl, 3-furyl, 2-thienyl, 3-thienyl, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-pyrimidyl, 4-pyrimidyl, 5-benzothiazolyl, purinyl, 2-benzimidazolyl, 5-indolyl, 1-isoquinolyl, 5-isoquinolyl, 2-quinoxalinyl, 5-quinoxalinyl, 3-quinolyl, and 6-quinolyl. Substituents for each of the above noted aryl and heteroaryl ring systems are selected from the group of acceptable substituents described below. "Aryl" and "heteroaryl" also encompass ring systems in which one or more non-aromatic ring systems are fused, or otherwise bound, to an aryl or heteroaryl system.

For brevity, the term "aryl" when used in combination with other terms (*e.g.*, aryloxy, arylthioxy, arylalkyl) includes both aryl and heteroaryl rings as defined above. Thus, the term "arylalkyl" is meant to include those radicals in which an aryl group is attached to an alkyl group (*e.g.*, benzyl, phenethyl, pyridylmethyl and the like) including those alkyl groups in which a carbon atom (*e.g.*, a methylene group) has been replaced by, for example, an oxygen atom (*e.g.*, phenoxymethyl, 2-pyridyloxymethyl, 3-(1-naphthyloxy)propyl, and the like).

Each of the above terms (*e.g.*, "alkyl," "heteroalkyl," "aryl" and "heteroaryl") include both substituted and unsubstituted forms of the indicated radical. Preferred substituents for each type of radical are provided below.

Substituents for the alkyl, and heteroalkyl radicals (including those groups often referred to as alkylene, alkenyl, heteroalkylene, heteroalkenyl, alkynyl, cycloalkyl, heterocycloalkyl, cycloalkenyl, and heterocycloalkenyl) are generally referred to as "alkyl substituents" and "heteroalkyl substituents," respectively, and they can be one or more of a variety of groups selected from, but not limited to: -OR', =O, =NR', =N-OR', -NR'R", -SR', -halogen, -SiR'R"R"', -OC(O)R', -C(O)R', -CO_2R', -CONR'R", -OC(O)NR'R", -NR"C(O)R', -NR'-C(O)NR"R"', -NR"$C(O)_2$R', -NR-C(NR'R"R"')=NR"", -NR-C(NR'R")=NR"', -S(O)R', -$S(O)_2$R', -$S(O)_2$NR'R", -$NRSO_2$R', -CN and -NO_2 in a number ranging from zero to (2m'+1), where m' is the total number of carbon atoms in

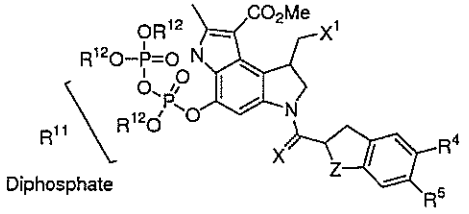
such radical. R', R", R"' and R"" each preferably independently refer to hydrogen, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, e.g., aryl substituted with 1-3 halogens, substituted or unsubstituted alkyl, alkoxy or thioalkoxy groups, or arylalkyl groups. When a compound of the invention includes more than one R group, for example, each of the R groups is independently selected as are each R', R", R"' and R"" groups when more than one of these groups is present. When R' and R" are attached to the same nitrogen atom, they can be combined with the nitrogen atom to form a 5-, 6-, or 7-membered ring. For example, -NR'R" is meant to include, but not be limited to, 1-pyrrolidinyl and 4-morpholinyl. From the above discussion of substituents, one of skill in the art will understand that the term "alkyl" is meant to include groups including carbon atoms bound to groups other than hydrogen groups, such as haloalkyl (*e.g.*, $-CF_3$ and $-CH_2CF_3$) and acyl (*e.g.*, $-C(O)CH_3$, $-C(O)CF_3$, $-C(O)CH_2OCH_3$, and the like).

Similar to the substituents described for the alkyl radical, the aryl substituents and heteroaryl substituents are generally referred to as "aryl substituents" and "heteroaryl substituents," respectively and are varied and selected from, for example: halogen, -OR', =O, =NR', =N-OR', -NR'R", -SR', -halogen, -SiR'R"R"', -OC(O)R', -C(O)R', $-CO_2R'$, -CONR'R", -OC(O)NR'R", -NR"C(O)R', -NR'-C(O)NR"R"', $-NR"C(O)_2R'$, -NR-C(NR'R")=NR"', -S(O)R', $-S(O)_2R'$, $-S(O)_2NR'R"$, $-NRSO_2R'$, -CN and $-NO_2$, -R', $-N_3$, $-CH(Ph)_2$, fluoro(C_1-C_4)alkoxy, and fluoro(C_1-C_4)alkyl, in a number ranging from zero to the total number of open valences on the aromatic ring system; and where R', R", R"' and R"" are preferably independently selected from hydrogen, (C_1-C_8)alkyl and heteroalkyl, unsubstituted aryl and heteroaryl, (unsubstituted aryl)-(C_1-C_4)alkyl, and (unsubstituted aryl)oxy-(C_1-C_4)alkyl. When a compound of the invention includes more than one R group, for example, each of the R groups is independently selected as are each R', R", R"' and R"" groups when more than one of these groups is present.

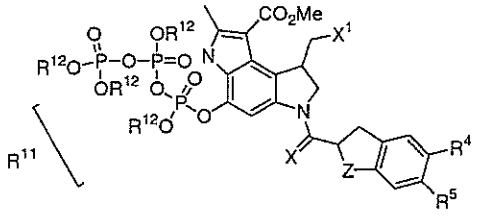
Two of the aryl substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula $-T-C(O)-(CRR')_q-U-$, wherein T and U are independently -NR-, -O-, -CRR'- or a single bond, and q is an integer of from 0 to 3. Alternatively, two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula $-A-(CH_2)_r-B-$, wherein A and B are independently -CRR'-, -O-, -NR-, -S-, -S(O)-, $-S(O)_2-$, $-S(O)_2NR'-$ or a single bond, and r is an integer of from 1 to 4. One of the single bonds of the new ring so

formed may optionally be replaced with a double bond. Alternatively, two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula $-(CRR')_s-X-(CR''R''')_d-$, where s and d are independently integers of from 0 to 3, and X is -O-, -NR'-, -S-, -S(O)-, $-S(O)_2-$, or $-S(O)_2NR'-$. The substituents R, R', R" and R"' are preferably independently selected from hydrogen or substituted or unsubstituted (C_1-C_6) alkyl.

As used herein, the term "diphosphate" includes but is not limited to an ester of phosphoric acid containing two phosphate groups. The term "triphosphate" includes but is not limited to an ester of phosphoric acid containing three phosphate groups. For example, particular drugs having a diphosphate or a triphosphate include:



Diphosphate



Triphosphate

As used herein, the term "heteroatom" includes oxygen (O), nitrogen (N), sulfur (S) and silicon (Si).

The symbol "R" is a general abbreviation that represents a substituent group that is selected from substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, and substituted or unsubstituted heterocyclyl groups.

The term "pharmaceutically acceptable carrier", as used herein means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting a chemical agent. Pharmaceutically acceptable carriers include pharmaceutically acceptable salts, where the term "pharmaceutically acceptable salts"

includes salts of the active compounds which are prepared with relatively nontoxic acids or bases, depending on the particular substituents found on the compounds described herein. When compounds of the present invention contain relatively acidic functionalities, base addition salts can be obtained by contacting the neutral form of such compounds with a sufficient amount of the desired base, either neat or in a suitable inert solvent. Examples of pharmaceutically acceptable base addition salts include sodium, potassium, calcium, ammonium, organic amino, or magnesium salt, or a similar salt. When compounds of the present invention contain relatively basic functionalities, acid addition salts can be obtained by contacting the neutral form of such compounds with a sufficient amount of the desired acid, either neat or in a suitable inert solvent. Examples of pharmaceutically acceptable acid addition salts include those derived from inorganic acids like hydrochloric, hydrobromic, nitric, carbonic, monohydrogencarbonic, phosphoric, monohydrogenphosphoric, dihydrogenphosphoric, sulfuric, monohydrogensulfuric, hydriodic, or phosphorous acids and the like, as well as the salts derived from relatively nontoxic organic acids like acetic, propionic, isobutyric, maleic, malonic, benzoic, succinic, suberic, fumaric, lactic, mandelic, phthalic, benzenesulfonic, p-tolylsulfonic, citric, tartaric, methanesulfonic, and the like. Also included are salts of amino acids such as arginate and the like, and salts of organic acids like glucuronic or galactunoric acids and the like (*see*, for example, Berge *et al.*, "Pharmaceutical Salts", *Journal of Pharmaceutical Science*, **1977**, *66*, 1-19). Certain specific compounds of the present invention contain both basic and acidic functionalities that allow the compounds to be converted into either base or acid addition salts.

The neutral forms of the compounds are preferably regenerated by contacting the salt with a base or acid and isolating the parent compound in the conventional manner. The parent form of the compound differs from the various salt forms in certain physical properties, such as solubility in polar solvents, but otherwise the salts are equivalent to the parent form of the compound for the purposes of the present invention.

In addition to salt forms, the present invention provides compounds, which are in a prodrug form. Prodrugs of the compounds described herein are those compounds that readily undergo chemical changes under physiological conditions to provide the compounds of the present invention. Additionally, prodrugs can be converted to the compounds of the present invention by chemical or biochemical methods in an *ex vivo* environment. For example, prodrugs can be slowly converted to the compounds of the

present invention when placed in a transdermal patch reservoir with a suitable enzyme or chemical reagent.

Certain compounds of the present invention can exist in unsolvated forms as well as solvated forms, including hydrated forms. In general, the solvated forms are equivalent to unsolvated forms and are encompassed within the scope of the present invention. Certain compounds of the present invention may exist in multiple crystalline or amorphous forms. In general, all physical forms are equivalent for the uses contemplated by the present invention and are intended to be within the scope of the present invention.

Certain compounds of the present invention possess asymmetric carbon atoms (optical centers) or double bonds; the racemates, diastereomers, geometric isomers and individual isomers are encompassed within the scope of the present invention.

The compounds of the present invention may also contain unnatural proportions of atomic isotopes at one or more of the atoms that constitute such compounds. For example, the compounds may be radiolabeled with radioactive isotopes, such as for example tritium (^{3}H), iodine-125 (^{125}I) or carbon-14 (^{14}C). All isotopic variations of the compounds of the present invention, whether radioactive or not, are intended to be encompassed within the scope of the present invention.

The term "attaching moiety" or "moiety for attaching a targeting group" refers to a moiety which allows for attachment of a targeting group to the linker. Typical attaching groups include, by way of illustration and not limitation, alkyl, aminoalkyl, aminocarbonylalkyl, carboxyalkyl, hydroxyalkyl, alkyl-maleimide, alkyl-N-hydroxylsuccinimide, poly(ethylene glycol)-maleimide and poly(ethylene glycol)-N-hydroxylsuccinimide, all of which may be further substituted. The linker can also have the attaching moiety be actually appended to the targeting group.

As used herein, the term "leaving group" refers to a portion of a substrate that is cleaved from the substrate in a reaction.

The term "antibody" as referred to herein includes whole antibodies and any antigen binding fragment (*i.e.*, "antigen-binding portion") or single chains thereof. An "antibody" refers to a glycoprotein comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds, or an antigen binding portion thereof. Each heavy chain is comprised of a heavy chain variable region (V_H) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, C_{H1},

C_{H2} and C_{H3}, and may be of the mu, delta, gamma, alpha or epsilon isotype. Each light chain is comprised of a light chain variable region (V_L) and a light chain constant region. The light chain constant region is comprised of one domain, C_L, which may be of the kappa or lambda isotype. The V_H and V_L regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin 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.

The terms "antibody fragment" or "antigen-binding portion" of an antibody (or simply "antibody portion"), as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen. It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antibody fragment" or "antigen-binding portion" of an antibody include (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 Fd fragment consisting of the V_H and C_{H1} domains; (iv) a Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, (1989) *Nature* 341:544-546), which consists of a V_H domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, V_L and V_H, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules (known as single chain Fv (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 intended to be encompassed within the term "antigen-binding portion" of an antibody. These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies.

The terms "monoclonal antibody" as used herein refers to a preparation of antibody molecules of single molecular composition. A monoclonal antibody composition displays a single binding specificity and affinity for a particular epitope.

For preparation of monoclonal or polyclonal antibodies, any technique known in the art can be used (*see, e.g.*, Kohler & Milstein, *Nature* 256:495-497 (1975); Kozbor *et al.*, *Immunology Today* 4: 72 (1983); Cole *et al.*, pp. 77-96 in MONOCLONAL ANTIBODIES AND CANCER THERAPY, Alan R. Liss, Inc. (1985)).

Methods of production of polyclonal antibodies are known to those of skill in the art. An inbred strain of mice (*e.g.*, BALB/C mice) or rabbits is immunized with the protein using a standard adjuvant, such as Freund's adjuvant, and a standard immunization protocol. The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the beta subunits. When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the protein can be done if desired.

Monoclonal antibodies may be obtained by various techniques familiar to those skilled in the art. Briefly, spleen cells from an animal immunized with a desired antigen are immortalized, commonly by fusion with a myeloma cell (*see* Kohler & Milstein, *Eur. J. Immunol.* **6**: 511-519 (1976)). Alternative methods of immortalization include transformation with Epstein Barr Virus, oncogenes, or retroviruses, or other methods well known in the art.

In a preferred embodiment, the antibody is a chimeric or humanized antibody. Chimeric or humanized antibodies of the present invention can be prepared based on the sequence of a murine monoclonal antibody. DNA encoding the heavy and light chain immunoglobulins can be obtained from the murine hybridoma of interest and engineered to contain non-murine (*e.g.*,. human) immunoglobulin sequences using standard molecular biology techniques. For example, to create a chimeric antibody, the murine variable regions can be linked to human constant regions using methods known in the art (see *e.g.*, U.S. Patent No. 4,816,567 to Cabilly *et al.*). To create a humanized antibody, the murine CDR regions can be inserted into a human framework using methods known in the art (see *e.g.*, U.S. Patent No. 5,225,539 to Winter, and U.S. Patent Nos. 5,530,101; 5,585,089; 5,693,762 and 6,180,370 to Queen *et al.*).

In another preferred embodiment, the antibody is a human antibody. Such human antibodies can be generated by immunizing transgenic or transchromosomic mice in which the endogenous mouse immunoglobulin genes have been inactivated and exogenous human immunoglobulin genes have been introduced. Such mice are known in the art (see *e.g.*, U.S. Patent Nos. 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,789,650; 5,877,397; 5,661,016; 5,814,318; 5,874,299; and 5,770,429; all to Lonberg and Kay; U.S. Patent Nos. 5,939,598; 6,075,181; 6,114,598; 6,150,584 and 6,162,963 to Kucherlapati *et al.*; and PCT Publication WO 02/43478 to Ishida *et al.*) Human antibodies can also be prepared using phage display methods for screening libraries of human immunoglobulin genes. Such phage display methods for isolating human antibodies also are know in the art (see *e.g.*, U.S. Patent Nos. 5,223,409; 5,403,484; and 5,571,698 to Ladner *et al.*; U.S. Patent Nos. 5,427,908 and 5,580,717 to Dower *et al.*; U.S. Patent Nos. 5,969,108 and 6,172,197 to McCafferty *et al.*; and U.S. Patent Nos. 5,885,793; 6,521,404; 6,544,731; 6,555,313; 6,582,915 and 6,593,081 to Griffiths *et al.*).

"Solid support," as used herein refers to a material that is substantially insoluble in a selected solvent system, or which can be readily separated (*e.g.*, by precipitation) from a selected solvent system in which it is soluble. Solid supports useful in practicing the present invention can include groups that are activated or capable of activation to allow selected species to be bound to the solid support. A solid support can also be a substrate, for example, a chip, wafer or well, onto which an individual, or more than one compound, of the invention is bound.

"Reactive functional group," as used herein refers to groups including, but not limited to, olefins, acetylenes, alcohols, phenols, ethers, oxides, halides, aldehydes, ketones, carboxylic acids, esters, amides, cyanates, isocyanates, thiocyanates, isothiocyanates, amines, hydrazines, hydrazones, hydrazides, diazo, diazonium, nitro, nitriles, mercaptans, sulfides, disulfides, sulfoxides, sulfones, sulfonic acids, sulfinic acids, acetals, ketals, anhydrides, sulfates, sulfenic acids isonitriles, amidines, imides, imidates, nitrones, hydroxylamines, oximes, hydroxamic acids thiohydroxamic acids, allenes, ortho esters, sulfites, enamines, ynamines, ureas, pseudoureas, semicarbazides, carbodiimides, carbamates, imines, azides, azo compounds, azoxy compounds, and nitroso compounds. Reactive functional groups also include those used to prepare bioconjugates, e.g., N-hydroxysuccinimide esters, maleimides and the like (*see*, for example, Hermanson, BIOCONJUGATE TECHNIQUES, Academic press, San Diego, 1996).

Methods to prepare each of these functional groups are well known in the art and their application to or modification for a particular purpose is within the ability of one of skill in the art (*see*, for example, Sandler and Karo, eds. ORGANIC FUNCTIONAL GROUP PREPARATIONS, Academic Press, San Diego, 1989). The reactive functional groups may be protected or unprotected.

The compounds of the invention are prepared as a single isomer (*e.g.*, enantiomer, cis-trans, positional, diastereomer) or as a mixture of isomers. In a preferred embodiment, the compounds are prepared as substantially a single isomer. Methods of preparing substantially isomerically pure compounds are known in the art. For example, enantiomerically enriched mixtures and pure enantiomeric compounds can be prepared by using synthetic intermediates that are enantiomerically pure in combination with reactions that either leave the stereochemistry at a chiral center unchanged or result in its complete inversion. Alternatively, the final product or intermediates along the synthetic route can be resolved into a single stereoisomer. Techniques for inverting or leaving unchanged a particular stereocenter, and those for resolving mixtures of stereoisomers are well known in the art and it is well within the ability of one of skill in the art to choose and appropriate method for a particular situation. *See*, generally, Furniss *et al.* (eds.),VOGEL'S ENCYCLOPEDIA OF PRACTICAL ORGANIC CHEMISTRY 5TH ED., Longman Scientific and Technical Ltd., Essex, 1991, pp. 809-816; and Heller, *Acc. Chem. Res.* **23**: 128 (1990).

LINKERS

The present invention provides for drug-ligand conjugates where the drug is linked to the ligand through a chemical linker. In some embodiments, the linker is a peptidyl linker, and is depicted herein as $(L^4)_p$—F— $(L^1)_m$. Other linkers include hydrazine and disulfide linkers, and is depicted herein as $(L^4)_p$—H— $(L^1)_m$ or $(L^4)_p$— J— $(L^1)_m$, respectively. In addition to the linkers as being attached to the drug, the present invention also provides cleaveable linker arms that are appropriate for attachment to essentially any molecular species. The linker arm aspect of the invention is exemplified herein by reference to their attachment to a therapeutic moiety. It will, however, be readily apparent to those of skill in the art that the linkers can be attached to diverse species including, but not limited to, diagnostic agents, analytical agents, biomolecules, targeting agents, detectable labels and the like.

The use of peptidyl and other linkers in drug-ligand conjugates is described in U.S. Provisional Patent Applications Serial Nos. 60/295,196; 60/295,259; 60/295342; 60/304,908; 60/572,667; 60/661,174; 60/669,871; 60/720,499; 60/730,804; and 60/735,657 and U.S. Patent Applications Serial Nos. 10/160,972; 10/161,234; 11/134,685; 11/134,826; and 11/398,854 and U.S. Patent No. 6,989,452 and PCT Patent Application No. PCT/US2006/37793, all of which are incorporated herein by reference.

In one aspect, the present invention relates to linkers that are useful to attach targeting groups to therapeutic agents and markers. In another aspect, the invention provides linkers that impart stability to compounds, reduce their *in vivo* toxicity, or otherwise favorably affect their pharmacokinetics, bioavailability and/or pharmacodynamics. It is generally preferred that in such embodiments, the linker is cleaved, releasing the active drug, once the drug is delivered to its site of action. Thus, in one embodiment of the invention, the linkers of the invention are traceless, such that once removed from the therapeutic agent or marker (such as during activation), no trace of the linker's presence remains.

In another embodiment of the invention, the linkers are characterized by their ability to be cleaved at a site in or near the target cell such as at the site of therapeutic action or marker activity. Such cleavage can be enzymatic in nature. This feature aids in reducing systemic activation of the therapeutic agent or marker, reducing toxicity and systemic side effects. Preferred cleaveable groups for enzymatic cleavage include peptide bonds, ester linkages, and disulfide linkages. In other embodiments, the linkers are sensitive to pH and are cleaved through changes in pH.

An important aspect of the current invention is the ability to control the speed with which the linkers cleave. Often a linker that cleaves quickly is desired. In some embodiments, however, a linker that cleaves more slowly may be preferred. For example, in a sustained release formulation or in a formulation with both a quick release and a slow release component, it may be useful to provide a linker which cleaves more slowly. WO 02/096910 provides several specific ligand-drug complexes having a hydrazine linker. However, there is no way to "tune" the linker composition dependent upon the rate of cyclization required, and the particular compounds described cleave the ligand from the drug at a slower rate than is preferred for many drug-linker conjugates. In contrast, the hydrazine linkers of the current invention provide for a range of cyclization rates, from very fast to very slow, thereby allowing for the selection of a

particular hydrazine linker based on the desired rate of cyclization. For example, very fast cyclization can be achieved with hydrazine linkers that produce a single 5-membered ring upon cleavage. Preferred cyclization rates for targeted delivery of a cytotoxic agent to cells are achieved using hydrazine linkers that produce, upon cleavage, either two 5-membered rings or a single 6-membered ring resulting from a linker having two methyls at the geminal position. The *gem*-dimethyl effect has been shown to accelerate the rate of the cyclization reaction as compared to a single 6-membered ring without the two methyls at the geminal position. This results from the strain being relieved in the ring. Sometimes, however, substitutents may slow down the reaction instead of making it faster. Often the reasons for the retardation can be traced to steric hindrance. As shown in Example 2.4, the *gem* dimethyl substitution allows for a much faster cyclization reaction to occur compared to when the geminal carbon is a CH_2.

It is important to note, however, that in some embodiments, a linker that cleaves more slowly may be preferred. For example, in a sustained release formulation or in a formulation with both a quick release and a slow release component, it may be useful to provide a linker which cleaves more slowly. In certain embodiments, a slow rate of cyclization is achieved using a hydrazine linker that produces, upon cleavage, either a single 6-membered ring, without the *gem*-dimethyl substitution, or a single 7-membered ring.

The linkers also serve to stabilize the therapeutic agent or marker against degradation while in circulation. This feature provides a significant benefit since such stabilization results in prolonging the circulation half-life of the attached agent or marker. The linker also serves to attenuate the activity of the attached agent or marker so that the conjugate is relatively benign while in circulation and has the desired effect, for example is toxic, after activation at the desired site of action. For therapeutic agent conjugates, this feature of the linker serves to improve the therapeutic index of the agent.

The stabilizing groups are preferably selected to limit clearance and metabolism of the therapeutic agent or marker by enzymes that may be present in blood or non-target tissue and are further selected to limit transport of the agent or marker into the cells. The stabilizing groups serve to block degradation of the agent or marker and may also act in providing other physical characteristics of the agent or marker. The stabilizing group may also improve the agent or marker's stability during storage in either a formulated or non-formulated form.

Ideally, the stabilizing group is useful to stabilize a therapeutic agent or marker if it serves to protect the agent or marker from degradation when tested by storage of the agent or marker in human blood at 37°C for 2 hours and results in less than 20%, preferably less than 10%, more preferably less than 5% and even more preferably less than 2%, cleavage of the agent or marker by the enzymes present in the human blood under the given assay conditions.

The present invention also relates to conjugates containing these linkers. More particularly, the invention relates to prodrugs that may be used for the treatment of disease, especially for cancer chemotherapy. Specifically, use of the linkers described herein provide for prodrugs that display a high specificity of action, a reduced toxicity, and an improved stability in blood relative to prodrugs of similar structure.

The linkers of the present invention as described herein may be present at a variety of positions within the cytotoxic conjugate.

Thus, there is provided a linker that may contain any of a variety of groups as part of its chain that will cleave *in vivo*, e.g., in the blood stream, at a rate which is enhanced relative to that of constructs that lack such groups. Also provided are conjugates of the linker arms with therapeutic and diagnostic agents. The linkers are useful to form prodrug analogs of therapeutic agents and to reversibly link a therapeutic or diagnostic agent to a targeting agent, a detectable label, or a solid support. The linkers may be incorporated into complexes that include the cytotoxins of the invention.

In addition to the cleaveable peptide, hydrazine, or disulfide group, one or more self-immolative linker groups L^1 are optionally introduced between the cytotoxin and the targeting agent. These linker groups may also be described as spacer groups and contain at least two reactive functional groups. Typically, one chemical functionality of the spacer group bonds to a chemical functionality of the therapeutic agent, *e.g.*, cytotoxin, while the other chemical functionality of the spacer group is used to bond to a chemical functionality of the targeting agent or the cleaveable linker. Examples of chemical functionalities of spacer groups include hydroxy, mercapto, carbonyl, carboxy, amino, ketone, and mercapto groups.

The self-immolative linkers, represented by L^1, are generally a substituted or unsubstituted alkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl or substituted or unsubstituted heteroalkyl group. In one embodiment, the

alkyl or aryl groups may comprise between 1 and 20 carbon atoms. They may also comprise a polyethylene glycol moiety.

Exemplary spacer groups include, for example, 6-aminohexanol, 6-mercaptohexanol, 10-hydroxydecanoic acid, glycine and other amino acids, 1,6-hexanediol, β-alanine, 2-aminoethanol, cysteamine (2-aminoethanethiol), 5-aminopentanoic acid, 6-aminohexanoic acid, 3-maleimidobenzoic acid, phthalide, α-substituted phthalides, the carbonyl group, aminal esters, nucleic acids, peptides and the like.

The spacer can serve to introduce additional molecular mass and chemical functionality into the cytotoxin-targeting agent complex. Generally, the additional mass and functionality will affect the serum half-life and other properties of the complex. Thus, through careful selection of spacer groups, cytotoxin complexes with a range of serum half-lives can be produced.

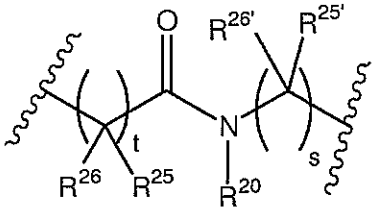
The spacer(s) located directly adjacent to the drug moiety is also denoted as $(L^1)_m$, wherein m is an integer selected from 0, 1, 2, 3, 4, 5, and 6. When multiple L^1 spacers are present, either identical or different spacers may be used. L^1 may be any self-immolative group.

L^4 is a linker moiety that preferably imparts increased solubility or decreased aggregation properties to conjugates utilizing a linker that contains the moiety or modifies the hydrolysis rate of the conjugate. The L^4 linker does not have to be self immolative. In one embodiment, the L^4 moiety is substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroalkyl, or unsubstituted heteroalkyl, any of which may be straight, branched, or cyclic. The substitutions may be, for example, a lower (C^1-C^6) alkyl, alkoxy, aklylthio, alkylamino, or dialkylamino. In certain embodiments, L^4 comprises a non-cyclic moiety . In another embodiment, L^4 comprises any positively or negatively charged amino acid polymer, such as polylysine or polyargenine. L^4 can comprise a polymer such as a polyethylene glycol moiety. Additionally the L^4 linker can comprise, for example, both a polymer component and a small chemical moiety.

In a preferred embodiment, L^4 comprises a polyethylene glycol (PEG) moiety. The PEG portion of L^4 may be between 1 and 50 units long. Preferably, the PEG will have 1-12 repeat units, more preferably 3-12 repeat units, more preferably 2-6 repeat units, or even more preferably 3-5 repeat units and most preferably 4 repeat units. L^4 may

consist solely of the PEG moiety, or it may also contain an additional substituted or unsubstituted alkyl or heteroalkyl. It is useful to combine PEG as part of the L^4 moiety to enhance the water solubility of the complex. Additionally, the PEG moiety reduces the degree of aggregation that may occur during the conjugation of the drug to the antibody.

In some embodiments, L^4 comprises

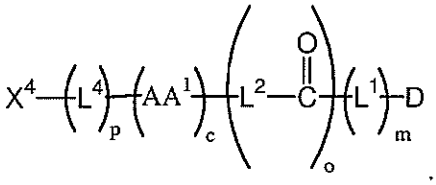


directly attached to the N-terminus of $(AA^1)_c$. R^{20} is is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl. Each R^{25}, $R^{25'}$, R^{26}, and $R^{26'}$ is independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, and substituted or unsubstituted heterocycloalkyl; and s and t are independently integers from 1 to 6. Preferably, R^{20}, R^{25}, $R^{25'}$, R^{26} and $R^{26'}$ are hydrophobic. In some embodiments, R^{20} is H or alkyl (preferably, unsubstituted lower alkyl). In some embodiments, R^{25}, $R^{25'}$, R^{26} and $R^{26'}$ are independently H or alkyl (preferably, unsubstituted C^1 to C^4 alkyl). In some embodiments, R^{25}, $R^{25'}$, R^{26} and $R^{26'}$ are all H. In some embodiments, t is 1 and s is 1 or 2.

Peptide Linkers (F)

As discussed above, the peptidyl linkers of the invention can be represented by the general formula: $(L^4)_p$—F— $(L^1)_m$, wherein F represents the linker portion comprising the peptidyl moiety. In one embodiment, the F portion comprises an optional additional self-immolative linker(s), L^2, and a carbonyl group. In another embodiment, the F portion comprises an amino group and an optional spacer group(s), L^3.

Accordingly, in one embodiment, the conjugate comprising the peptidyl linker comprises a structure of the Formula 4:

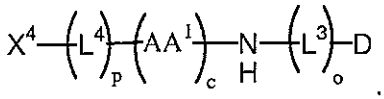


(4)

In this embodiment, $L^1$is a self-immolative linker, as described above, and L^4 is a moiety that preferably imparts increased solubility, or decreased aggregation properties, or modifies the hydrolysis rate, as described above. L^2 represents a self-immolative linker(s). In addition, m is 0, 1, 2, 3, 4, 5, or 6; and o and p are independently 0 or 1. AA^1 represents one or more natural amino acids, and/or unnatural α-amino acids; c is an integer in the range from 1 and 20. In some embodiments, c is in the range of 2 to 5 or c is 2 or 3.

In the peptide linkers of the invention of the above Formula 4, AA^1 is linked, at its amino terminus, either directly to L^4 or, when L^4 is absent, directly to the X^4 group (*i.e.*, the targeting agent, detectable label, protected reactive functional group or unprotected reactive functional group). In some embodiments, when L^4 is present, L^4 does not comprise a carboxylic acyl group directly attached to the N-terminus of $(AA^1)_c$. Thus, it is not necessary in these embodiments for there to be a carboxylic acyl unit directly between either L^4 or X^4 and AA^1, as is necessary in the peptidic linkers of U.S. Patent No. 6,214,345.

In another embodiment, the conjugate comprising the peptidyl linker comprises a structure of the Formula 5:



(5)

In this embodiment, L^4 is a moiety that preferably imparts increased solubility, or decreased aggregation properties, or modifies the hydrolysis rate, as described above; L^3 is a spacer group comprising a primary or secondary amine or a carboxyl functional group, and either the amine of L^3 forms an amide bond with a pendant carboxyl functional group of D or the carboxyl of L^3 forms an amide bond with a pendant amine functional group of D; and o and p are independently 0 or 1. AA^1 represents one or more natural amino acids, and/or unnatural α-amino acids; c is an integer from 1 and 20. In this embodiment, L^1 is absent (*i.e.*, m is 0 is the general formula).

In the peptide linkers of the invention of the above Formula 5, AA^1 is linked, at its amino terminus, either directly to L^4 or, when L^4 is absent, directly to the X^4 group (*i.e.*, the targeting agent, detectable label, protected reactive functional group or unprotected reactive functional group). In some embodiments, when L^4 is present, L^4 does not comprise a carboxylic acyl group directly attached to the N-terminus of $(AA^1)_c$. Thus, it is not necessary in these embodiments for there to be a carboxylic acyl unit directly between either L^4 or X^4 and AA^1, as is necessary in the peptidic linkers of U.S. Patent No. 6,214,345.

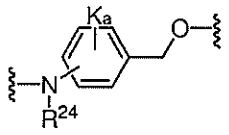
The self-immolative linker L^2

The self-immolative linker L^2 is a bifunctional chemical moiety which is capable of covalently linking together two spaced chemical moieties into a normally stable tripartate molecule, releasing one of said spaced chemical moieties from the tripartate molecule by means of enzymatic cleavage; and following said enzymatic cleavage, spontaneously cleaving from the remainder of the molecule to release the other of said spaced chemical moieties. In accordance with the present invention, the self-immolative spacer is covalently linked at one of its ends to the peptide moiety and covalently linked at its other end to the chemically reactive site of the drug moiety whose derivatization inhibits pharmacological activity, so as to space and covalently link together the peptide moiety and the drug moiety into a tripartate molecule which is stable and pharmacologically inactive in the absence of the target enzyme, but which is enzymatically cleavable by such target enzyme at the bond covalently linking the spacer moiety and the peptide moiety to thereby effect release of the peptide moiety from the tripartate molecule. Such enzymatic cleavage, in turn, will activate the self-immolating

character of the spacer moiety and initiate spontaneous cleavage of the bond covalently linking the spacer moiety to the drug moiety, to thereby effect release of the drug in pharmacologically active form.

The self-immolative linker L^2 may be any self-immolative group. Preferably L^2 is a substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, unsubstituted heterocycloalkyl, substituted heterocycloalkyl, substituted and unsubstituted aryl, and substituted and unsubstituted heteroaryl.

One particularly preferred self-immolative spacer L^2 may be represented by the formula 6:



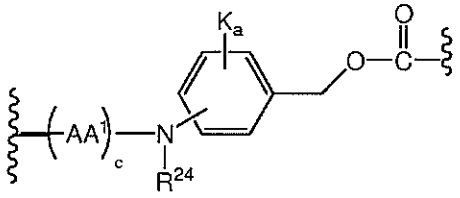
(6)

The aromatic ring of the aminobenzyl group may be substituted with one or more "K" groups. A "K" group is a substituent on the aromatic ring that replaces a hydrogen otherwise attached to one of the four non-substituted carbons that are part of the ring structure. The "K" group may be a single atom, such as a halogen, or may be a multi-atom group, such as alkyl, heteroalkyl, amino, nitro, hydroxy, alkoxy, haloalkyl, and cyano. Each K is independently selected from the group consisting of substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{21}R^{22}$, $NR^{21}COR^{22}$, $OCONR^{21}R^{22}$, $OCOR^{21}$, and OR^{21}, wherein R^{21} and R^{22} are independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl and unsubstituted heterocycloalkyl. Exemplary K substituents include, but are not limited to, F, Cl, Br, I, NO_2, OH, OCH_3, $NHCOCH_3$, $N(CH_3)_2$, $NHCOCF_3$ and methyl. For "K_a", a is an integer of 0, 1, 2, 3, or 4. In one preferred embodiment, a is 0.

The ether oxygen atom of the structure shown above is connected to a carbonyl group. The line from the NR^{24} functionality into the aromatic ring indicates that the amine functionality may be bonded to any of the five carbons that both form the ring and are not substituted by the $-CH_2-O-$ group. Preferably, the NR^{24} functionality of X is covalently

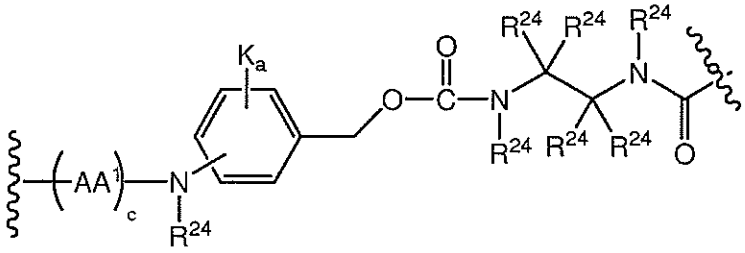
bound to the aromatic ring at the para position relative to the $-CH_2-O-$ group. R^{24} is a member selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, and unsubstituted heteroalkyl. In a specific embodiment, R^{24} is hydrogen.

In one embodiment, the invention provides a peptide linker of formula (4) above, wherein F comprises the structure:



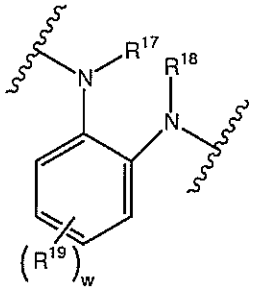
where R^{24} is selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, and unsubstituted heteroalkyl. Each K is a member independently selected from the group consisting of substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{21}R^{22}$, $NR^{21}COR^{22}$, $OCONR^{21}R^{22}$, $OCOR^{21}$, and OR^{21} where R^{21} and R^{22} are independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl; and a is an integer of 0,1, 2, 3, or 4.

In another embodiment, the peptide linker of formula (4) above comprises a $-F-(L^1)_m-$ that comprises the structure:



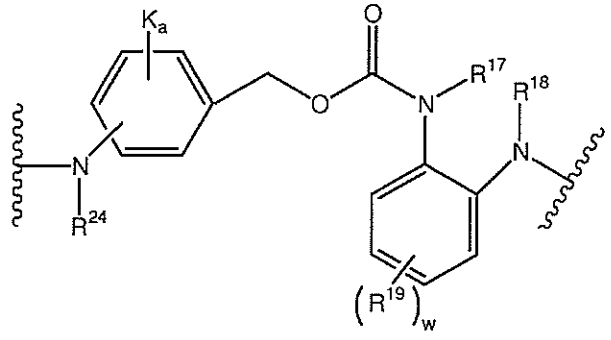
where each R^{24} is a member independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, and unsubstituted heteroalkyl.

In some embodiments, the self-immolative spacer L^1 or L^2 includes



where each R^{17}, R^{18}, and R^{19} is independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl, and w is an integer from 0 to 4. In some embodiments, R^{17} and R^{18} are independently H or alkyl (preferably, unsubstituted C1-4 alkyl). Preferably, R^{17} and R^{18} are C1-4 alkyl, such as methyl or ethyl. In some embodiments, w is 0. While not wishing to be bound to any particular theory, it has been found experimentally that this particular self-immolative spacer cyclizes relatively quickly.

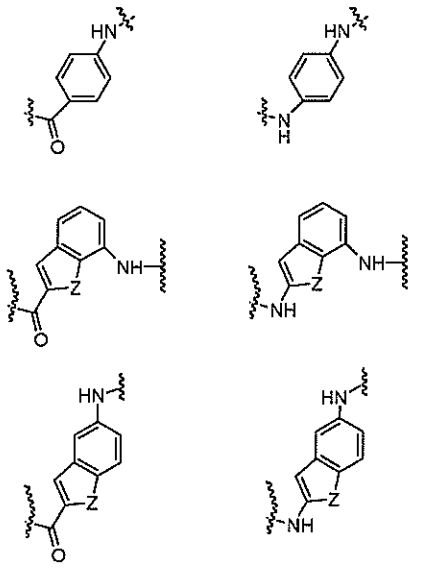
In some embodiments, L^1 or L^2 includes



The Spacer Group L^3

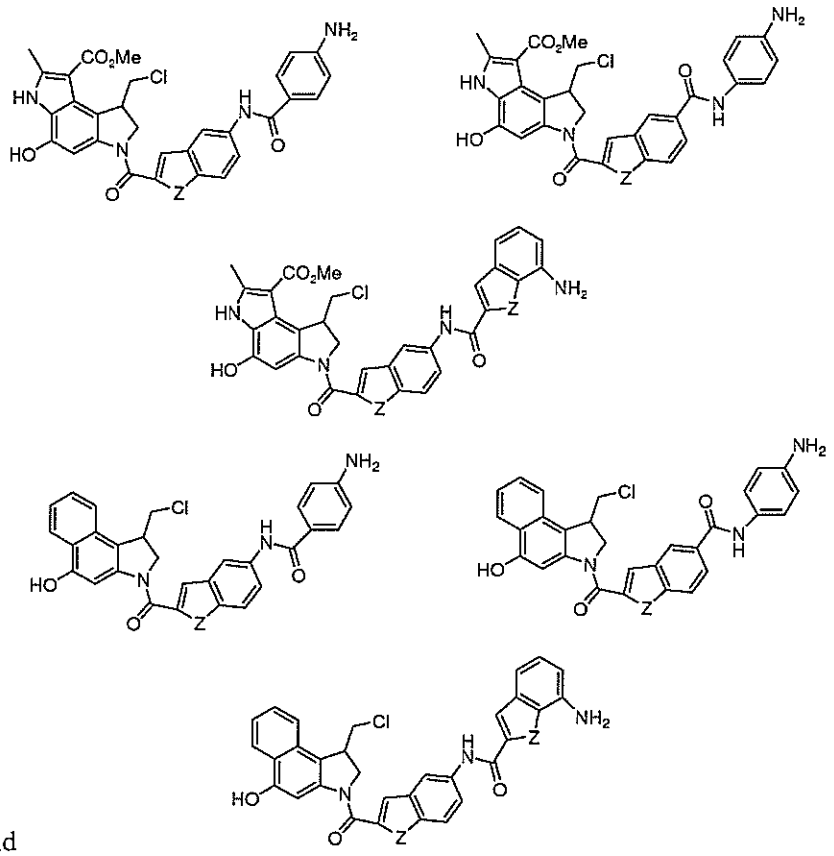
The spacer group L^3 is characterized in that it comprises a primary or secondary amine or a carboxyl functional group, and either the amine of the L^3 group forms an amide bond with a pendant carboxyl functional group of D or the carboxyl of L^3 forms an amide bond with a pendant amine functional group of D. L^3 can be selected from the group consisting of substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted hteroaryl, or substituted or unsubstituted heterocycloalkyl. In a preferred embodiment, L^3 comprises an aromatic group. More preferably, L^3 comprises a benzoic acid group, an aniline group

or indole group. Non-limiting examples of structures that can serve as an -L^3-NH- spacer include the following structures:



where Z is a member selected from O, S and NR^{23}, and where R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl.

Upon cleavage of the linker of the invention containing L^3, the L^3 moiety remains attached to the drug, D. Accordingly, the L^3 moiety is chosen such that its presence attached to D does not significantly alter the activity of D. In another embodiment, a portion of the drug D itself functions as the L^3 spacer. For example, in one embodiment, the drug, D, is a duocarmycin derivative in which a portion of the drug functions as the L^3 spacer. Non-limiting examples of such embodiments include those in which NH_2-(L^3)-D has a structure selected from the group consisting of:



and

where Z is a member selected from O, S and NR^{23}, where R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl; and where the NH_2 group on each structure reacts with $(AA^1)_c$ to form $-(AA^1)_c$-NH-.

The Peptide Sequence AA^1

The group AA^1 represents a single amino acid or a plurality of amino acids that are joined together by amide bonds. The amino acids may be natural amino acids and/or unnatural α-amino acids.

The peptide sequence $(AA^1)_c$ is functionally the amidification residue of a single amino acid (when c=1) or a plurality of amino acids joined together by amide bonds. The peptide of the current invention is selected for directing enzyme-catalyzed cleavage of the peptide by an enzyme in a location of interest in a biological system. For example, for conjugates that are targeted to a cell using a targeting agent, and then taken up by the cell, a peptide is chosen that is cleaved by one or more lysosomal proteases such that the peptide is cleaved intracellularly within the lysosome. The number of amino acids within

the peptide can range from 1 to 20; but more preferably there will be 2-8 amino acids, 2-6 amino acids or 2, 3 or 4 amino acids comprising $(AA^1)_c$. Peptide sequences that are susceptible to cleavage by specific enzymes or classes of enzymes are well known in the art.

Many peptide sequences that are cleaved by enzymes in the serum, liver, gut, etc. are known in the art. An exemplary peptide sequence of the invention includes a peptide sequence that is cleaved by a protease. The focus of the discussion that follows on the use of a protease-sensitive sequence is for clarity of illustration and does not serve to limit the scope of the present invention.

When the enzyme that cleaves the peptide is a protease, the linker generally includes a peptide containing a cleavage recognition sequence for the protease. A cleavage recognition sequence for a protease is a specific amino acid sequence recognized by the protease during proteolytic cleavage. Many protease cleavage sites are known in the art, and these and other cleavage sites can be included in the linker moiety. *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); Bouvier *et al. Meth. Enzymol.* **248**: 614 (1995), Hardy *et al.*, in AMYLOID PROTEIN PRECURSOR IN DEVELOPMENT, AGING, AND ALZHEIMER'S DISEASE, ed. Masters *et al.* pp. 190-198 (1994).

The amino acids of the peptide sequence $(AA^1)_c$ are chosen based on their suitability for selective enzymatic cleavage by particular molecules such as tumor-associated protease. The amino acids used may be natural or unnatural amino acids. They may be in the L or the D configuration. In one embodiment, at least three different amino acids are used. In another embodiment, only two amino acids are used.

In a preferred embodiment, the peptide sequence $(AA^1)_c$ is chosen based on its ability to be cleaved by a lysosomal proteases, non-limiting examples of which include cathepsins B, C, D, H, L and S. Preferably, the peptide sequence $(AA^1)_c$ is capable of being cleaved by cathepsin B *in vitro*, which can be tested using *in vitro* protease cleavage assays known in the art.

In another embodiment, the peptide sequence $(AA^1)_c$ is chosen based on its ability to be cleaved by a tumor-associated protease, such as a protease that is found extracellularly in the vicinity of tumor cells, non-limiting examples of which include

thimet oligopeptidase (TOP) and CD10. The ability of a peptide to be cleaved by TOP or CD10 can be tested using *in vitro* protease cleavage assays known in the art.

Suitable, but non-limiting, examples of peptide sequences suitable for use in the conjugates of the invention include Val-Cit, Cit-Cit, Val-Lys, Phe-Lys, Lys-Lys, Ala-Lys, Phe-Cit, Leu-Cit, Ile-Cit, Trp, Cit, Phe-Ala, Phe-N^9-tosyl-Arg, Phe-N^9-nitro-Arg, Phe-Phe-Lys, D-Phe-Phe-Lys, Gly-Phe-Lys, Leu-Ala-Leu, Ile-Ala-Leu, Val-Ala-Val, Ala-Leu-Ala-Leu (SEQ. ID NO: 1), β-Ala-Leu-Ala-Leu (SEQ. ID NO: 2), Gly-Phe-Leu-Gly (SEQ. ID NO: 3), Val-Ala, Leu-Leu-Gly-Leu (SEQ. ID NO: 14), Leu-Asn-Ala, and Lys-Leu-Val. Preferred peptides sequences are Val-Cit and Val-Lys.

In another embodiment, the amino acid located the closest to the drug moiety is selected from the group consisting of: Ala, Asn, Asp, Cit, Cys, Gln, Glu, Gly, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val. In yet another embodiment, the amino acid located the closest to the drug moiety is selected from the group consisting of: Ala, Asn, Asp, Cys, Gln, Glu, Gly, Ile, Leu, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val.

Proteases have been implicated in cancer metastasis. Increased synthesis of the protease urokinase was correlated with an increased ability to metastasize in many cancers. Urokinase activates plasmin from plasminogen, which is ubiquitously located in the extracellular space and its activation can cause the degradation of the proteins in the extracellular matrix through which the metastasizing tumor cells invade. Plasmin can also activate the collagenases thus promoting the degradation of the collagen in the basement membrane surrounding the capillaries and lymph system thereby allowing tumor cells to invade into the target tissues (Dano, *et al. Adv. Cancer. Res.*, **44**: 139 (1985)). Thus, it is within the scope of the present invention to utilize as a linker a peptide sequence that is cleaved by urokinase.

The invention also provides the use of peptide sequences that are sensitive to cleavage by tryptases. Human mast cells express at least four distinct tryptases, designated α βI, βII, and βIII. These enzymes are not controlled by blood plasma proteinase inhibitors and only cleave a few physiological substrates *in vitro*. The tryptase family of serine proteases has been implicated in a variety of allergic and inflammatory diseases involving mast cells because of elevated tryptase levels found in biological fluids from patients with these disorders. However, the exact role of tryptase in the pathophysiology of disease remains to be delineated. The scope of biological functions

and corresponding physiological consequences of tryptase are substantially defined by their substrate specificity.

Tryptase is a potent activator of pro-urokinase plasminogen activator (uPA), the zymogen form of a protease associated with tumor metastasis and invasion. Activation of the plasminogen cascade, resulting in the destruction of extracellular matrix for cellular extravasation and migration, may be a function of tryptase activation of pro-urokinase plasminogen activator at the P4-P1 sequence of Pro-Arg-Phe-Lys (SEQ. ID NO: 4) (Stack, *et al.*, *Journal of Biological Chemistry* **269** (13): 9416-9419 (1994)). Vasoactive intestinal peptide, a neuropeptide that is implicated in the regulation of vascular permeability, is also cleaved by tryptase, primarily at the Thr-Arg-Leu-Arg (SEQ. ID NO: 5) sequence (Tam, *et al.*, *Am. J. Respir. Cell Mol. Biol.* **3**: 27-32 (1990)). The G-protein coupled receptor PAR-2 can be cleaved and activated by tryptase at the Ser-Lys-Gly-Arg (SEQ. ID NO: 6) sequence to drive fibroblast proliferation, whereas the thrombin activated receptor PAR-1 is inactivated by tryptase at the Pro-Asn-Asp-Lys (SEQ. ID NO: 7) sequence (Molino *et al.*, *Journal of Biological Chemistry* **272**(7): 4043-4049 (1997)). Taken together, this evidence suggests a central role for tryptase in tissue remodeling as a consequence of disease. This is consistent with the profound changes observed in several mast cell-mediated disorders. One hallmark of chronic asthma and other long-term respiratory diseases is fibrosis and thickening of the underlying tissues that could be the result of tryptase activation of its physiological targets. Similarly, a series of reports have shown angiogenesis to be associated with mast cell density, tryptase activity and poor prognosis in a variety of cancers (Coussens *et al.*, *Genes and Development* **13**(11): 1382-97 (1999)); Takanami *et al.*, *Cancer* **88**(12): 2686-92 (2000); Toth-Jakatics *et al.*, *Human Pathology* **31**(8): 955-960 (2000); Ribatti *et al.*, *International Journal of Cancer* **85**(2): 171-5 (2000)).

Methods are known in the art for evaluating whether a particular protease cleaves a selected peptide sequence. For example, the use of 7-amino-4-methyl coumarin (AMC) fluorogenic peptide substrates is a well-established method for the determination of protease specificity (Zimmerman, M., *et al.*, (1977) *Analytical Biochemistry* **78**:47-51). Specific cleavage of the anilide bond liberates the fluorogenic AMC leaving group allowing for the simple determination of cleavage rates for individual substrates. More recently, arrays (Lee, D., *et al.*, (1999) *Bioorganic and Medicinal Chemistry Letters* **9**:1667-72) and positional-scanning libraries (Rano, T.A., *et al.*, (1997) *Chemistry and*

Biology **4**:149-55) of AMC peptide substrate libraries have been employed to rapidly profile the N-terminal specificity of proteases by sampling a wide range of substrates in a single experiment. Thus, one of skill in the art may readily evaluate an array of peptide sequences to determine their utility in the present invention without resort to undue experimentation.

The drug-ligand conjugate of the current invention may optionally contain two or more linkers. These linkers may be the same or different. For example, a peptidyl linker may be used to connect the drug to the ligand and a second peptidyl linker may attach a diagnostic agent to the complex. Other uses for additional linkers include linking analytical agents, biomolecules, targeting agents, and detectable labels to the drug-ligand complex.

Also within the scope of the present invention are compounds of the invention that are poly- or multi-valent species, including, for example, species such as dimers, trimers, tetramers and higher homologs of the compounds of the invention or reactive analogues thereof. The poly- and multi-valent species can be assembled from a single species or more than one species of the invention. For example, a dimeric construct can be "homo-dimeric" or "heterodimeric." Moreover, poly- and multi-valent constructs in which a compound of the invention or a reactive analogue thereof, is attached to an oligomeric or polymeric framework (*e.g.*, polylysine, dextran, hydroxyethyl starch and the like) are within the scope of the present invention. The framework is preferably polyfunctional (*i.e.* having an array of reactive sites for attaching compounds of the invention). Moreover, the framework can be derivatized with a single species of the invention or more than one species of the invention.

Moreover, the present invention includes compounds that are functionalized to afford compounds having water-solubility that is enhanced relative to analogous compounds that are not similarly functionalized. Thus, any of the substituents set forth herein can be replaced with analogous radicals that have enhanced water solubility. For example, it is within the scope of the invention to, for example, replace a hydroxyl group with a diol, or an amine with a quaternary amine, hydroxy amine or similar more water-soluble moiety. In a preferred embodiment, additional water solubility is imparted by substitution at a site not essential for the activity towards the ion channel of the compounds set forth herein with a moiety that enhances the water solubility of the parent compounds. Methods of enhancing the water-solubility of organic compounds are known

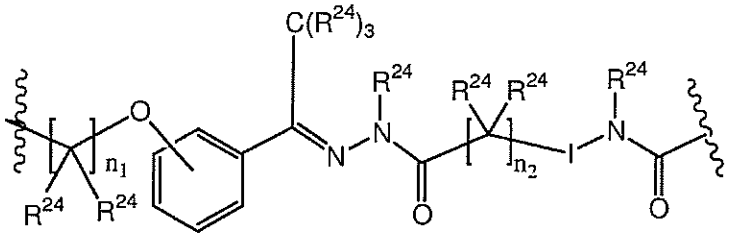
in the art. Such methods include, but are not limited to, functionalizing an organic nucleus with a permanently charged moiety, e.g., quaternary ammonium, or a group that is charged at a physiologically relevant pH, e.g. carboxylic acid, amine. Other methods include, appending to the organic nucleus hydroxyl- or amine-containing groups, e.g. alcohols, polyols, polyethers, and the like. Representative examples include, but are not limited to, polylysine, polyethyleneimine, poly(ethyleneglycol) and poly(propyleneglycol). Suitable functionalization chemistries and strategies for these compounds are known in the art. *See*, for example, Dunn, R.L., *et al.*, Eds. POLYMERIC DRUGS AND DRUG DELIVERY SYSTEMS, ACS Symposium Series Vol. 469, American Chemical Society, Washington, D.C. 1991.

Hydrazine Linkers (H)

In a second embodiment, the conjugate of the invention comprises a hydrazine self-immolative linker, wherein the conjugate has the structure:

$$X^4\text{——}(L^4)_p\text{—H—}(L^1)_m\text{——D}$$

wherein D, L^1, L^4, and X^4 are as defined above and described further herein, and H is a linker comprising the structure:



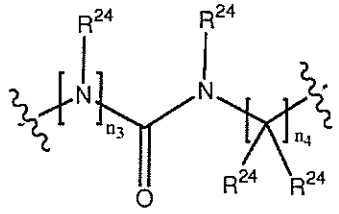
wherein

n_1 is an integer from 1 – 10;

n_2 is 0, 1, or 2;

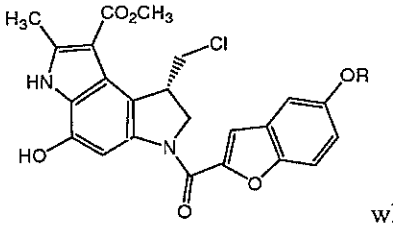
each R^{24} is a member independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, and unsubstituted heteroalkyl; and

I is either a bond (*i.e.*, the bond between the carbon of the backbone and the adjacent nitrogen) or:



wherein n_3 is 0 or 1, with the proviso that when n_3 is 0, n_2 is not 0; and n_4 is 1, 2, or 3,

wherein when I is a bond, n_1 is 3 and n_2 is 1, D can not be

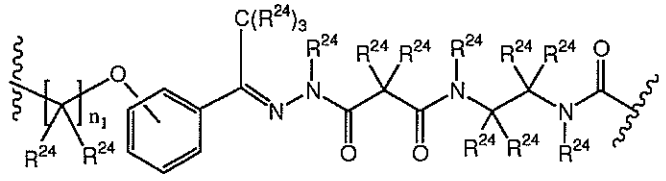


where R is Me or CH_2- CH_2-NMe_2.

In one embodiment, the substitution on the phenyl ring is a para substitution. In preferred embodiments, n_1 is 2, 3, or 4 or n_1 is 3. In preferred embodiments, n_2 is 1. In preferred embodiments, I is a bond (*i.e.*, the bond between the carbon of the backbone and the adjacent nitrogen). In one aspect, the hydrazine linker, H, can form a 6-membered self immolative linker upon cleavage, for example, when n_3 is 0 and n_4 is 2. In another aspect, the hydrazine linker, H, can form two 5-membered self immolative linkers upon cleavage. In yet other aspects, H forms a 5-membered self immolative linker, H forms a 7-membered self immolative linker, or H forms a 5-membered self immolative linker and a 6-membered self immolative linker, upon cleavage. The rate of cleavage is affected by the size of the ring formed upon cleavage. Thus, depending upon the rate of cleavage desired, an appopriate size ring to be formed upon cleavage can be selected.

Five Membered Hydrazine Linkers

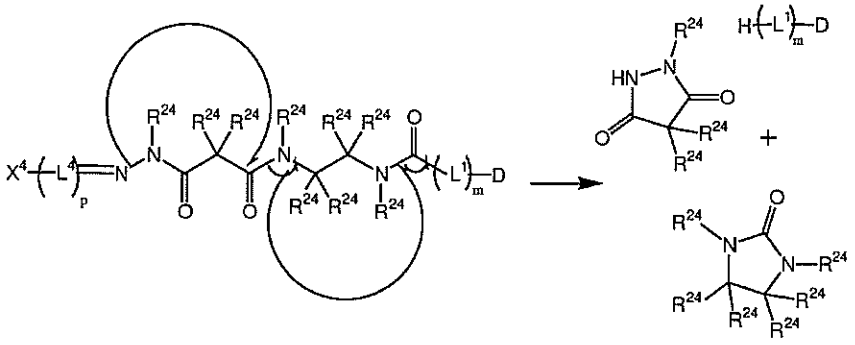
In one embodiment, the hydrazine linker comprises a 5-membered hydrazine linker, wherein H comprises the structure:



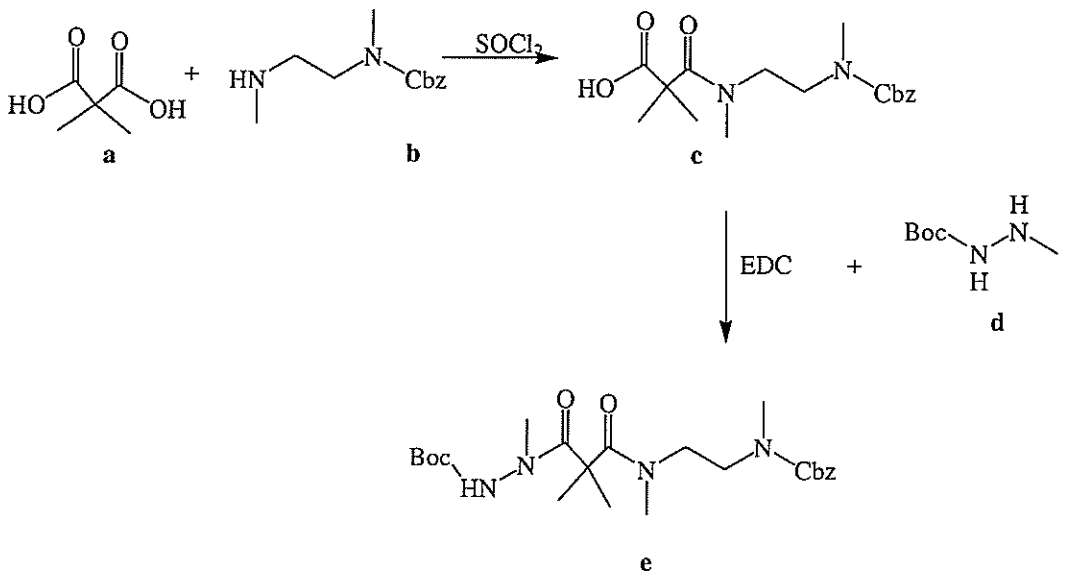
In a preferred embodiment, n_1 is 2, 3, or 4. In another preferred embodiment, n_1 is 3.

In the above structure, each R^{24} is a member independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, and unsubstituted heteroalkyl. In one embodiment, each R^{24} is independently H or a $C_1 - C_6$ alkyl. In another embodiment, each R^{24} is independently H or a $C_1 - C_3$ alkyl, more preferably H or CH_3. In another embodiment, at least one R^{24} is a methyl group. In another embodiment, each R_{24} is H. Each R^{24} is selected to tailor the compounds steric effects and for altering solubility.

The 5-membered hydrazine linkers can undergo one or more cyclization reactions that separate the drug from the linker, and can be described, for example, by:



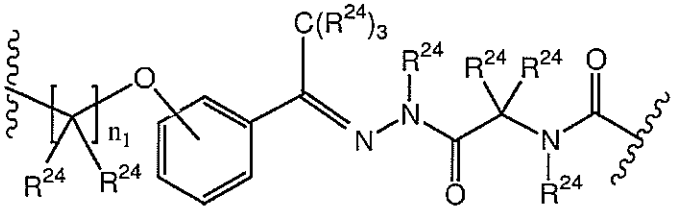
An exemplary synthetic route for preparing a five membered linker of the invention is:



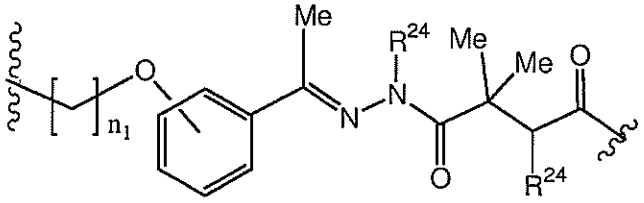
The Cbz-protected DMDA **b** is reacted with 2,2-Dimethyl-malonic acid **a** in solution with thionyl chloride to form a Cbz-DMDA-2,2-dimethylmalonic acid **c**. Compound **c** is reacted with Boc-N-methyl hydrazine **d** in the presence of EDC to form DMDA-2,2-dimetylmalonic-Boc-N-methylhydrazine **e**.

Six Membered Hydrazine Linkers

In another embodiment, the hydrazine linker comprises a 6-membered hydrazine linker, wherein H comprises the structure:

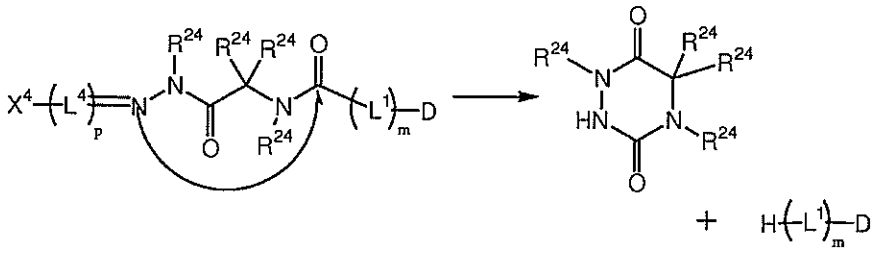


In a preferred embodiment, n_1 is 3. In the above structure, each R^{24} is a member independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, and unsubstituted heteroalkyl. In one embodiment, each R^{24} is independently H or a $C_1 - C_6$ alkyl. In another embodiment, each R^{24} is independently H or a $C_1 - C_3$ alkyl, more preferably H or CH_3. In another embodiment, at least one R^{24} is a methyl group. In another embodiment, each R_{24} is H. Each R^{24} is selected to tailor the compounds steric effects and for altering solubility. In a preferred embodiment, H comprises the structure:

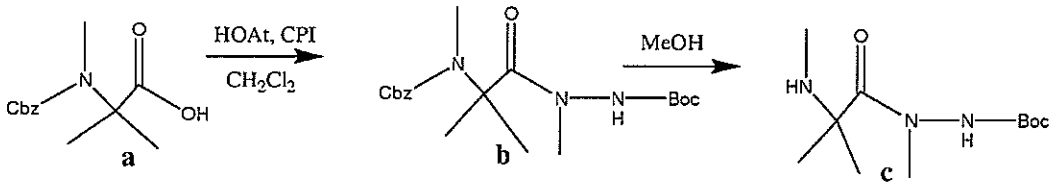


In one embodiment, H comprises a geminal dimethyl substitution. In one embodiment of the above structure, each R^{24} is independently an H or a substituted or unsubstituted alkyl.

The 6-membered hydrazine linkers will undergo a cyclization reaction that separates the drug from the linker, and can be described as:



An exemplary synthetic route for preparing a six membered linker of the invention is:

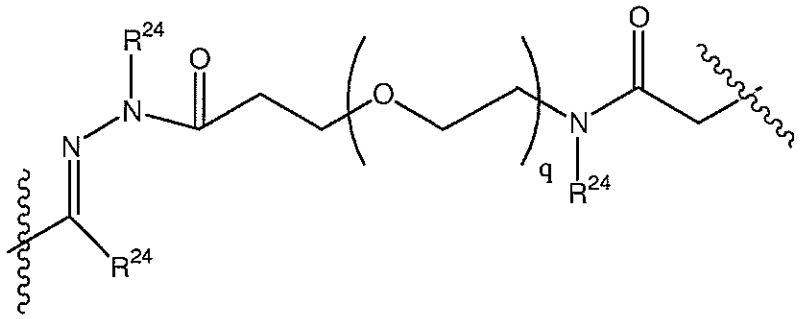


The Cbz-protected dimethyl alanine **a** in solution with dichlormethane, was reacted with HOAt, and CPI to form a Cbz-protected dimethylalanine hydrazine **b**. The hydrazine **b** is deprotected by the action of methanol, forming compound **c**.

Other Hydrazine Linkers

It is contemplated that the invention comprises a linker having seven members. This linker would likely not cyclize as quickly as the five or six membered linkers, but this may be preferred for some drug-ligand conjugates. Similarly, the hydrazine linker may comprise two six membered rings or a hydrazine linker having one six and one five membered cyclization products. A five and seven membered linker as well as a six and seven membered linker are also contemplated.

Another hydrazine structure, H, has the formula:

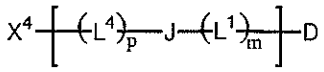


where q is 0, 1,2, 3, 4, 5, or 6; and

each R^{24} is a member independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, and unsubstituted heteroalkyl. This hydrazine structure can also form five-, six-, or seven-membered rings and additional components can be added to form multiple rings.

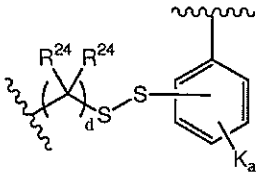
Disulfide Linkers (J)

In yet another embodiment, the linker comprises an enzymatically cleavable disulfide group. In one embodiment, the invention provides a cytotoxic drug-ligand compound having a structure according to Formula 3:



(3)

wherein D, L^1, L^4, and X^4 are as defined above and described further herein, and J is a disulfide linker comprising a group having the structure:



wherein

each R^{24} is a member independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, and unsubstituted heteroalkyl;

each K is a member independently selected from the group consisting of substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{21}R^{22}$, $NR^{21}COR^{22}$, $OCONR^{21}R^{22}$, $OCOR^{21}$, and OR^{21}

wherein

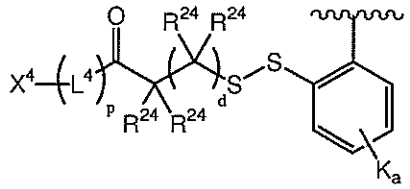
R^{21} and R^{22} are independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl and unsubstituted heterocycloalkyl;

a is an integer of 0,1, 2, 3, or 4; and

d is an integer of 0, 1, 2, 3, 4, 5, or 6.

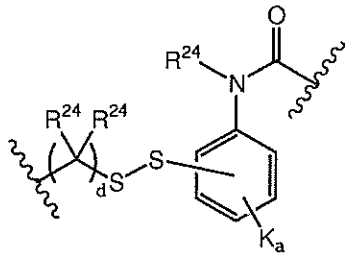
The aromatic ring of the disulfides linker may be substituted with one or more "K" groups. A "K" group is a substituent on the aromatic ring that replaces a hydrogen otherwise attached to one of the four non-substituted carbons that are part of the ring structure. The "K" group may be a single atom, such as a halogen, or may be a multi-atom group, such as alkyl, heteroalkyl, amino, nitro, hydroxy, alkoxy, haloalkyl, and cyano. Exemplary K substituents independently include, but are not limited to, F, Cl, Br, I, NO_2, OH, OCH_3, $NHCOCH_3$, $N(CH_3)_2$, $NHCOCF_3$ and methyl. For "Ka", a is an integer of 0, 1, 2, 3, or 4. In a specific embodiment, a is 0.

In a preferred embodiment, the linker comprises an enzymatically cleavable disulfide group of the following formula:

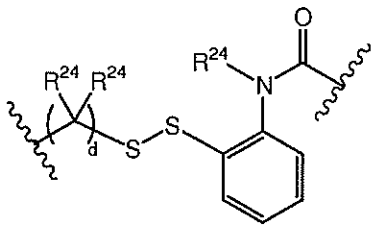


In this embodiment, the identities of L^4, X^4, p, and R^{24} are as described above, and d is 0, 1, 2, 3, 4, 5, or 6. In a particular embodiment, d is 1 or 2.

A more specific disulfide linker is shown in the formula below:

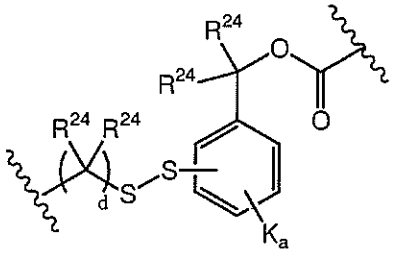


A specific example of this embodiment is as follows:

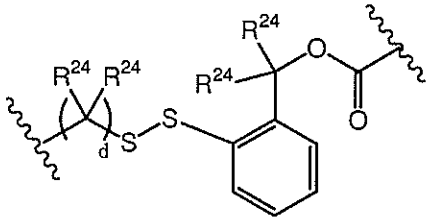


Preferably, d is 1 or 2.

Another disulfide linker is shown in the formula below:



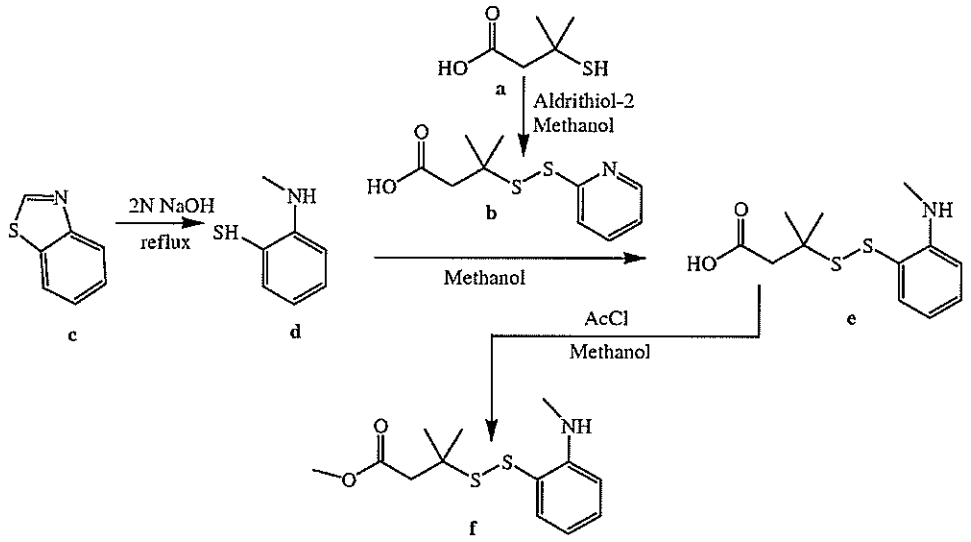
A specific example of this embodiment is as follows:



Preferably, d is 1 or 2.

In various embodiments, the disulfides are ortho to the amine. In another specific embodiment, a is 0. In preferred embodiments, R^{24} is independently selected from H and CH_3.

An exemplary synthetic route for preparing a disulfide linker of the invention is as follows:



A solution of 3-mercaptopropionic acid **a** is reacted with aldrithiol-2 to form 3-methyl benzothiazolium iodide **b**. 3-methylbenzothiazolium iodide **c** is reacted with sodium hydroxide to form compound **d**. A solution of compound **d** with methanol is further reacted with compound **b** to form compound **e**. Compound **e** deprotected by the action of acetyl chloride and methanol forming compound **f**.

DRUG-CLEAVABLE SUBSTRATE CONJUGATES

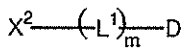
The drugs, depicted as "D" herein, can be provided in the current invention as part of a drug-cleavable substrate conjugate where the drug is linked to a cleavable substrate, X^2, optionally via a self-immolative linker, L^1. This conjugate can be a prodrug. The drug typically possesses a desired biological activity and contains a reactive functional group to link to the cleavable substrate. The desired biological activity includes the diagnosis, cure, mitigation, treatment, or prevention of disease in an animal such as a human. Preferred reactive functional groups include primary or secondary amines, hydroxyls, sulfhydryls, carboxyls, aldehydes, and ketones. More preferred reactive functional groups include hydroxyls, primary or secondary amines, sulfhydryls and carboxylic acid functional groups. Even more preferred reactive functional groups include hydroxyls, primary and secondary amines and carboxylic acid functional groups. The drug typically has at least one, but may have 2, 3, 4, 5, 6 or more reactive functional groups.

The drug-cleavable substrate conjugate is effective for the usual purposes for which the corresponding drugs are effective, but may have superior efficacy because of the ability to transport the drug to the desired cell (e.g, a tumor cell) where it is of particular benefit. For example, the drug may be selected to be activated at a site of tumor cells by conjugation to a tumor-specific cleavable substrate. These tumor specific drug-cleavable substrate conjugates have tumor specificity arising from the specificity of the cleavable substrate. The specificity can arise when the cleavable substrate is preferentially cleaved in or around the tumor cells. Examples include conjugates that are highly selective substrates for tumor specific enzymes or enzymes which are associated, naturally or artificially, with the tumor. These enzymes are present in the proximity of the tumor in sufficient amounts to generate cytotoxic levels of free drug in the vicinity of the tumor.

In another approach, referred to as antibody-directed enzyme prodrug therapy (ADEPT), an enzyme is attached to an antibody specific for a tumor antigen, to thereby direct the enzyme to the site of tumor cells. The drug is then conjugated to a substrate cleavable by the enzyme attached to the tumor-specific antibody. Thus, these drug-cleavable substrate conjugates have tumor specificity arising from the localization of the enzyme at the site of tumor cells through the attachment of the enzyme to the tumor-specific antibody.

One advantage of the drug-cleavable substrate complex is that they can be less toxic than the corresponding free drug; additionally, the specificity of the complex may allow for lower overall concentrations to be used relative to the free drug since the increased specificity will result in a higher percentage of the complex to be present at the tumor site.

In one embodiment, the invention provides a cytotoxic drug-cleavable substrate compound having a structure according to Formulas 2:



(2)

The symbol L^1 represents a self-immolative spacer where m is an integer of 0, 1, 2, 3, 4, 5, or 6. Preferably, M is 0, 1, or 2.

The symbol X^2 represents a cleavable substrate, preferably, an enzyme cleavable substrate.

DRUGS

Drugs, depicted as "D" herein, are provided in the current invention as part of a drug-ligand conjugate, where the drug is linked to a ligand through either a peptidyl or other linker, or as a conjugate with a cleavable substrate. The drug must possess a desired biological activity and contain a reactive functional group in order to link to the ligand. The desired biological activity includes the diagnosis, cure, mitigation, treatment, or prevention of disease in an animal such as a human. Thus, so long as it has the needed reactive functional group, the term "drug" refers to chemicals recognized as drugs in the official United States Pharmacopeia, official Homeopathic Pharmacopeia of the United States, or official National Formulary, or any supplement thereof. Exemplary drugs are set forth in the Physician's Desk Reference (PDR) and in the Orange Book maintained by the U.S. Food and Drug Administration (FDA). New drugs are being continually being discovered and developed, and the present invention provides that these new drugs may also be incorporated into the drug-ligand complex of the current invention.

Preferred functional groups include primary or secondary amines, hydroxyls, sulfhydryls, carboxyls, aldehydes, and ketones. More preferred functional groups include hydroxyls, primary or secondary amines, sulfhydryls and carboxylic acid functional groups. Even more preferred functional groups include hydroxyls, primary and secondary amines and carboxylic acid functional groups. The drug must have at least one, but may have 2, 3, 4, 5, 6 or more reactive functional groups. Additionally, a self-immolative spacer, L^1, may be incorporated between the reactive functional group of the drug and the peptide or other linker or cleavable substrate.

The drug-ligand conjugate is effective for the usual purposes for which the corresponding drugs are effective, but have superior efficacy because of the ability, inherent for at least some ligands, to transport the drug to the desired cell where it is of particular benefit.

Exemplary drugs include proteins, peptides, and small molecule drugs containing a functional group for linkage to the ligand. More specifically, these drugs include, for example, the enzyme inhibitors such as dihydrofolate reductase inhibitors, thymidylate synthase inhibitors, DNA intercalators, DNA cleavers, topoisomerase inhibitors, the anthracycline family of drugs, the vinca drugs, the mitomycins, the bleomycins, the cytotoxic nucleosides, the pteridine family of drugs, diynenes, the podophyllotoxins, differentiation inducers, and taxols.

Preferred drugs of the current invention include cytotoxic drugs useful in cancer therapy and other small molecules, proteins or polypeptides with desired biological activity, such as a toxin. The drug may be selected to be activated at a tumor cells by conjugation to a tumor-specific ligand. These tumor specific drug-ligand conjugates have tumor specificity arising from the specificity of the ligand. Examples of this are drug-ligand conjugates that are highly selective substrates for tumor specific enzymes, where these enzymes are present in the proximity of the tumor in sufficient amounts to generate cytotoxic levels of free drug in the vicinity of the tumor. One advantage of these tumor-specific drug-ligand complexes is that they are stable to adventitious proteases in the human serum. Another advantage of the drug-ligand complex is that they are less toxic than the corresponding free drug; additionally, the specificity of the complex may allow for lower overall concentrations to be used relative to the free drug since the increased specificity will result in a higher percentage of the complex to be present at the tumor site.

Cytotoxins

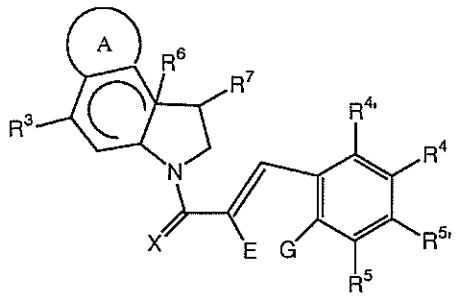
Cytotoxic drugs useful in the current invention include, for example, duocarmycins and CC-1065, and analogues thereof, including CBI (1,2,9,9a-tetrahydrocyclopropa[c]benz[e]indol-4-one)-based analogues, MCBI (7-methoxy-1,2,9,9a-tetra-hydrocyclopropa[c]benz[e]indol-4-one)-based analogues and CCBI (7-cyano-1,2,9,9a-tetra-hydrocyclo-propa[c]benz[e]-indol-4-one)-based analogues of the duocarmycins and CC-1065, doxorubicin and doxorubicin conjugates such as morpholino-doxorubicin and cyanomorpholino-doxorubicin, dolastatins such as dolastatin-10, combretastatin, calicheamicin, maytansine, maytansine analogs, DM-1, auristatin E, auristatin EB (AEB), auristatin EFP (AEFP), monomethyl auristatin E (MMAE), 5-benzoylvaleric acid-AE ester (AEVB), tubulysins, disorazole, epothilones , Paclitaxel, docetaxel, SN-38, Topotecan, rhizoxin, echinomycin, colchicine, vinblastin, vindesine, estramustine, cemadotin, eleutherobin, methotrexate, methopterin, dichloromethotrexate, 5-fluorouracil, 6-mercaptopurine, cytosine arabinoside, melphalan, leurosine, leurosideine, actinomycin, daunorubicin and daunorubicin conjugates, mitomycin C, mitomycin A, carminomycin, aminopterin, tallysomycin, podophyllotoxin and podophyllotoxin derivatives such as etoposide or etoposide phosphate, vincristine, taxol, taxotere retinoic acid, butyric acid, N^8 -acetyl spermidine, camptothecin, and their

analogues. Other known drugs may be modified in order to provide a functional group for conjugation to the linker described herein. Such chemical modification is known in the art.

Preferred cytotoxins for use in the current invention include: duocarmycins, CC-1065, and CCBI-based and MCBI-based analogues thereof, morpholino-doxorubicin, cyanomorpholino-doxorubicin, dolastatin-10, combretastatin, calicheamicin, maytansine, DM-1, auristatin E, AEB, AEFP, MMAE, Tubulysin A, Disorazole, epothilone A and epothilone B.

Particularly preferred cytotoxins of the present invention are active, potent duocarmycin derivatives and CC-1065. The parent agents are exceptionally potent antitumor antibiotics that derive their biological effects through the reversible, stereoelectronically controlled sequence selective alkylation of DNA (Boger *et al. J. Org. Chem.* **55**: 4499 (1990); Boger *et al. J. Am. Chem. Soc.* **112**: 8961 (1990); Boger *et al., J. Am. Chem. Soc.* **113**: 6645 (1991); Boger *et al. J. Am. Chem. Soc.* **115**: 9872 (1993); Boger *et al., Bioorg. Med. Chem. Lett.* **2**: 759 (1992)). Subsequent to the initial disclosure of the duocarmycins, extensive efforts have been devoted to elucidating the DNA alkylation selectivity of the duocarmycins and its structural origin.

A particularly preferred aspect of the current invention provides a cytotoxic compound having a structure according to Formula 7:



(7)

in which ring system A is a member selected from substituted or unsubstituted aryl substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl groups. Exemplary ring systems include phenyl and pyrrole.

The symbols E and G are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, a heteroatom, a single bond or E and G are optionally joined to form a ring system selected from substituted or

unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl.

The symbol X represents a member selected from O, S and NR^{23}. R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl.

The symbol R^3 represents a member selected from (=O), SR^{11}, NHR^{11} and OR^{11}, in which R^{11} is H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, monophosphates, diphosphates, triphosphates, sulfonates, acyl, $C(O)R^{12}$ R^{13}, $C(O)OR^{12}$, $C(O)NR^{12}R^{13}$, $P(O)(OR^{12})_2$, $C(O)CHR^{12}R^{13}$, SR^{12} or $SiR^{12}R^{13}R^{14}$. The symbols R^{12}, R^{13}, and R^{14} independently represent H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl, where R^{12} and R^{13} together with the nitrogen or carbon atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms. One or more of R^{12}, R^{13}, or R^{14} can include a cleaveable group within its structure.

R^4, $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, $CR^{15}{=}NR^{16}$, and $O(CH_2)_nN(CH_3)_2$, where n is an integer from 1 to 20, or any adjacent pair of R^4, $R^{4'}$, R^5 and $R^{5'}$, together with the carbon atoms to which they are attached, are joined to form a substituted or unsubstituted cycloalkyl or heterocycloalkyl ring system having from 4 to 6 members. R^{15} and R^{16} independently represent H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl and substituted or unsubstituted peptidyl, where R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms. One exemplarly structure is aniline.

R^4, $R^{4'}$, R^5, $R^{5'}$, R^{11}, R^{12}, R^{13}, R^{15} and R^{16} optionally contain one or more cleaveable groups within their structure, such as a cleavable linker or cleavable substrate. Exemplary cleaveable groups include, but are not limited to peptides, amino acids, hydrazines, disulfides, and cephalosporin derivatives.

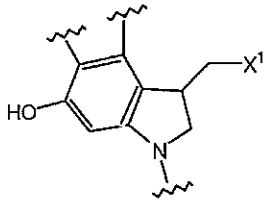
In some embodiments, at least one of R^4, $R^{4'}$, R^5, $R^{5'}$, R^{11}, R^{12}, R^{13}, R^{15} and R^{16} is used to join the drug to a linker or enzyme cleavable substrate of the present invention, as described herein, for example to L^1, if present or to F, H, J, or X^2.

In a still further exemplary embodiment, at least one of R^4, $R^{4'}$, R^5, $R^{5'}$, R^{11}, R^{12}, R^{13}, R^{15} and R^{16} bears a reactive group appropriate for conjugating the compound. In a further exemplary embodiment, R^4, $R^{4'}$, R^5, $R^{5'}$, R^{11}, R^{12}, R^{13}, R^{15} and R^{16} are independently selected from H, substituted alkyl and substituted heteroalkyl and have a reactive functional group at the free terminus of the alkyl or heteroalkyl moiety. One or more of R^4, $R^{4'}$, R^5, $R^{5'}$, R^{11}, R^{12}, R^{13}, R^{15} and R^{16} may be conjugated to another species, *e.g*, targeting agent, detectable label, solid support, etc.

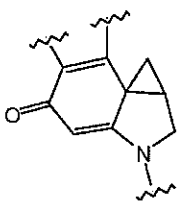
R^6 is a single bond which is either present or absent. When R^6 is present, R^6 and R^7 are joined to form a cyclopropyl ring. R^7 is CH_2-X^1 or -CH_2-. When R^7 is -CH_2- it is a component of the cyclopropane ring. The symbol X^1 represents a leaving group such as a halogen, for example Cl, Br or F. The combinations of R^6 and R^7 are interpreted in a manner that does not violate the principles of chemical valence.

X^1 may be any leaving group. Useful leaving groups include, but are not limited to, halogens, azides, sulfonic esters (e.g., alkylsulfonyl, arylsulfonyl), oxonium ions, alkyl perchlorates, ammonioalkanesulfonate esters, alkylfluorosulfonates and fluorinated compounds (*e.g*., triflates, nonaflates, tresylates) and the like. Particular halogens useful as leaving groups are F, Cl and Br. The choice of these and other leaving groups appropriate for a particular set of reaction conditions is within the abilities of those of skill in the art (*see*, for example, March J, ADVANCED ORGANIC CHEMISTRY, 2nd Edition, John Wiley and Sons, 1992; Sandler SR, Karo W, ORGANIC FUNCTIONAL GROUP PREPARATIONS, 2nd Edition, Academic Press, Inc., 1983; and Wade LG, COMPENDIUM OF ORGANIC SYNTHETIC METHODS, John Wiley and Sons, 1980).

The curved line within the six-membered ring indicates that the ring may have one or more degrees of unsaturation, and it may be aromatic. Thus, ring structures such as those set forth below, and related structures, are within the scope of Formula (8):

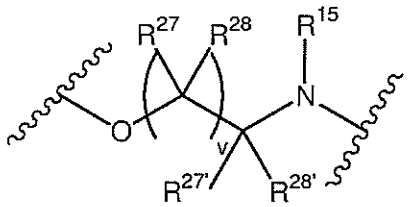


and



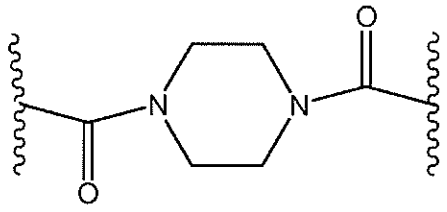
(8).

In some embodiments, at least one of R^4, $R^{4'}$, R^5, and $R^{5'}$ links said drug to L^1, if present, or to F, H, J, or X^2, and includes



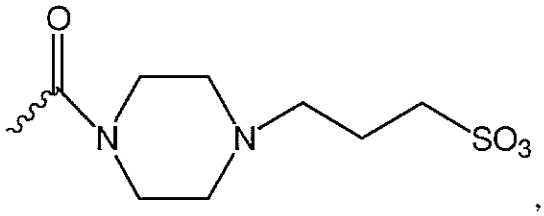
where v is an integer from 1 to 6; and each R^{27}, $R^{27'}$, R^{28}, and $R^{28'}$ is independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, and substituted or unsubstituted heterocycloalkyl. In some embodiments, R^{27}, $R^{27'}$, R^{28}, and $R^{28'}$ are all H. In some embodiments, v is an integer from 1 to 3 (preferably, 1). This unit can be used to separate aryl substituents from the drug and thereby resist or avoid generating compounds that are substrates for multi-drug resistance.

In one embodiment, R^{11} includes a moiety, X^5, that does not self-cyclize and links the drug to L^1, if present, or to F, H, J, or X^2. The moiety, X^5, is preferably cleavable using an enzyme and, when cleaved, provides the active drug. As an example, R^{11} can have the following structure (with the right side coupling to the remainder of the drug):

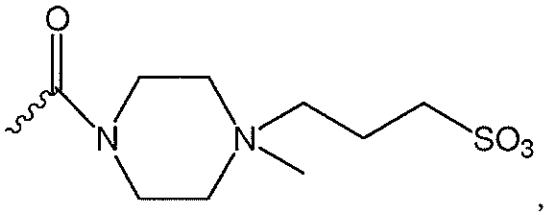


In an exemplary embodiment, ring system A of formula 7 is a substituted or unsubstituted phenyl ring. Ring system A may be substituted with one or more aryl group substituents as set forth in the definitions section herein. In some embodiments, the phenyl ring is substituted with a CN or methoxy moiety.

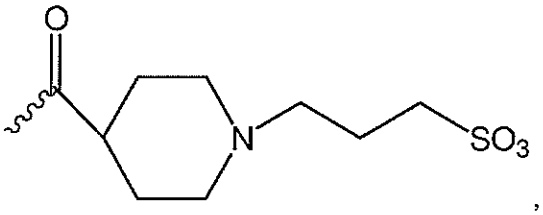
In some embodiments, at least one of R^4, $R^{4'}$, R^5, and $R^{5'}$ links said drug to L^1, if present, or to F, H, J, or X^2, and R^3 is selected from SR^{11}, NHR^{11} and OR^{11}. R^{11} is selected from $-SO(OH)_2$, $-PO(OH)_2$, $-AA_n$, $-Si(CH_3)_2C(CH_3)_3$, $-C(O)OPhNH(AA)_m$,



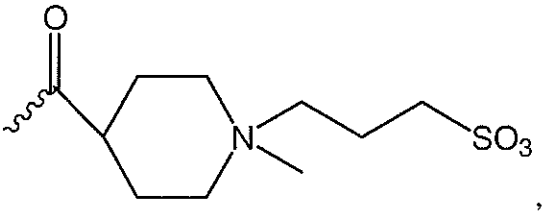
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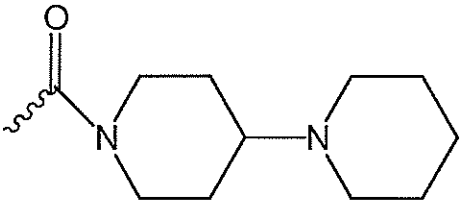
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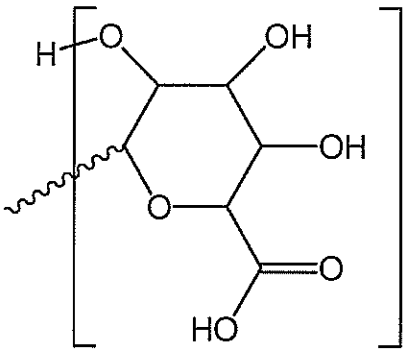
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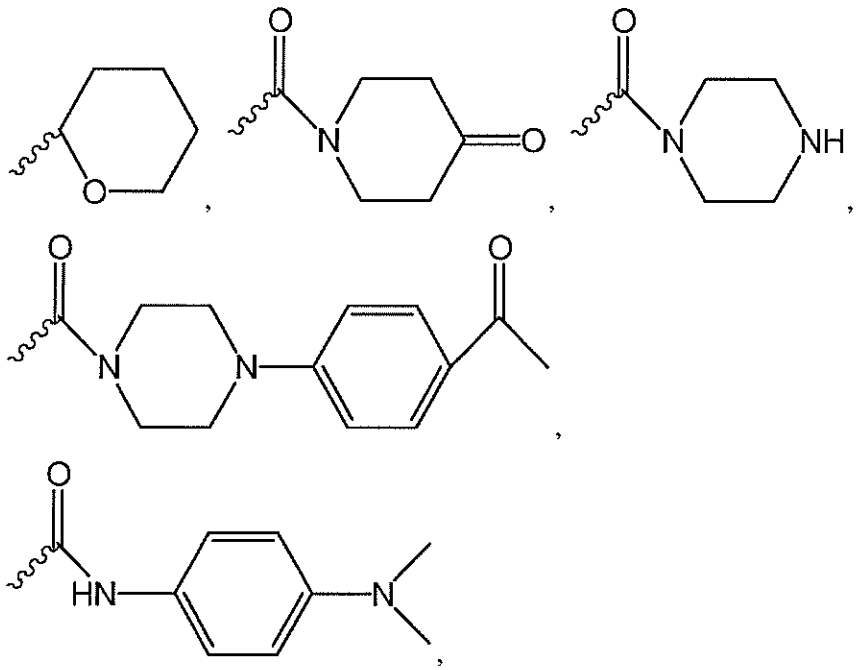
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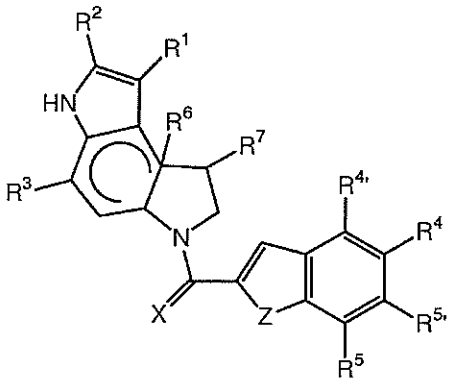


or any other sugar or combination of sugars,



and pharmaceutically acceptable salts thereof, where n is any integer in the range of 1 to 10, m is any integer in the range of 1 to 4, p is any integer in the range of 1 to 6, and AA is any natural or non-natural amino acid. In some embodiments, AA_n or AA_m is selected from the same amino acid sequences described above for the peptide linkers (F) and optionally is the same as the amino acid sequence used in the linker portion of R^4, $R^{4'}$, R^5, or $R^{5'}$. In at least some embodiments, R^3 is cleavable *in vivo* to provide an active drug compound. In at least some embodiments, R^3 increases *in vivo* solublility of the compound. In some embodiments, the rate of decrease of the concentration of the active drug in the blood is substantially faster than the rate of cleavage of R^3 to provide the active drug. This may be particularly useful where the toxicity of the active drug is substantially higher than that of the prodrug form. In other embodiments, the rate of cleavage of R^3 to provide the active drug is faster than the rate of decrease of concentration of the active drug in the blood.

In another exemplary embodiment, the invention provides a compound having a structure according to Formula 9:



(9).

In this embodiment, the identities of the substituents R^3, R^4, $R^{4'}$, R^5, $R^{5'}$, R^6, R^7 and X are substantially as described above for Formula 7, as well as preferences for particular embodiments. The symbol Z is a member independently selected from O, S and NR^{23}. The symbol R^{23} represents a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl. Each R^{23} is independently selected. The symbol R^1 represents H, substituted or unsubstituted lower alkyl, or $C(O)R^8$ or CO_2R^8. R^8 is a member selected from substituted alkyl, unsubstituted alkyl, NR^9R^{10}, NR^9NHR^{10} and OR^9. R^9 and R^{10} are independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl. R^2 is H, or substituted or unsubstituted lower alkyl. It is generally preferred that when R^2 is substituted alkyl, it is other than a perfluoroalkyl, e.g., CF_3. In one embodiment, R^2 is a substituted alkyl wherein the substitution is not a halogen. In another embodiment, R^2 is an unsubstituted alkyl.

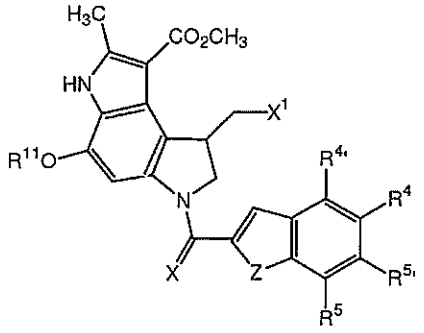
In some embodiments R^1 is an ester moiety, such as CO_2CH_3. In some embodiments, R^2 is a lower alkyl group, which may be substituted or unsubstituted. A presently preferred lower alkyl group is CH_3. In some preferred embodiments, R^1 is CO_2CH_3 and R^2 is CH_3.

In some embodiments, R^4, $R^{4'}$, R^5, and $R^{5'}$ are members independently selected from H, halogen, NH_2, OMe, $O(CH_2)_2N(R^{29})_2$ and NO_2. Each R^{29} is independently H or lower alkyl (e.g., methyl).

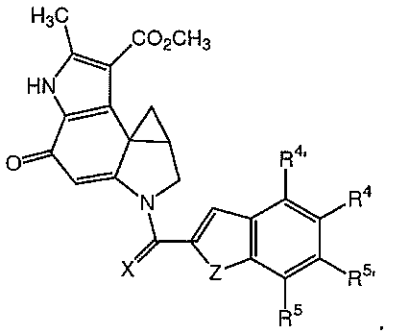
In some embodiments, the drug is selected such that the leaving group X^1 is a member selected from the group consisting of halogen, alkylsulfonyl, arylsulfonyl, and azide. In some embodiments, X^1 is F, Cl, or Br.

In some embodiments, Z is O or NH. In some embodiments, X is O.

In yet another exemplary embodiment, the invention provides compounds having a structure according to Formula 10 or 11:



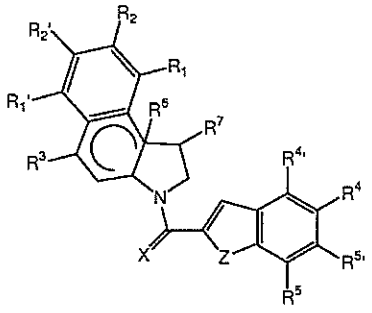
and



(10) (11)

Another preferred structure of the duocarmycin analog of Formula 7 is a structure in which the ring system A is an unsubstituted or substituted phenyl ring. The preferred substituents on the drug molecule described hereinabove for the structure of Formula 7 when the ring system A is a pyrrole are also preferred substituents when the ring system A is an unsubstituted or substituted phenyl ring.

For example, in a preferred embodiment, the drug (D) comprises a structure (12):



(12)

In this structure, R^3, R^6, R^7, X are as described above for Formula 7. Furthermore, Z is a member selected from O, S and NR^{23}, wherein R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^1 is H, substituted or unsubstituted lower alkyl, $C(O)R^8$, or CO_2R^8, wherein R^8 is a member selected from NR^9R^{10} and OR^9, in which R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

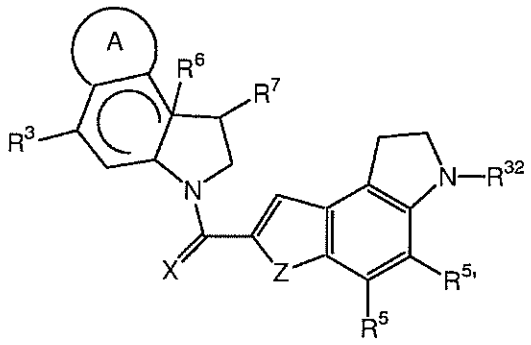
$R^{1'}$ is H, substituted or unsubstituted lower alkyl, or $C(O)R^8$, wherein R^8 is a member selected from NR^9R^{10} and OR^9, in which R^9 and R^{10} are members independently

selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

R^2 is H, or substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl or cyano or alkoxy; and $R^{2'}$ is H, or substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl.

At least one of R^4, $R^{4'}$, R^5, $R^{5'}$, R^{11}, R^{12}, R^{13}, R^{15} or R^{16} links the drug to L^1, if present, or to F, H, J, or X^2.

Another embodiment of the drug (D) comprises a structure (13) where R^4 and $R^{4'}$ have been joined to from a heterocycloalkyl:



(13)

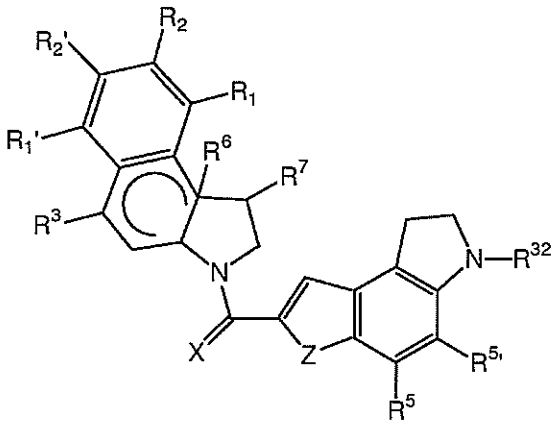
In this structure, R^3, R^5, $R^{5'}$, R^6, R^7, X are as described above for Formula 7. Furthermore, Z is a member selected from O, S and NR^{23}, wherein R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^{32} is selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, $CR^{15}{=}NR^{16}$, and $O(CH_2)_nN(CH_3)_2$, where n is an integer from 1 to 20. R^{15} and R^{16} independently represent H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl and substituted or unsubstituted peptidyl, where R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms. R^{32} optionally contains one or more cleaveable groups within its structure, such as a cleavable linker or cleavable substrate. Exemplary cleaveable groups include, but are not limited

to, peptides, amino acids, hydrazines, disulfides, and cephalosporin derivatives. Moreover, any selection of substituents described herein for R^4, $R^{4'}$, R^5, $R^{5'}$, R^{15}, and R^{16} is also applicable to R^{32}.

At least one of R^5, $R^{5'}$, R^{11}, R^{12}, R^{13}, R^{15}, R^{16}, or R^{32} links the drug to L^1, if present, or to F, H, J, or X^2. In at least some embodiments, R^{32} links the drug to L^1, if present, or to F, H, J, or X^2.

One preferred embodiment of this compound is:

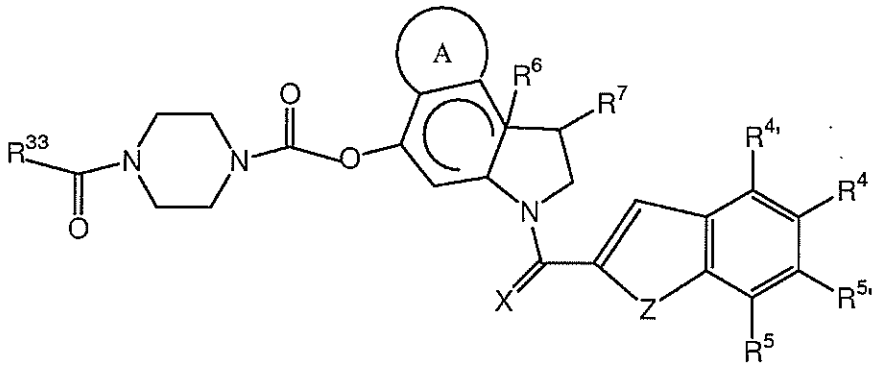


R^1 is H, substituted or unsubstituted lower alkyl, $C(O)R^8$, or CO_2R^8, wherein R^8 is a member selected from NR^9R^{10} and OR^9, in which R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

$R^{1'}$ is H, substituted or unsubstituted lower alkyl, or $C(O)R^8$, wherein R^8 is a member selected from NR^9R^{10} and OR^9, in which R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

R^2 is H, or substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl or cyano or alkoxy; and $R^{2'}$ is H, or substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl.

A further embodiment has the formula:



In this structure, A, R^6, R^7, X, R^4, $R^{4'}$, R^5, and $R^{5'}$ are as described above for Formula 7. Furthermore, Z is a member selected from O, S and NR^{23}, where R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

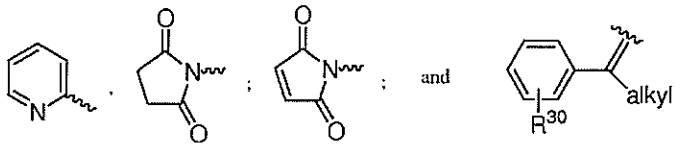
R^{33} is selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, $CR^{15}{=}NR^{16}$, and $O(CH_2)_nN(CH_3)_2$, where n is an integer from 1 to 20. R^{15} and R^{16} independently represent H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl and substituted or unsubstituted peptidyl, where R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms. R^{33} links the drug to L^1, if present, or to F, H, J, or X^2.

Preferably, A is substituted or unsubstituted phenyl or substituted or unsubstituted pyrrole. Moreover, any selection of substituents described herein for R^{11} is also applicable to R^{33}.

LIGANDS

X^4 represents a ligand selected from the group consisting of protected reactive functional groups, unprotected reactive functional groups, detectable labels, and targeting agents. Preferred ligands are targeting agents, such as antibodies and fragments thereof.

In some embodiments, the group X^4 can be described as a member selected from R^{29}, $COOR^{29}$, $C(O)NR^{29}$, and $C(O)NNR^{29}$ wherein R^{29} is a member selected from substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted heteroaryl. In yet another exemplary embodiment, R^{29} is a member selected from H; OH; $NHNH_2$;



where R^{30} represents substituted or unsubstituted alkyl terminated with a reactive functional group, substituted or unsubstituted heteroaryl terminated with a functional group. The above structures act as reactive protective groups that can be reacted with, for example, a side chain of an amino acid of a targeting agent, such as an antibody, to thereby link the targeting agent to the linker-drug moiety.

Targeting Agents

The linker arms and cytotoxins of the invention can be linked to targeting agents that selectively deliver a payload to a cell, organ or region of the body. Exemplary targeting agents such as antibodies (e.g., chimeric, humanized and human), ligands for receptors, lectins, saccharides, antibodies, and the like are recognized in the art and are useful without limitation in practicing the present invention. Other targeting agents include a class of compounds that do not include specific molecular recognition motifs include macromolecules such as poly(ethylene glycol), polysaccharide, polyamino acids and the like, which add molecular mass to the cytotoxin. The additional molecular mass affects the pharmacokinetics of the cytotoxin, e.g., serum half-life.

In an exemplary embodiment, the invention provides a cytotoxin, linker or cytotoxin-linker conjugate with a targeting agent that is a biomolecule, e.g, an antibody, receptor, peptide, lectin, saccharide, nucleic acid or a combination thereof.

Biomolecules useful in practicing the present invention can be derived from any source. The biomolecules can be isolated from natural sources or can be produced by synthetic methods. Proteins can be natural proteins or mutated proteins. Mutations can be effected by chemical mutagenesis, site-directed mutagenesis or other means of inducing mutations known to those of skill in the art. Proteins useful in practicing the

instant invention include, for example, enzymes, antigens, antibodies and receptors. Antibodies can be either polyclonal or monoclonal, but most preferably are monoclonal. Peptides and nucleic acids can be isolated from natural sources or can be wholly or partially synthetic in origin.

In a preferred embodiment, the targeting agent is an antibody, or antibody fragment, that is selected based on its specificity for an antigen expressed on a target cell, or at a target site, of interest. A wide variety of tumor-specific or other disease-specific antigens have been identified and antibodies to those antigens have been used or proposed for use in the treatment of such tumors or other diseases. The antibodies that are known in the art can be used in the conjugates of the invention, in particular for the treatment of the disease with which the target antigen is associated. Non-limiting examples of target antigens (and their associated diseases) to which an antibody-linker-drug conjugate of the invention can be targeted include: Her2 (breast cancer), CD20 (lymphomas), EGFR (solid tumors), CD22 (lymphomas, including non-Hodgkin's lymphoma), CD52 (chronic lymphocytic leukemia), CD33 (acute myelogenous leukemia), CD4 (lymphomas, autoimmune diseases, including rheumatoid arthritis), CD30 (lymphomas, including non-Hodgkin's lymphoma), Muc18 (melanoma), integrins (solid tumors), PSMA (prostate cancer, benign prostatic hyperplasia), CEA (colorectal cancer), CD11a (psoriasis), CD80 (psoriasis), CD23 (asthma), CD40L (immune thromobcytopenic purpura), CTLA4 (T cell lymphomas) and BLys (autoimmune diseases, including systemic lupus erythematosus).

In those embodiments wherein the recognition moiety is a protein or antibody, the protein can be tethered to a surface or a self assembled monolayer (SAM) component or connected through a spacer arm by any reactive peptide residue available on the surface of the protein. In preferred embodiments, the reactive groups are amines or carboxylates. In particularly preferred embodiments, the reactive groups are the ε-amine groups of lysine residues. Furthermore, these molecules can be adsorbed onto the surface of the substrate or SAM by non-specific interactions (*e.g.*, chemisorption, physisorption).

Recognition moieties which are antibodies can be used to recognize analytes which are proteins, peptides, nucleic acids, saccharides or small molecules such as drugs, herbicides, pesticides, industrial chemicals and agents of war. Methods of raising antibodies for specific molecules are well-known to those of skill in the art. *See*, United States Patents No. 5/147,786, issued to Feng *et al.* on September 15, 1992; No. 5/334,528, issued to Stanker *et al.* on August 2, 1994; No. 5/686,237, issued to Al-Bayati,

M.A.S. on November 11, 1997; and No. 5/573,922, issued to Hoess *et al.* on November 12, 1996. Methods for attaching antibodies to surfaces are also art-known. *See*, Delamarche *et al. Langmuir* **12:**1944-1946 (1996).

Targeting agents can be attached to the linkers of the invention by any available reactive group. For example, peptides can be attached through an amine, carboxyl, sulfhydryl, or hydroxyl group. Such a group can reside at a peptide terminus or at a site internal to the peptide chain. Nucleic acids can be attached through a reactive group on a base (*e.g.*, exocyclic amine) or an available hydroxyl group on a sugar moiety (*e.g.*, 3'- or 5'-hydroxyl). The peptide and nucleic acid chains can be further derivatized at one or more sites to allow for the attachment of appropriate reactive groups onto the chain. *See*, Chrisey *et al. Nucleic Acids Res.* **24:**3031-3039 (1996).

When the peptide or nucleic acid is a fully or partially synthetic molecule, a reactive group or masked reactive group can be incorporated during the process of the synthesis. Many derivatized monomers appropriate for reactive group incorporation in both peptides and nucleic acids are know to those of skill in the art. *See*, for example, THE PEPTIDES: ANALYSIS, SYNTHESIS, BIOLOGY, Vol. 2: "Special Methods in Peptide Synthesis," Gross, E. and Melenhofer, J., Eds., Academic Press, New York (1980). Many useful monomers are commercially available (Bachem, Sigma, *etc.*). This masked group can then be unmasked following the synthesis, at which time it becomes available for reaction with a component of a compound of the invention.

Exemplary nucleic acid targeting agents include aptamers, antisense compounds, and nucleic acids that form triple helices. Typically, a hydroxyl group of a sugar residue, an amino group from a base residue, or a phosphate oxygen of the nucleotide is utilized as the needed chemical functionality to couple the nucleotide-based targeting agent to the cytotoxin. However, one of skill in the art will readily appreciate that other "non-natural" reactive functionalities can be appended to a nucleic acid by conventional techniques. For example, the hydroxyl group of the sugar residue can be converted to a mercapto or amino group using techniques well known in the art.

Aptamers (or nucleic acid antibody) are single- or double-stranded DNA or single-stranded RNA molecules that bind specific molecular targets. Generally, aptamers function by inhibiting the actions of the molecular target, *e.g.*, proteins, by binding to the pool of the target circulating in the blood. Aptamers possess chemical functionality and thus, can covalently bond to cytotoxins, as described herein.

Although a wide variety of molecular targets are capable of forming non-covalent but specific associations with aptamers, including small molecules drugs, metabolites, cofactors, toxins, saccharide-based drugs, nucleotide-based drugs, glycoproteins, and the like, generally the molecular target will comprise a protein or peptide, including serum proteins, kinins, eicosanoids, cell surface molecules, and the like. Examples of aptamers include Gilead's antithrombin inhibitor GS 522 and its derivatives (Gilead Science, Foster City, Calif.). *See also*, Macaya *et al. Proc. Natl. Acad. Sci. USA* **90**:3745-9 (1993); Bock *et al. Nature (London)* **355**:564-566 (1992) and Wang *et al. Biochem.* **32**:1899-904 (1993).

Aptamers specific for a given biomolecule can be identified using techniques known in the art. *See, e.g.*, Toole *et al.* (1992) PCT Publication No. WO 92/14843; Tuerk and Gold (1991) PCT Publication No. WO 91/19813; Weintraub and Hutchinson (1992) PCT Publication No. 92/05285; and Ellington and Szostak, *Nature* **346**:818 (1990). Briefly, these techniques typically involve the complexation of the molecular target with a random mixture of oligonucleotides. The aptamer-molecular target complex is separated from the uncomplexed oligonucleotides. The aptamer is recovered from the separated complex and amplified. This cycle is repeated to identify those aptamer sequences with the highest affinity for the molecular target.

For diseases that result from the inappropriate expression of genes, specific prevention or reduction of the expression of such genes represents an ideal therapy. In principle, production of a particular gene product may be inhibited, reduced or shut off by hybridization of a single-stranded deoxynucleotide or ribodeoxynucleotide complementary to an accessible sequence in the mRNA, or a sequence within the transcript that is essential for pre-mRNA processing, or to a sequence within the gene itself. This paradigm for genetic control is often referred to as antisense or antigene inhibition. Additional efficacy is imparted by the conjugation to the nucleic acid of an alkylating agent, such as those of the present invention.

Antisense compounds are nucleic acids designed to bind and disable or prevent the production of the mRNA responsible for generating a particular protein. Antisense compounds include antisense RNA or DNA, single or double stranded, oligonucleotides, or their analogs, which can hybridize specifically to individual mRNA species and prevent transcription and/or RNA processing of the mRNA species and/or translation of the encoded polypeptide and thereby effect a reduction in the amount of the respective

encoded polypeptide. Ching *et al. Proc. Natl. Acad. Sci. U.S.A.* **86**:10006-10010 (1989); Broder *et al. Ann. Int. Med.* **113**:604-618 (1990); Loreau *et al. FEBS Letters* **274**:53-56 (1990); Holcenberg *et al.* WO91/11535; WO91/09865; WO91/04753; WO90/13641; WO 91/13080, WO 91/06629, and EP 386563). Due to their exquisite target sensitivity and selectivity, antisense oligonucleotides are useful for delivering therapeutic agents, such as the cytotoxins of the invention to a desired molecular target.

Others have reported that nucleic acids can bind to duplex DNA via triple helix formation and inhibit transcription and/or DNA synthesis. Triple helix compounds (also referred to as triple strand drugs) are oligonucleotides that bind to sequences of double-stranded DNA and are intended to inhibit selectively the transcription of disease-causing genes, such as viral genes, *e.g.*, HIV and herpes simplex virus, and oncogenes, *i.e.*, they stop protein production at the cell nucleus. These drugs bind directly to the double stranded DNA in the cell's genome to form a triple helix and prevent the cell from making a target protein. *See, e.g.*, PCT publications Nos. WO 92/10590, WO 92/09705, WO91/06626, and U.S. Pat. No. 5,176,996. Thus, the cytotoxins of the present invention are also conjugated to nucleic acid sequences that form triple helices.

The site specificity of nucleic acids (*e.g.*, antisense compounds and triple helix drugs) is not significantly affected by modification of the phosphodiester linkage or by chemical modification of the oligonucleotide terminus. Consequently, these nucleic acids can be chemically modified; enhancing the overall binding stability, increasing the stability with respect to chemical degradation, increasing the rate at which the oligonucleotides are transported into cells, and conferring chemical reactivity to the molecules. The general approach to constructing various nucleic acids useful in antisense therapy has been reviewed by van der Krol *et al.*, *Biotechniques* **6**:958-976 (1988) and Stein *et al. Cancer Res.* **48**:2659-2668 (1988). Therefore, in an exemplary embodiment, the cytotoxins of the invention are conjugated to a nucleic acid by modification of the phosphodiester linkage.

Moreover, aptamers, antisense compounds and triple helix drugs bearing cytotoxins of the invention can also can include nucleotide substitutions, additions, deletions, or transpositions, so long as specific hybridization to or association with the relevant target sequence is retained as a functional property of the oligonucleotide. For example, some embodiments will employ phosphorothioate analogs which are more resistant to degradation by nucleases than their naturally occurring phosphate diester

counterparts and are thus expected to have a higher persistence in vivo and greater potency (*see, e.g.*, Campbell *et al., J. Biochem. Biophys. Methods* **20**:259-267(1990)). Phosphoramidate derivatives of oligonucleotides also are known to bind to complementary polynucleotides and have the additional capability of accommodating covalently attached ligand species and will be amenable to the methods of the present invention. *See*, for example, Froehler *et al., Nucleic Acids Res.* **16**(11):4831 (1988).

In some embodiments the aptamers, antisense compounds and triple helix drugs will comprise O-methylribonucleotides (EP Publication No. 360609). Chimeric oligonucleotides may also be used (Dagle *et al., Nucleic Acids Res.* **18**: 4751 (1990)). For some applications, antisense oligonucleotides and triple helix may comprise polyamide nucleic acids (Nielsen *et al., Science* **254**: 1497 (1991) and PCT publication No. WO 90/15065) or other cationic derivatives (Letsinger *et al., J. Am. Chem. Soc.* **110**: 4470-4471 (1988)). Other applications may utilize oligonucleotides wherein one or more of the phosphodiester linkages has been substituted with an isosteric group, such as a 2-4 atom long internucleoside linkage as described in PCT publication Nos. WO 92/05186 and 91/06556, or a formacetal group (Matteucci *et al., J. Am. Chem. Soc.* **113**: 7767-7768 (1991)) or an amide group (Nielsen *et al., Science* **254**: 1497-1500 (1991)).

In addition, nucleotide analogs, for example wherein the sugar or base is chemically modified, can be employed in the present invention. "Analogous" forms of purines and pyrimidines are those generally known in the art, many of which are used as chemotherapeutic agents. An exemplary but not exhaustive list includes 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl- 2-thiouracil, 5-carboxymethylaminomethyluracil, dihydrouracil, inosine, N^6 -isopentenyladenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N^6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, β-D-mannosylqueosine, 5'methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N^6 -isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), pseudouracil, queosine, 2-thiocytosine, and 2,6-diaminopurine. In addition, the conventional bases by halogenated bases. Furthermore, the 2'-furanose position on the

base can have a non-charged bulky group substitution. Examples of non-charged bulky groups include branched alkyls, sugars and branched sugars.

Terminal modification also provides a useful procedure to conjugate the cytotoxins to the nucleic acid, modify cell type specificity, pharmacokinetics, nuclear permeability, and absolute cell uptake rate for oligonucleotide pharmaceutical agents. For example, an array of substitutions at the 5' and 3' ends to include reactive groups are known, which allow covalent attachment of the cytotoxins. See, *e.g.*, OLIGODEOXYNUCLEOTIDES: ANTISENSE INHIBITORS OF GENE EXPRESSION, (1989) Cohen, Ed., CRC Press; PROSPECTS FOR ANTISENSE NUCLEIC ACID THERAPEUTICS FOR CANCER AND AIDS, (1991), Wickstrom, Ed., Wiley-Liss; GENE REGULATION: BIOLOGY OF ANTISENSE RNA AND DNA, (1992) Erickson and Izant, Eds., Raven Press; and ANTISENSE RNA AND DNA, (1992), Murray, Ed., Wiley-Liss. For general methods relating to antisense compounds, *see*, ANTISENSE RNA AND DNA, (1988), D. A. Melton, Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.).

In at least one embodiment, the C-terminal end of an antibody is modified by the introduction of a cysteine residue as is described in U.S. Provisional Application Serial No. 60/957,271, which is hereby incorporated by reference in its entirety. Such modifications include, but are not limited to, the replacement of an existing amino acid residue at or near the C-terminus of a full-length heavy chain sequence, as well as the introduction of a cysteine-containing extension to the c-terminus of a full-length heavy chain sequence. In one embodiment, the cysteine-containing extension comprises the sequence alanine-alanine-cysteine (from N-terminal to C-terminal).

In at least some embodiments the presence of such C-terminal cysteine modifications provide a location for conjugation of a compound, such as a therapeutic agent or a marker molecule. In particular, the presence of a reactive thiol group, due to the C-terminal cysteine modification, can be used to conjugate a compound employing the linkers described in detail above. Conjugation of the antibody to a partner molecule in this manner allows for increased control over the specific site of attachment. Furthermore, by introducing the site of attachment at or near the C-terminus, conjugation can be optimized such that it reduces or eliminates interference with the antibody's functional properties, and allows for simplified analysis and quality control of conjugate preparations.

Detectable Labels

The particular label or detectable group used in conjunction with the compounds and methods of the invention is generally not a critical aspect of the invention, as long as it does not significantly interfere with the activity or utility of the compound of the invention. The detectable group can be any material having a detectable physical or chemical property. Such detectable labels have been well developed in the field of immunoassays and, in general, most any label useful in such methods can be applied to the present invention. Thus, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include magnetic beads (e.g., DYNABEADS™), fluorescent dyes (*e.g.*, fluorescein isothiocyanate, Texas red, rhodamine, and the like), radiolabels (*e.g.*, ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P), enzymes (*e.g.*, horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic beads (*e.g.*, polystyrene, polypropylene, latex, etc.).

The label may be coupled directly or indirectly to a compound of the invention according to methods well known in the art. As indicated above, a wide variety of labels may be used, with the choice of label depending on sensitivity required, ease of conjugation with the compound, stability requirements, available instrumentation, and disposal provisions.

When the compound of the invention is conjugated to a detectable label, the label is preferably a member selected from the group consisting of radioactive isotopes, fluorescent agents, fluorescent agent precursors, chromophores, enzymes and combinations thereof. Methods for conjugating various groups to antibodies are well known in the art. For example, a detectable label that is frequently conjugated to an antibody is an enzyme, such as horseradish peroxidase, alkaline phosphatase, β-galactosidase, and glucose oxidase.

Non-radioactive labels are often attached by indirect means. Generally, a ligand molecule (*e.g.*, biotin) is covalently bound to a component of the conjugate. The ligand then binds to another molecules (*e.g.*, streptavidin) molecule, which is either inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound.

Components of the conjugates of the invention can also be conjugated directly to signal generating compounds, *e.g.*, by conjugation with an enzyme or fluorophore. Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidotases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, *etc*. Chemiluminescent compounds include luciferin, and 2,3-dihydrophthalazinediones, *e.g.*, luminol. For a review of various labeling or signal producing systems that may be used, *see*, U.S. Patent No. 4,391,904.

Means of detecting labels are well known to those of skill in the art. Thus, for example, where the label is a radioactive label, means for detection include a scintillation counter or photographic film as in autoradiography. Where the label is a fluorescent label, it may be detected by exciting the fluorochrome with the appropriate wavelength of light and detecting the resulting fluorescence. The fluorescence may be detected visually, by means of photographic film, by the use of electronic detectors such as charge coupled devices (CCDs) or photomultipliers and the like. Similarly, enzymatic labels may be detected by providing the appropriate substrates for the enzyme and detecting the resulting reaction product. Finally simple colorimetric labels may be detected simply by observing the color associated with the label. Thus, in various dipstick assays, conjugated gold often appears pink, while various conjugated beads appear the color of the bead.

Fluorescent labels are presently preferred as they have the advantage of requiring few precautions in handling, and being amenable to high-throughput visualization techniques (optical analysis including digitization of the image for analysis in an integrated system comprising a computer). Preferred labels are typically characterized by one or more of the following: high sensitivity, high stability, low background, low environmental sensitivity and high specificity in labeling. Many fluorescent labels are commercially available from the SIGMA chemical company (Saint Louis, MO), Molecular Probes (Eugene, OR), R&D systems (Minneapolis, MN), Pharmacia LKB Biotechnology (Piscataway, NJ), CLONTECH Laboratories, Inc. (Palo Alto, CA), Chem Genes Corp., Aldrich Chemical Company (Milwaukee, WI), Glen Research, Inc., GIBCO BRL Life Technologies, Inc. (Gaithersburg, MD), Fluka Chemica- Biochemika Analytika (Fluka Chemie AG, Buchs, Switzerland), and Applied Biosystems (Foster City, CA), as well as many other commercial sources known to one of skill. Furthermore, those of skill in the art will recognize how to select an appropriate fluorophore for a particular

application and, if it not readily available commercially, will be able to synthesize the necessary fluorophore de novo or synthetically modify commercially available fluorescent compounds to arrive at the desired fluorescent label.

In addition to small molecule fluorophores, naturally occurring fluorescent proteins and engineered analogues of such proteins are useful in the present invention. Such proteins include, for example, green fluorescent proteins of cnidarians (Ward *et al.*, *Photochem. Photobiol.* **35**:803-808 (1982); Levine *et al.*, *Comp. Biochem. Physiol.*, **72B**:77-85 (1982)), yellow fluorescent protein from Vibrio *fischeri* strain (Baldwin *et al.*, *Biochemistry* **29**:5509-15 (1990)), Peridinin-chlorophyll from the dinoflagellate Symbiodinium sp. (Morris *et al.*, *Plant Molecular Biology* **24**:673:77 (1994)), phycobiliproteins from marine cyanobacteria, such as Synechococcus, *e.g.*, phycoerythrin and phycocyanin (Wilbanks *et al.*, *J. Biol. Chem.* **268**:1226-35 (1993)), and the like.

Generally, prior to forming the linkage between the cytotoxin and the targeting (or other) agent, and optionally, the spacer group, at least one of the chemical functionalities will be activated. One skilled in the art will appreciate that a variety of chemical functionalities, including hydroxy, amino, and carboxy groups, can be activated using a variety of standard methods and conditions. For example, a hydroxyl group of the cytotoxin or targeting agent can be activated through treatment with phosgene to form the corresponding chloroformate, or p-nitrophenylchloroformate to form the corresponding carbonate.

In an exemplary embodiment, the invention makes use of a targeting agent that includes a carboxyl functionality. Carboxyl groups may be activated by, for example, conversion to the corresponding acyl halide or active ester. This reaction may be performed under a variety of conditions as illustrated in March, supra pp. 388-89. In an exemplary embodiment, the acyl halide is prepared through the reaction of the carboxyl-containing group with oxalyl chloride. The activated agent is reacted with a cytotoxin or cytotoxin-linker arm combination to form a conjugate of the invention. Those of skill in the art will appreciate that the use of carboxyl-containing targeting agents is merely illustrative, and that agents having many other functional groups can be conjugated to the linkers of the invention.

Reactive Functional Groups

For clarity of illustration the succeeding discussion focuses on the conjugation of a cytotoxin of the invention to a targeting agent. The focus exemplifies one embodiment of the invention from which, others are readily inferred by one of skill in the art. No limitation of the invention is implied, by focusing the discussion on a single embodiment.

Exemplary compounds of the invention bear a reactive functional group, which is generally located on a substituted or unsubstituted alkyl or heteroalkyl chain, allowing their facile attachment to another species. A convenient location for the reactive group is the terminal position of the chain.

Reactive groups and classes of reactions useful in practicing the present invention are generally those that are well known in the art of bioconjugate chemistry. The reactive functional group may be protected or unprotected, and the protected nature of the group may be changed by methods known in the art of organic synthesis. Currently favored classes of reactions available with reactive cytotoxin analogues are those which proceed under relatively mild conditions. These include, but are not limited to nucleophilic substitutions (*e.g.*, reactions of amines and alcohols with acyl halides, active esters), electrophilic substitutions (*e.g.*, enamine reactions) and additions to carbon-carbon and carbon-heteroatom multiple bonds (*e.g.*, Michael reaction, Diels-Alder addition). These and other useful reactions are discussed in, for example, March, ADVANCED ORGANIC CHEMISTRY, 3rd Ed., John Wiley & Sons, New York, 1985; Hermanson, BIOCONJUGATE TECHNIQUES, Academic Press, San Diego, 1996; and Feeney *et al.*, MODIFICATION OF PROTEINS; Advances in Chemistry Series, Vol. 198, American Chemical Society, Washington, D.C., 1982.

Exemplary reaction types include the reaction of carboxyl groups and various derivatives thereof including, but not limited to, N-hydroxysuccinimide esters, N-hydroxybenztriazole esters, acid halides, acyl imidazoles, thioesters, p-nitrophenyl esters, alkyl, alkenyl, alkynyl and aromatic esters. Hydroxyl groups can be converted to esters, ethers, aldehydes, *etc*. Haloalkyl groups are converted to new species by reaction with, for example, an amine, a carboxylate anion, thiol anion, carbanion, or an alkoxide ion. Dienophile (e.g., maleimide) groups participate in Diels-Alder. Aldehyde or ketone groups can be converted to imines, hydrazones, semicarbazones or oximes, or via such mechanisms as Grignard addition or alkyllithium addition. Sulfonyl halides react readily with amines, for example, to form sulfonamides. Amine or sulfhydryl groups are, for

example, acylated, alkylated or oxidized. Alkenes, can be converted to an array of new species using cycloadditions, acylation, Michael addition, *etc.* Epoxides react readily with amines and hydroxyl compounds.

One skilled in the art will readily appreciate that many of these linkages may be produced in a variety of ways and using a variety of conditions. For the preparation of esters, *see, e.g.*, March *supra* at 1157; for thioesters, *see*, March, *supra* at 362-363, 491, 720-722, 829, 941, and 1172; for carbonates, *see*, March, *supra* at 346-347; for carbamates, *see*, March, *supra* at 1156-57; for amides, *see*, March *supra* at 1152; for ureas and thioureas, *see*, March *supra* at 1174; for acetals and ketals, *see*, Greene *et al.* *supra* 178-210 and March *supra* at 1146; for acyloxyalkyl derivatives, *see*, PRODRUGS: TOPICAL AND OCULAR DRUG DELIVERY, K. B. Sloan, ed., Marcel Dekker, Inc., New York, 1992; for enol esters, *see*, March supra at 1160; for N-sulfonylimidates, *see*, Bundgaard *et al.*, *J. Med. Chem.*, **31**:2066 (1988); for anhydrides, *see*, March *supra* at 355-56, 636-37, 990-91, and 1154; for N-acylamides, *see*, March *supra* at 379; for N-Mannich bases, *see*, March *supra* at 800-02, and 828; for hydroxymethyl ketone esters, *see*, Petracek *et al. Annals NY Acad. Sci.*, **507**:353-54 (1987); for disulfides, *see*, March *supra* at 1160; and for phosphonate esters and phosphonamidates.

The reactive functional groups can be unprotected and chosen such that they do not participate in, or interfere with, the reactions. Alternatively, a reactive functional group can be protected from participating in the reaction by the presence of a protecting group. Those of skill in the art will understand how to protect a particular functional group from interfering with a chosen set of reaction conditions. For examples of useful protecting groups, *See* Greene *et al.*, PROTECTIVE GROUPS IN ORGANIC SYNTHESIS, John Wiley & Sons, New York, 1991.

Typically, the targeting agent is linked covalently to a cytotoxin using standard chemical techniques through their respective chemical functionalities. Optionally, the linker or agent is coupled to the agent through one or more spacer groups. The spacer groups can be equivalent or different when used in combination.

Generally, prior to forming the linkage between the cytotoxin and the reactive functional group, and optionally, the spacer group, at least one of the chemical functionalities will be activated. One skilled in the art will appreciate that a variety of chemical functionalities, including hydroxy, amino, and carboxy groups, can be activated using a variety of standard methods and conditions. In an exemplary embodiment, the

invention comprises a carboxyl functionality as a reactive functional group. Carboxyl groups may be activated as described hereinabove.

CLEAVABLE SUBSTRATE

The cleavable substrates of the current invention are depicted as "X^2". Preferably, the cleavable substrate is a cleavable enzyme substrate that can be cleaved by an enzyme. Preferably, the enzyme is preferentially associated, directly or indirectly, with the tumor or other target cells to be treated. The enzyme may be generated by the tumor or other target cells to be treated. For example, the cleavable substrate can be a peptide that is preferentially cleavable by an enzyme found around or in a tumor or other target cell. Additionally or alternatively, the enzyme can be attached to a targeting agent that binds specifically to tumor cells, such as an antibody specific for a tumor antigen.

As examples of enzyme cleavable substrates suitable for coupling to the drugs described above, PCT Patent Applications Publication Nos. WO 00/33888, WO 01/95943, WO 01/95945, WO 02/00263, and WO 02/100353, all of which are incorporated herein by reference, disclose attachment of a cleavable peptide to a drug. The peptide is cleavable by an enzyme, such as a trouase (such as thimet oligopeptidase), CD10 (neprilysin), a matrix metalloprotease (such as MMP2 or MMP9), a type II transmembrane serine protease (such as Hepsin, testisin, TMPRSS4, or matriptase/MT-SP1), or a cathepsin, associated with a tumor. In this embodiment, a prodrug includes the drug as described above, a peptide, a stabilizing group, and optionally a linking group between the drug and the peptide. The stabilizing group is attached to the end of the peptide to protect the prodrug from degradation before arriving at the tumor or other target cell. Examples of suitable stabilizing groups include non-amino acids, such as succinic acid, diglycolic acid, maleic acid, polyethylene glycol, pyroglutamic acid, acetic acid, naphthylcarboxylic acid, terephthalic acid, and glutaric acid derivatives; as well as non-genetically-coded amino acids or aspartic acid or glutamic acid attached to the N-terminus of the peptide at the β-carboxy group of aspartic acid or the γ-carboxyl group of glutamic acid.

The peptide typically includes 3-12 (or more) amino acids. The selection of particular amino acids will depend, at least in part, on the enzyme to be used for cleaving the peptide, as well as, the stability of the peptide *in vivo*. One example of a suitable cleavable peptide is β–AlaLeuAlaLeu (SEQ ID NO. 2). This can be combined with a

stabilizing group to form succinyl-β–AlaLeuAlaLeu (SEQ ID NO: 2). Other examples of suitable cleavable peptides are provided in the references cited above.

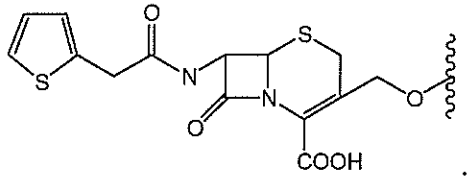
As one illustrative example, CD10, also known as neprilysin, neutral endopeptidase (NEP), and common acute lymphoblastic leukemia antigen (CALLA), is a type II cell-surface zinc-dependent metalloprotease. Cleavable substrates suitable for use with CD10 include LeuAlaLeu and IleAlaLeu. Other known substrates for CD10 include peptides of up to 50 amino acids in length, although catalytic efficiency often declines as the substrate gets larger.

Another illustrative example is based on matrix metalloproteases (MMP). Probably the best characterized proteolytic enzymes associated with tumors, there is a clear correlation of activation of MMPs within tumor microenvironments. In particular, the soluble matrix enzymes MMP2 (gelatinase A) and MMP9 (gelatinase B), have been intensively studied, and shown to be selectively activated during tissue remodeling including tumor growth. Peptide sequences designed to be cleaved by MMP2 and MMP9 have been designed and tested for conjugates of dextran and methotrexate (Chau *et al.*, *Bioconjugate Chem.* 15:931-941 (2004)); PEG (polyethylene glycol) and doxorubicin (Bae *et al.*, *Drugs Exp. Clin. Res.* 29:15-23 (2004)); and albumin and doxorubicin (Kratz *et al.*, *Bioorg. Med. Chem. Lett.* 11:2001-2006 (2001)). Examples of suitable sequences for use with MMPs include, but are not limited to, ProValGlyLeuIleGly (SEQ. ID NO. 8), GlyProLeuGlyVal (SEQ. ID NO. 9), GlyProLeuGlyIleAlaGlyGln (SEQ. ID NO. 10), ProLeuGlyLeu (SEQ. ID NO. 11), GlyProLeuGlyMetLeuSerGln (SEQ. ID NO. 12), and GlyProLeuGlyLeuTrpAlaGln (SEQ. ID NO. 13). (*See, e.g.*, the previously cited references as well as Kline *et al.*, *Mol. Pharmaceut.* 1:9-22 (2004) and Liu *et al.*, *Cancer Res.* 60:6061-6067 (2000).) Other cleavable substrates can also be used.

Yet another example is type II transmembrane serine proteases. This group of enzymes includes, for example, hepsin, testisin, and TMPRSS4. GlnAlaArg is one substrate sequence that is useful with matriptase/MT-SP1 (which is over-expressed in breast and ovarian cancers) and LeuSerArg is useful with hepsin (over-expressed in prostate and some other tumor types). (*See, e.g.*, Lee *et.al.*, *J. Biol. Chem.* 275:36720-36725 and Kurachi and Yamamoto, Handbook of Proeolytic Enzymes Vol. 2, 2nd edition (Barrett AJ, Rawlings ND & Woessner JF, eds) pp. 1699-1702 (2004).) Other cleavable substrates can also be used.

Another type of cleavable substrate arrangement includes preparing a separate enzyme capable of cleaving the cleavable substrate that becomes associated with the tumor or cells. For example, an enzyme can be coupled to a tumor-specific antibody (or other entity that is preferentially attracted to the tumor or other target cell such as a receptor ligand) and then the enzyme-antibody conjugate can be provided to the patient. The enzyme-antibody conjugate is directed to, and binds to, antigen associated with the tumor. Subsequently, the drug-cleavable substrate conjugate is provided to the patient as a prodrug. The drug is only released in the vicinity of the tumor when the drug-cleavable substrate conjugate interacts with the enzyme that has become associated with the tumor so that the cleavable substrate is cleaved and the drug is freed. For example, U.S. Patents Nos. 4,975,278; 5,587,161; 5,660,829; 5,773,435; and 6,132,722, all of which are incorporated herein by reference, disclose such an arrangement. Examples of suitable enzymes and substrates include, but are not limited to, β-lactamase and cephalosporin derivatives, carboxypeptidase G2 and glutamic and aspartic folate derivatives.

In one embodiment, the enzyme-antibody conjugate includes an antibody, or antibody fragment, that is selected based on its specificity for an antigen expressed on a target cell, or at a target site, of interest. A discussion of antibodies is provided hereinabove. One example of a suitable cephalosporin-cleavable substrate is



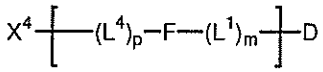
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EXAMPLES OF CONJUGATES

The linkers and cleavable substrates of the invention can be used in conjugates containing duocarmycin or CBI analogs as cytotoxic agents. Examples of conjugates of the invention are described in further detail below. Unless otherwise indicated, substituents are defined as set forth above in the sections regarding cytotoxins, linkers, and cleavable substrates.

A. Linker Conjugates

One example of a suitable conjugate is a compound of the formula

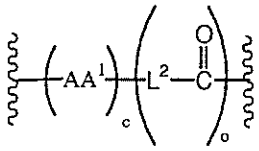


wherein

L^1 is a self-immolative linker;

m is an integer 0, 1, 2, 3, 4, 5, or 6;

F is a linker comprising the structure:

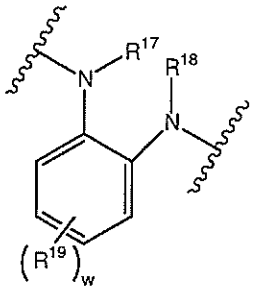


wherein

AA^1 is one or more members independently selected from the group consisting of natural amino acids and unnatural α-amino acids;

c is an integer from 1 to 20;

L^2 is a self-immolative linker and comprises



wherein each R^{17}, R^{18}, and R^{19} is independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl,

and w is an integer from 0 to 4;

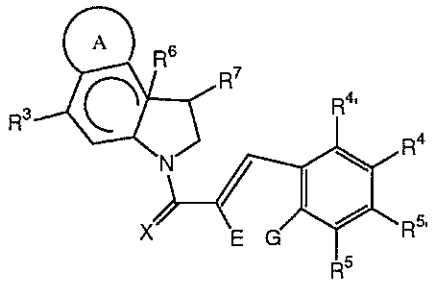
o is 1;

L^4 is a linker member;

p is 0 or 1;

X^4 is a member selected from the group consisting of protected reactive functional groups, unprotected reactive functional groups, detectable labels, and targeting agents; and

D comprises a structure:



wherein the ring system A is a member selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl groups;

E and G are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, a heteroatom, a single bond, or E and G are joined to form a ring system selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl;

X is a member selected from O, S and NR^{23};

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^3 is OR^{11},

wherein R^{11} is a member selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, monophosphates, diphosphates, triphosphates, sulfonates, acyl, $C(O)R^{12}R^{13}$, $C(O)OR^{12}$, $C(O)NR^{12}R^{13}$, $P(O)(OR^{12})_2$, , $C(O)CHR^{12}R^{13}$, SR^{12} and $SiR^{12}R^{13}R^{14}$,

R^4 , $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, $CR^{15}{=}NR^{16}$, and $O(CH_2)_nN(CH_3)_2$, or any adjacent pair of R^4, $R^{4'}$, R^5 and $R^{5'}$, together with the carbon atoms to which they are attached, are joined to form a substituted or unsubstituted cycloalkyl or heterocycloalkyl ring system having from 4 to 6 members;

wherein

n is an integer from 1 to 20;

R^{15} and R^{16} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, and substituted or unsubstituted peptidyl, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

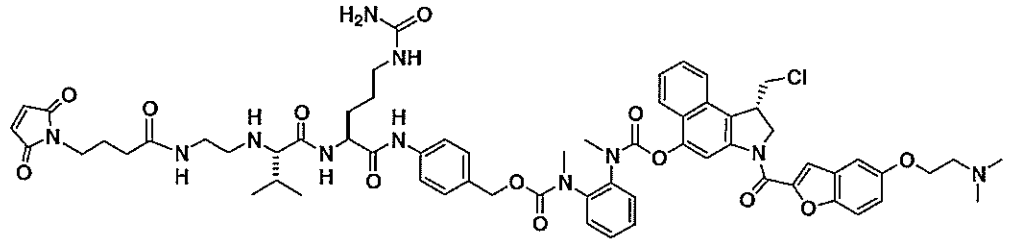
R^6 is a single bond which is either present or absent and when present R^6 and R^7 are joined to form a cyclopropyl ring; and

R^7 is CH_2-X^1 or –CH_2- joined in said cyclopropyl ring with R^6, wherein

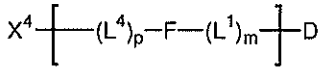
X^1 is a leaving group,

wherein R^{11} links said drug to L^1, if present, or to F.

In some embodiment, the drug has structure (9) or (12) above. One specific example of a compound suitable for use as a conjugate is



Another example of a type of conjugate is a compound of the formula

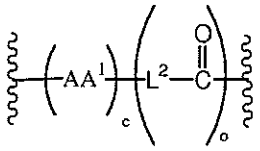


wherein

L^1 is a self-immolative linker;

m is an integer 0, 1, 2, 3, 4, 5, or 6;

F is a linker comprising the structure:



wherein

AA^1 is one or more members independently selected from the group consisting of natural amino acids and unnatural α-amino acids;

c is an integer from 1 to 20;

L^2 is a self-immolative linker;

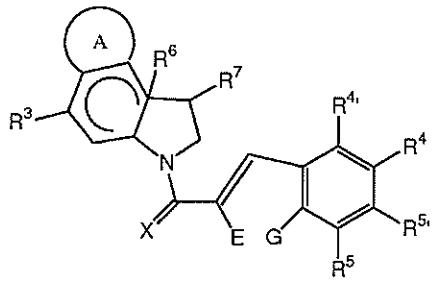
o is 0 or 1;

L^4 is a linker member;

p is 0 or 1;

X^4 is a member selected from the group consisting of protected reactive functional groups, unprotected reactive functional groups, detectable labels, and targeting agents; and

D comprises a structure:



wherein the ring system A is a member selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl groups;

E and G are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, a heteroatom, a single bond, or E and G are joined to form a ring system selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl;

X is a member selected from O, S and NR^{23};

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^3 is a member selected from the group consisting of (=O), SR^{11}, NHR^{11} and OR^{11},

wherein R^{11} is a member selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted

heteroalkyl, monophosphates, diphosphates, triphosphates, sulfonates, acyl, $C(O)R^{12}R^{13}$, $C(O)OR^{12}$, $C(O)NR^{12}R^{13}$, $P(O)(OR^{12})_2$, , $C(O)CHR^{12}R^{13}$, SR^{12} and $SiR^{12}R^{13}R^{14}$,

in which R^{12}, R^{13}, and R^{14} are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl, wherein R^{12} and R^{13} together with the nitrogen or carbon atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

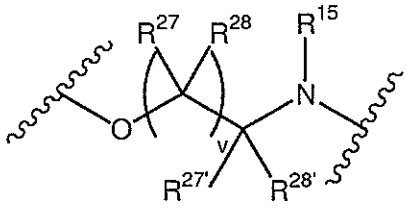
R^4 , $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, $CR^{15}{=}NR^{16}$, and $O(CH_2)_nN(CH_3)_2$, or any adjacent pair of R^4, $R^{4'}$, R^5 and $R^{5'}$, together with the carbon atoms to which they are attached, are joined to form a substituted or unsubstituted cycloalkyl or heterocycloalkyl ring system having from 4 to 6 members,

wherein

n is an integer from 1 to 20;

R^{15} and R^{16} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, and substituted or unsubstituted peptidyl, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

wherein at least one of R^4 , $R^{4'}$, R^5 and $R^{5'}$ links said drug to L^1, if present, or to F, and comprises



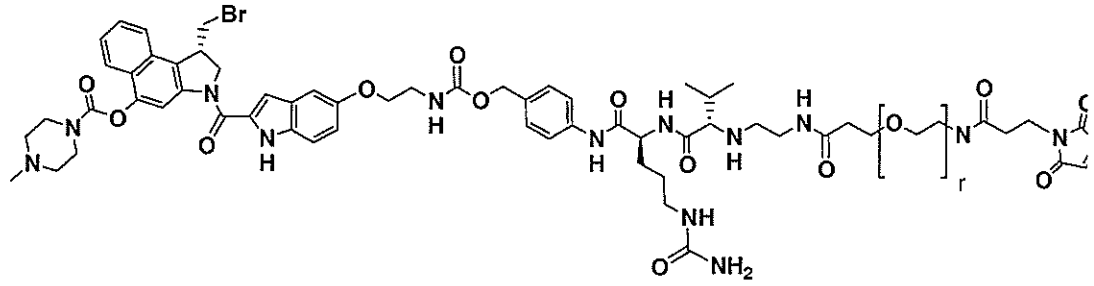
wherein v is an integer from 1 to 6; and

each R^{27}, $R^{27'}$, R^{28}, and $R^{28'}$ is independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, and substituted or unsubstituted heterocycloalkyl;

R^6 is a single bond which is either present or absent and when present R^6 and R^7 are joined to form a cyclopropyl ring; and

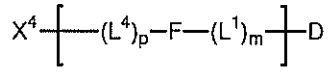
R^7 is CH_2-X^1 or –CH_2- joined in said cyclopropyl ring with R^6, wherein X^1 is a leaving group.

In some embodiment, the drug has structure (9) or (12) above. One specific example of a compound suitable for use as a conjugate is



where r is an integer in the range from 0 to 24.

Another example of a suitable conjugate is a compound of the formula

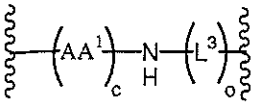


wherein

L^1 is a self-immolative linker;

m is an integer 0, 1, 2, 3, 4, 5, or 6;

F is a linker comprising the structure:



wherein

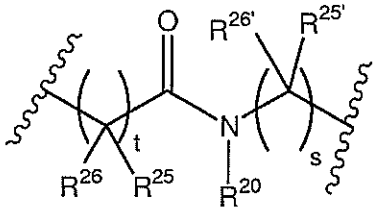
AA^1 is one or more members independently selected from the group consisting of natural amino acids and unnatural α-amino acids;

c is an integer from 1 to 20;

L^3 is a spacer group comprising a primary or secondary amine or a carboxyl functional group; wherein if L^3 is present, m is 0 and either the amine of L^3 forms an amide bond with a pendant carboxyl functional group of D or the carboxyl of L^3 forms an amide bond with a pendant amine functional group of D;

o is 0 or 1;

L^4 is a linker member, wherein L^4 comprises



directly attached to the N-terminus of $(AA^1)_c$, wherein

R^{20} is is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl,

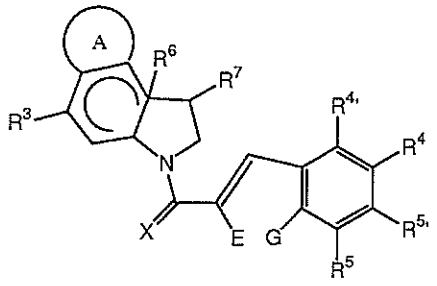
each R^{25}, $R^{25'}$, R^{26}, and $R^{26'}$ is independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, and substituted or unsubstituted heterocycloalkyl;

and s and t are independently integers from 1 to 6;

p is 1;

X^4 is a member selected from the group consisting of protected reactive functional groups, unprotected reactive functional groups, detectable labels, and targeting agents; and

D comprises a structure:



wherein the ring system A is a member selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl groups;

E and G are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, a heteroatom, a single bond, or E and G are joined to form a ring system selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl;

X is a member selected from O, S and NR^{23};

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^3 is a member selected from the group consisting of (=O), SR^{11}, NHR^{11} and OR^{11},

wherein

R^{11} is a member selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, monophosphates, diphosphates, triphosphates, sulfonates, acyl, $C(O)R^{12}R^{13}$, $C(O)OR^{12}$, $C(O)NR^{12}R^{13}$, $P(O)(OR^{12})_2$, , $C(O)CHR^{12}R^{13}$, SR^{12} and $SiR^{12}R^{13}R^{14}$,

in which

R^{12}, R^{13}, and R^{14} are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl, wherein R^{12} and R^{13} together with the nitrogen or carbon atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

R^4 , $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, $CR^{15}{=}NR^{16}$, and $O(CH_2)_nN(CH_3)_2$, or any adjacent pair of R^4, $R^{4'}$, R^5 and $R^{5'}$, together with the carbon atoms to which they are attached, are joined to form a substituted or unsubstituted cycloalkyl or heterocycloalkyl ring system having from 4 to 6 members,

wherein

n is an integer from 1 to 20;

R^{15} and R^{16} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, and substituted or unsubstituted peptidyl, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

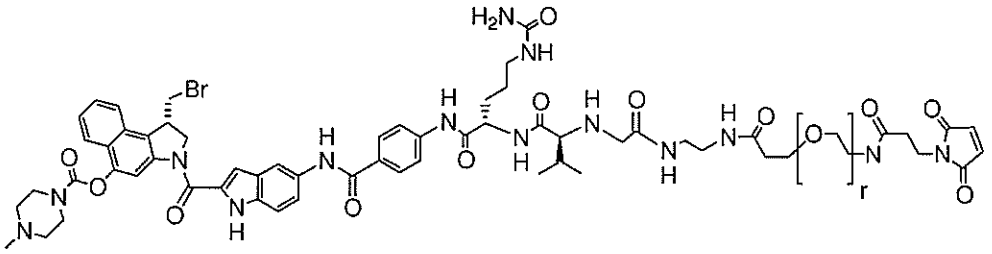
R^6 is a single bond which is either present or absent and when present R^6 and R^7 are joined to form a cyclopropyl ring; and

R^7 is CH_2-X^1 or –CH_2- joined in said cyclopropyl ring with R^6, wherein

X^1 is a leaving group,

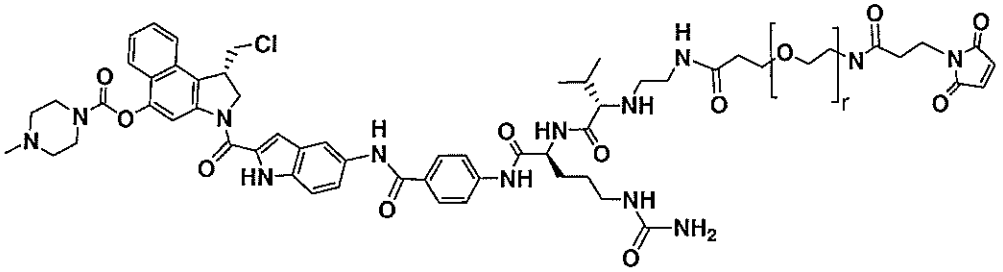
wherein at least one of R^4 , $R^{4'}$, R^5, $R^{5'}$, R^{15} or R^{16} links said drug to L^1, if present, or to F.

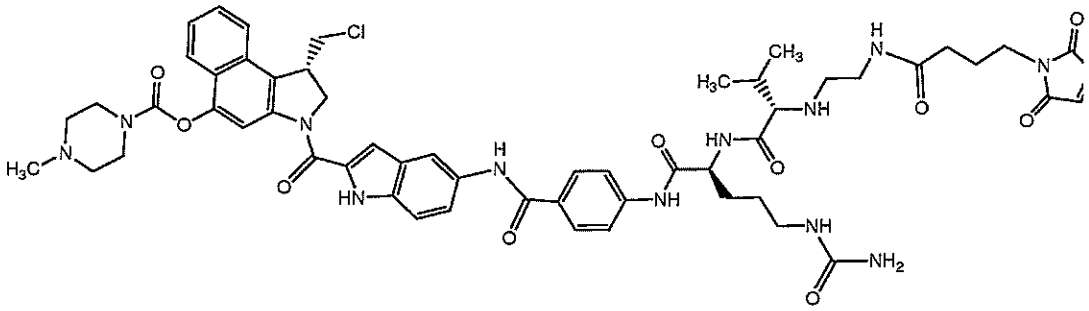
In some embodiment, the drug has structure (9) or (12) above.One specific example of a compound suitable for use as conjugate is

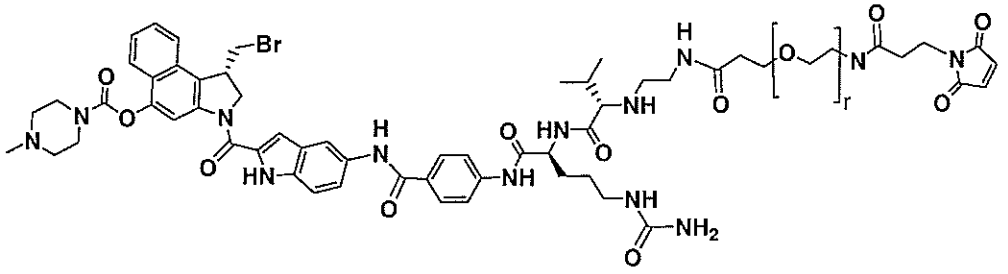


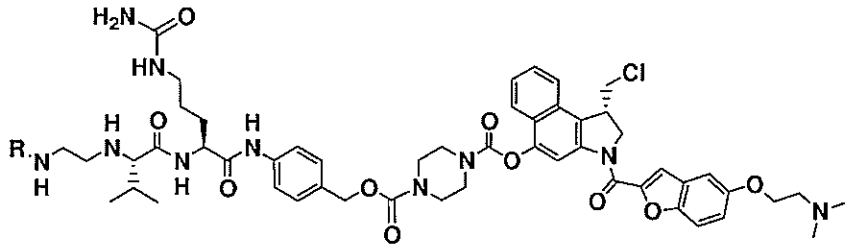
where r is an integer in the range from 0 to 24.

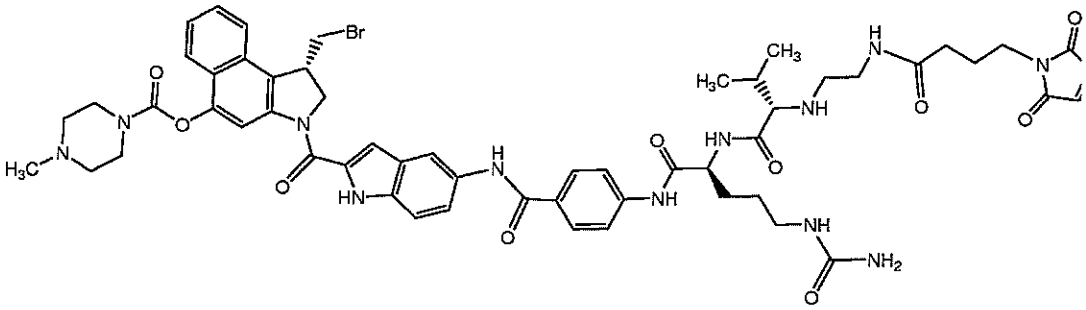
Other examples of suitable compounds for use as conjugates include:

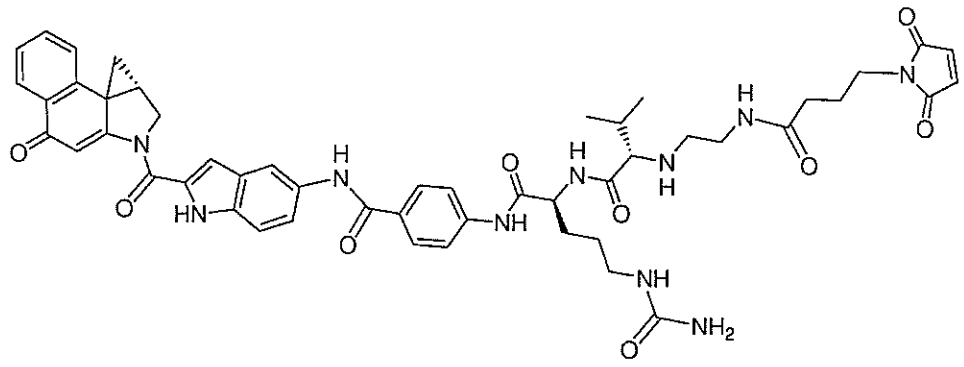


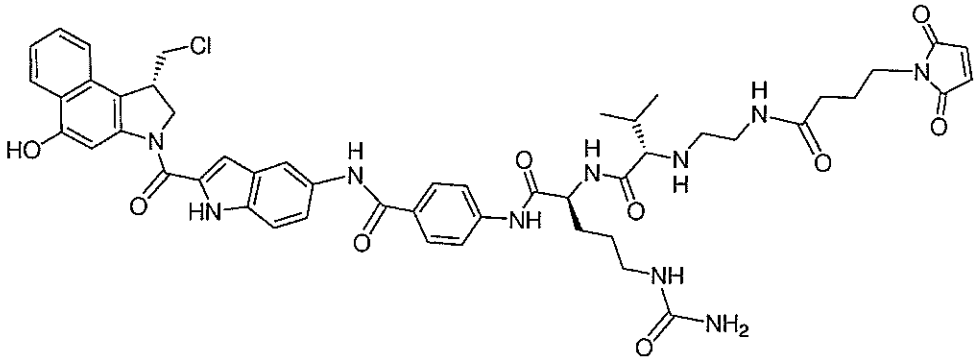


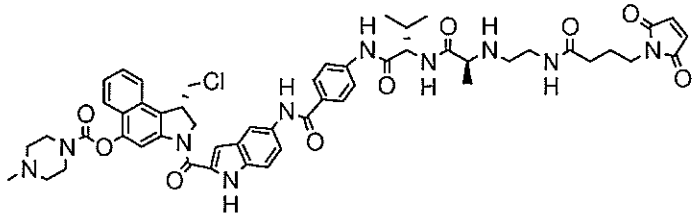




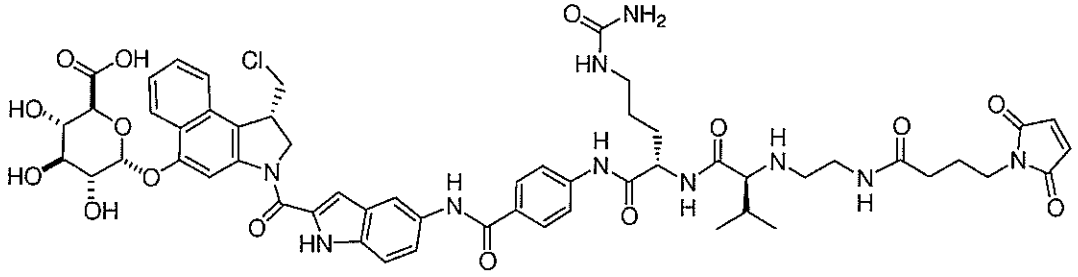


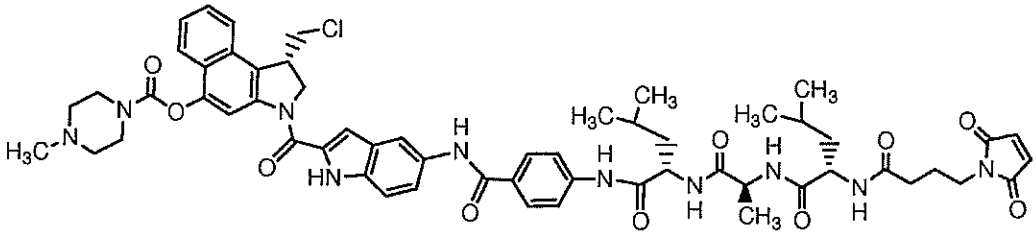


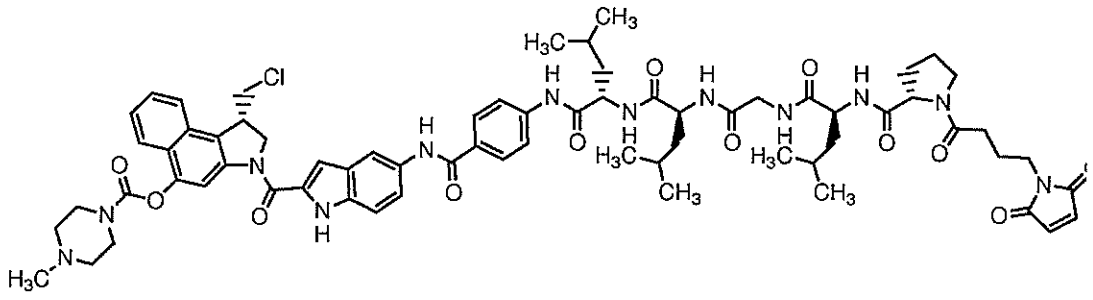


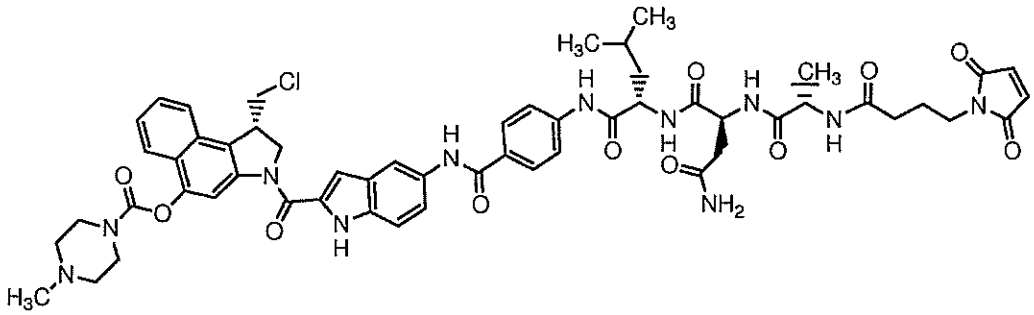


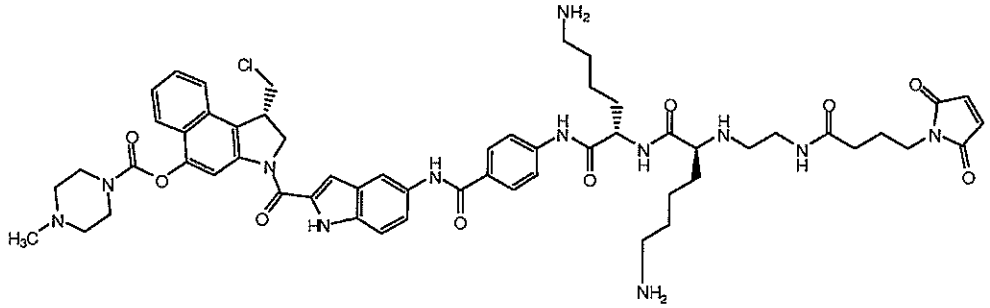
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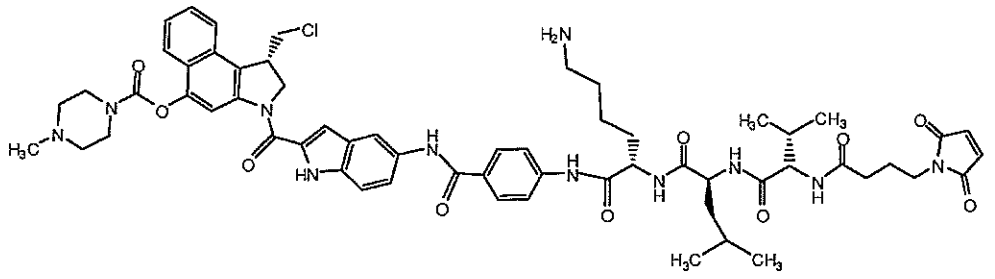


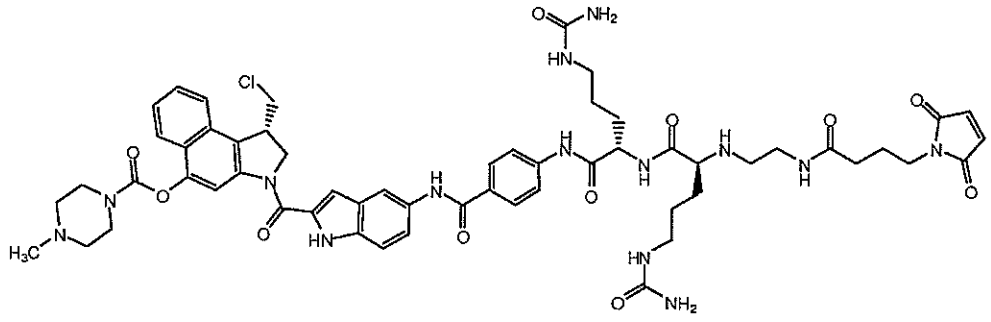


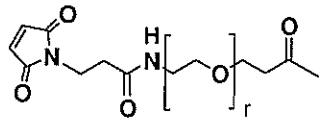
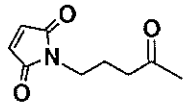




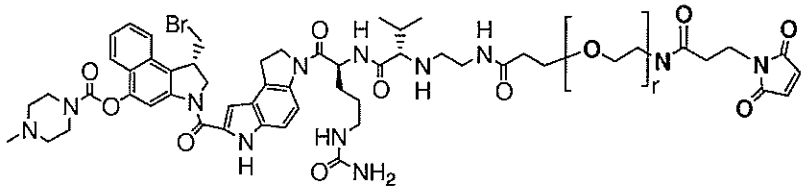


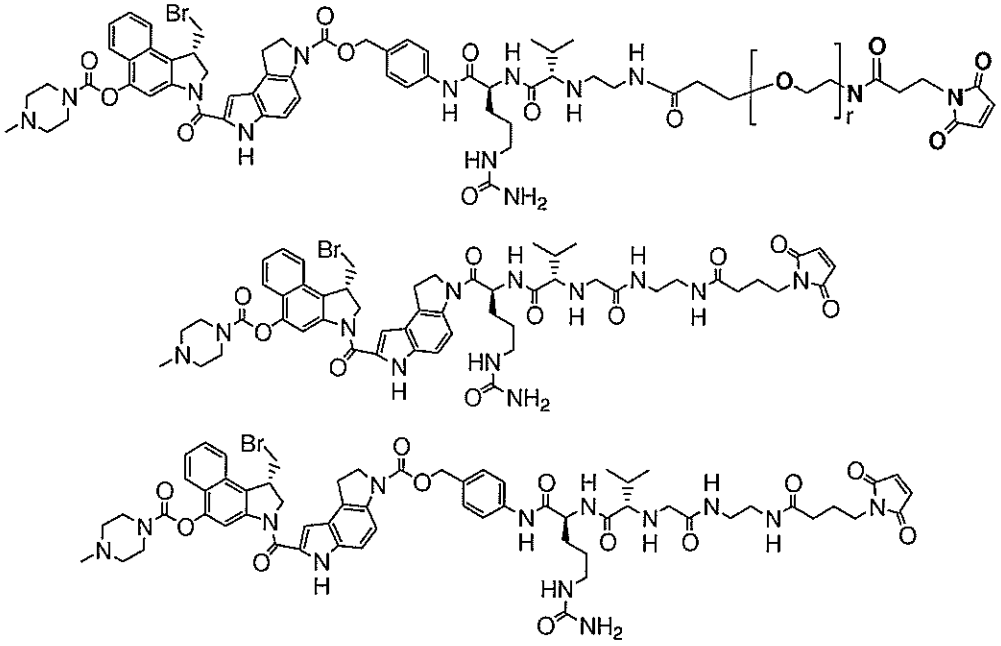


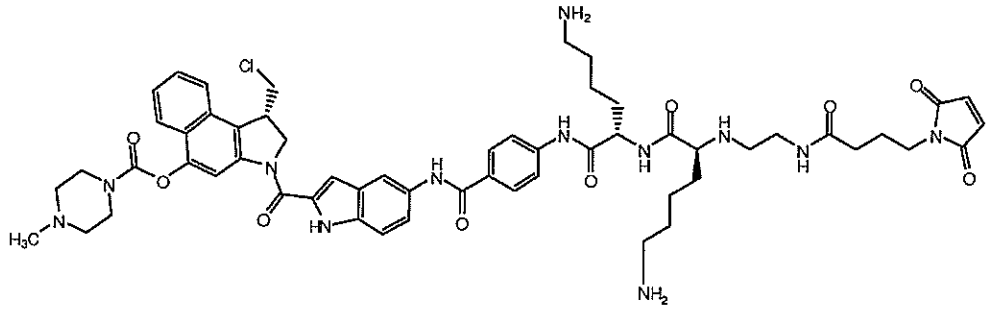


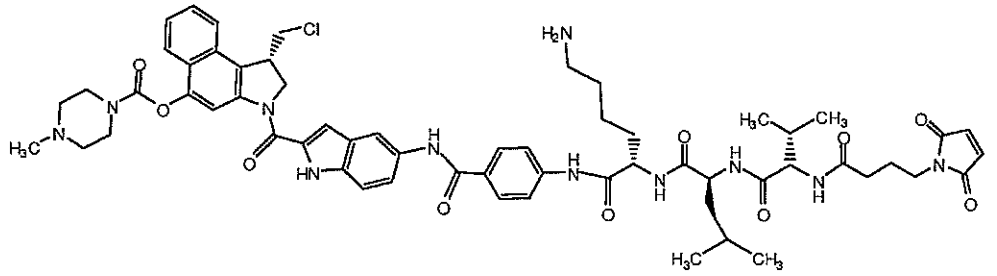
where R is  or  and r is an integer in the range from 0 to 24

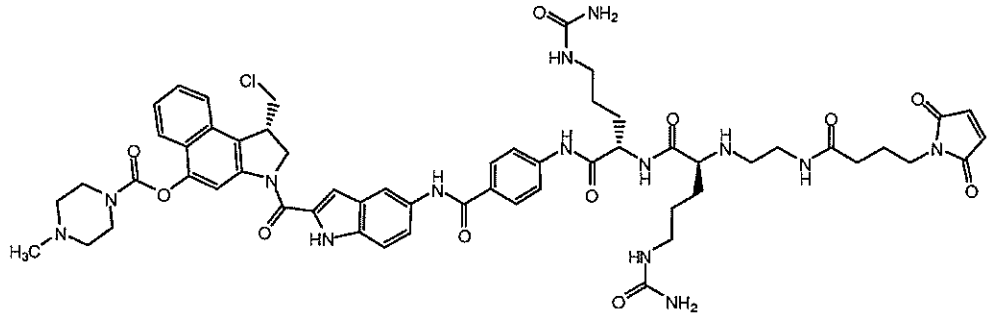
Conjugates can also be formed using the drugs having structure (13), such as the following compounds:











where r is an integer in the range from 0 to 24.

A. Cleavable Linker Conjugates

One example of a suitable conjugate is a compound having the following structure:

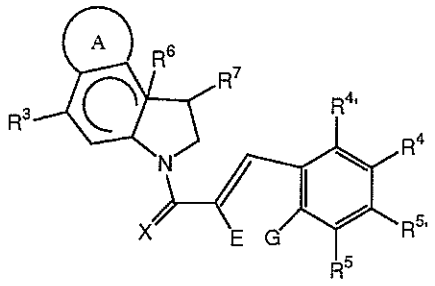
$$X^2-(L^1)_m-D \qquad (2)$$

wherein L^1 is a self-immolative spacer;

m is an integer of 0, 1, 2, 3, 4, 5, or 6;

X^2 is a cleavable substrate; and

D comprises a structure:



wherein the ring system A is a member selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl groups;

E and G are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, a heteroatom, a single bond, or E and G are joined to form a ring system selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl;

X is a member selected from O, S and NR^{23};

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^3 is a member selected from the group consisting of (=O), SR^{11}, NHR^{11} and OR^{11},

wherein

R^{11} is a member selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, monophosphates, diphosphates, triphosphates, sulfonates, acyl, $C(O)R^{12}R^{13}$, $C(O)OR^{12}$, $C(O)NR^{12}R^{13}$, $P(O)(OR^{12})_2$, , $C(O)CHR^{12}R^{13}$, SR^{12} and $SiR^{12}R^{13}R^{14}$,

in which

R^{12}, R^{13}, and R^{14} are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl, wherein R^{12} and R^{13} together with the nitrogen or carbon atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

R^6 is a single bond which is either present or absent and when present R^6 and R^7 are joined to form a cyclopropyl ring; and

R^7 is CH_2-X^1 or –CH_2- joined in said cyclopropyl ring with R^6, wherein

X^1 is a leaving group

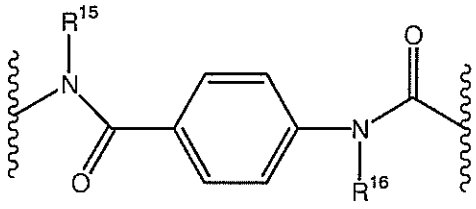
R^4 , $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, CR^{15}=NR^{16}, and $O(CH_2)_nN(CH_3)_2$, or any adjacent pair of R^4, $R^{4'}$, R^5 and $R^{5'}$, together with the carbon atoms to which they are attached, are joined to form a substituted or unsubstituted cycloalkyl or heterocycloalkyl ring system having from 4 to 6 members,

wherein

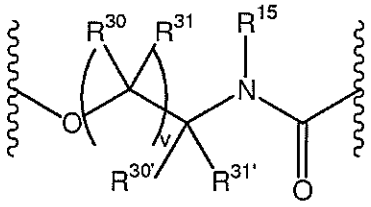
n is an integer from 1 to 20;

R^{15} and R^{16} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, and substituted or unsubstituted peptidyl, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

wherein at least one of members R^4 , $R^{4'}$, R^5 and $R^{5'}$ links said drug to L^1, if present, or to X^2, and is selected from the group consisting of



and

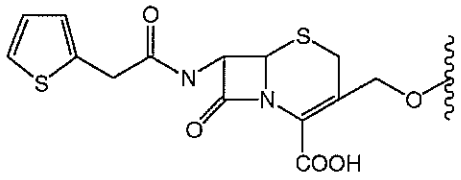


wherein

R^{30}, $R^{30'}$, R^{31}, and $R^{31'}$ are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, and substituted or unsubstituted heterocycloalkyl; and

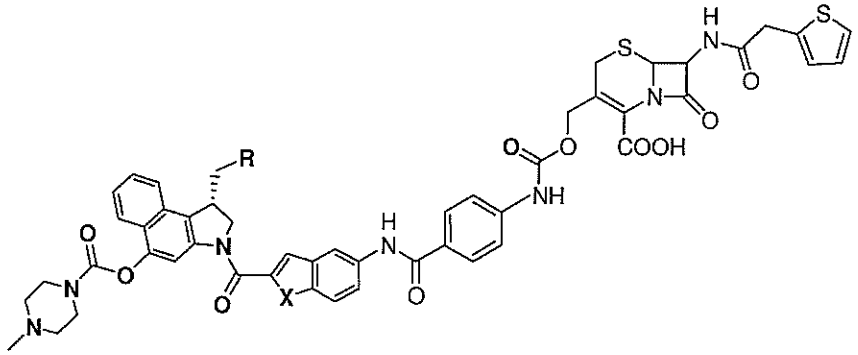
v is an integer from 1 to 6.

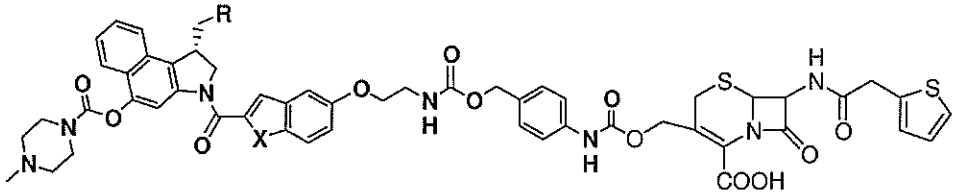
Examples of suitable cleavable linkers include β-AlaLeuAlaLeu and

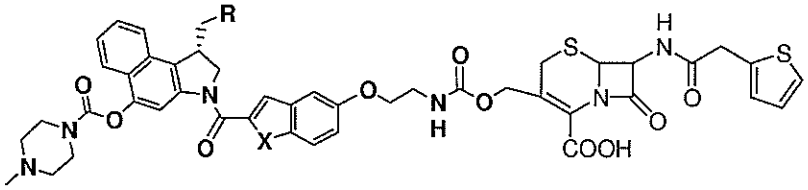


.

In some embodiment, the drug has structure (9) or (12) above. Examples of the type of compound include:







where R is F, Cl, Br, or I.

PHARMACEUTICAL FORMULATIONS AND ADMINISTRATION

In another preferred embodiment, the present invention provides a pharmaceutical formulation comprising a compound of the invention and a pharmaceutically acceptable carrier.

The compounds described herein including pharmaceutically acceptable carriers such as addition salts or hydrates thereof, can be delivered to a patient using a wide variety of routes or modes of administration. Suitable routes of administration include, but are not limited to, inhalation, transdermal, oral, rectal, transmucosal, intestinal and parenteral administration, including intramuscular, subcutaneous and intravenous injections. Preferably, the conjugates of the invention are administered parenterally, more preferably intravenously.

As used herein, the terms "administering" or "administration" are intended to encompass all means for directly and indirectly delivering a compound to its intended site of action.

The compounds described herein, or pharmaceutically acceptable salts and/or hydrates thereof, may be administered singly, in combination with other compounds of the invention, and/or in cocktails combined with other therapeutic agents. Of course, the choice of therapeutic agents that can be co-administered with the compounds of the invention will depend, in part, on the condition being treated.

For example, when administered to patients suffering from a disease state caused by an organism that relies on an autoinducer, the compounds of the invention can be administered in cocktails containing agents used to treat the pain, infection and other symptoms and side effects commonly associated with the disease. Such agents include, *e.g.*, analgesics, antibiotics, *etc.*

When administered to a patient undergoing cancer treatment, the compounds may be administered in cocktails containing anti-cancer agents and/or supplementary potentiating agents. The compounds may also be administered in cocktails containing agents that treat the side-effects of radiation therapy, such as anti-emetics, radiation protectants, *etc.*

Supplementary potentiating agents that can be co-administered with the compounds of the invention include, *e.g.*, tricyclic anti-depressant drugs (*e.g.*, imipramine, desipramine, amitriptyline, clomipramine, trimipramine, doxepin, nortriptyline, protriptyline, amoxapine and maprotiline); non-tricyclic and anti-depressant

drugs (*e.g.*, sertraline, trazodone and citalopram); Ca^{+2} antagonists (*e.g.*, verapamil, nifedipine, nitrendipine and caroverine); amphotericin; triparanol analogues (*e.g.*, tamoxifen); antiarrhythmic drugs (*e.g.*, quinidine); antihypertensive drugs (*e.g.*, reserpine); thiol depleters (*e.g.*, buthionine and sulfoximine); and calcium leucovorin.

The active compound(s) of the invention are administered *per se* or in the form of a pharmaceutical composition wherein the active compound(s) is in admixture with one or more pharmaceutically acceptable carriers, excipients or diluents. Pharmaceutical compositions for use in accordance with the present invention are typically formulated in a conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active compounds into preparations which, can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the compounds can be formulated readily by combining the active compound(s) with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. Pharmaceutical preparations for oral use can be obtained solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired. to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxyniethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to

the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations, which can be used orally, include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for such administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of *e.g.*, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Injection is a preferred method of administration for the compositions of the current invention. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions

may contain substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents, which increase the solubility of the compounds to allow for the preparation of highly, concentrated solutions. For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation or transcutaneous delivery (*e.g.*, subcutaneously or intramuscularly), intramuscular injection or a transdermal patch. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (*e.g.*, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

A preferred pharmaceutical composition is a composition formulated for injection such as intravenous injection and includes about 0.01% to about 100% by weight of the drug conjugate, based upon 100% weight of total pharmaceutical composition. The drug conjugate may be an antibody-cytotoxin conjugate where the antibody has been selected to target a particular cancer.

LIBRARIES

Also within the scope of the present invention are libraries of the cytotoxin, cytotoxin-linker and agent-linker conjugates of the cytotoxins, linkers of the invention and cytotoxin-cleavable substrates of the invention. Exemplary libraries include at least

10 compounds, more preferably at least 100 compound, even more preferably at least 1000 compounds and still more preferably at least 100,000 compounds. The libraries are in a form that is readily queried for a particular property, e.g., cytotoxicity, cleavage of a linker or a substrate by an enzyme, or other cleavage reagent. Exemplary forms include chip formats, microarrays, and the like.

Parallel, or combinatorial, synthesis has as its primary objective the generation of a library of diverse molecules which all share a common feature, referred to throughout this description as a scaffold. By substituting different moieties at each of the variable parts of the scaffold molecule, the amount of space explorable in a library grows. Theories and modern medicinal chemistry advocate the concept of occupied space as a key factor in determining the efficacy of a given compound against a given biological target. By creating a diverse library of molecules, which explores a large percentage of the targeted space, the odds of developing a highly efficacious lead compound increase dramatically.

Parallel synthesis is generally conducted on a solid phase support, such as a polymeric resin. The scaffold, or other suitable intermediate is cleavably tethered to the resin by a chemical linker. Reactions are carried out to modify the scaffold while tethered to the particle. Variations in reagents and/or reaction conditions produce the structural diversity, which is the hallmark of each library.

Parallel synthesis of "small" molecules (non-oligomers with a molecular weight of 200-1000) was rarely attempted prior to 1990. *See*, for example, Camps. *et al.*, *Annaks de Quimica,* **70**: 848 (1990). Recently, Ellmann disclosed the solid phase-supported parallel (also referred to as "combinatorial") synthesis of eleven benzodiazepine analogs along with some prostaglandins and beta-turn mimetics. These disclosures are exemplified in U.S. Pat. No. 5,288,514. Another relevant disclosure of parallel synthesis of small molecules may be found in U.S. Pat. No. 5,324,483. This patent discloses the parallel synthesis of between 4 and 40 compounds in each of sixteen different scaffolds. Chen *et al.* have also applied organic synthetic strategies to develop non-peptide libraries synthesized using multi-step processes on a polymer support. (Chen *et al., J. Am. Chem. Soc.*, **116**: 2661-2662 (1994)).

Once a library of unique compounds is prepared, the preparation of a library of immunoconjugates, or antibodies can be prepared using the library of autoinducers as a starting point and using the methods described herein.

KITS

In another aspect, the present invention provides kits containing one or more of the compounds or compositions of the invention and directions for using the compound or composition. In an exemplary embodiment, the invention provides a kit for conjugating a linker arm of the invention to another molecule. The kit includes the linker, and directions for attaching the linker to a particular functional group. The kit may also, or alternatively, include one or more of a cytotoxic drug, a targeting agent, a detectable label, a cleavable substrate, pharmaceutical salts or buffers. The kit may also include a container and optionally one or more vial, test tube, flask, bottle, or syringe. Other formats for kits will be apparent to those of skill in the art and are within the scope of the present invention.

PURIFICATION

In another exemplary embodiment, the present invention provides a method for isolating a molecular target for a ligand-cytotoxin or cleavable substrate-cytotoxin of the invention, which binds to the cleavable substrate X^2 or ligand X^4. The method preferably comprises, contacting a cellular preparation that includes the target with an immobilized compound, thereby forming a complex between the receptor and the immobilized compound.

The cytotoxin of the invention can be immobilized on an affinity support by any art-recognized means. Alternatively, the cytotoxin can be immobilized using one or more of the cleavable substrates or linkers of the invention.

In yet another exemplary embodiment, the invention provides an affinity purification matrix.

The method of the invention for isolating a target will typically utilize one or more affinity chromatography techniques. Affinity chromatography enables the efficient isolation of species such as biological molecules or biopolymers by utilizing their recognition sites for certain supported chemical structures with a high degree of selectivity. The literature is replete with articles, monographs, and books on the subject of affinity chromatography, including such topics as affinity chromatography supports, crosslinking members, ligands and their preparation and use. A sampling of those references includes: Ostrove, *Methods Enzymol.* **182**: 357-71 (1990); Ferment, *Bioeng.*

70: 199-209 (1990). Huang *et al.*, *J. Chromatogr.* **492**: 431-69 (1989); "Purification of enzymes by heparin-Sepharose affinity chromatography," *J. Chromatogr.*, **184**: 335-45 (1980); Farooqi, *Enzyme Eng.*, **4**: 441-2 (1978); Nishikawa, *Chem. Technol.*, **5(9)**: 564-71 (1975); Guilford *et al.*, in, PRACT. HIGH PERFORM. LIQ. CHROMATOGR., Simpson (ed.), 193-206 (1976); Nishikawa, *Proc. Int. Workshop Technol. Protein Sep. Improv. Blood Plasma Fractionation*, Sandberg (ed.), 422-35; (1977) "Affinity chromatography of enzymes," *Affinity Chromatogr., Proc. Int. Symp.* 25-38, (1977) (Pub. 1978); and AFFINITY CHROMATOGRAPHY: A PRACTICAL APPROACH, Dean *et al.* (ed.), IRL Press Limited, Oxford, England (1985). Those of skill in the art have ample guidance in developing particular affinity chromatographic methods utilizing the materials of the invention.

In the present method, affinity chromatographic media of varying chemical structures can be used as supports. For example, agarose gels and cross-linked agarose gels are useful as support materials, because their hydrophilicity makes them relatively free of nonspecific binding. Other useful supports include, for example, controlled-pore glass (CPG) beads, cellulose particles, polyacrylamide gel beads and Sephadex™ gel beads made from dextran and epichlorohydrin.

DRUG CONJUGATE METHODS OF USE

In addition to the compositions and constructs described above, the present invention also provides a number of methods that can be practiced utilizing the compounds and conjugates of the invention. Methods for using the drug-ligand conjugate and drug-cleavable substrate conjugate of the current invention include: killing or inhibiting the growth or replication of a tumor cell or cancer cell, treating cancer, treating a pre-cancerous condition, killing or inhibiting the growth or replication of a cell that expresses an auto-immune antibody, treating an autoimmune disease, treating an infectious disease, preventing the multiplication of a tumor cell or cancer cell, preventing cancer, preventing the multiplication of a cell that expresses an auto-immune antibody, preventing an autoimmune disease, and preventing an infectious disease. These methods of use comprise administering to an animal such as a mammal or a human in need thereof an effective amount of a drug-ligand conjugate or drug-cleavable substrate conjugate. In some embodiments, an enzyme is separately administered so that it becomes associated with the tumor or target cell.

The drug-ligand conjugate or drug-cleavable substrate complex of the current invention is useful for treating, for example, cancer, autoimmune disease or infectious disease in an animal. Compositions and methods for treating tumors by providing a subject the composition in a pharmaceutically acceptable manner, with a pharmaceutically effective amount of a composition of the present invention are provided.

The current invention is particularly useful for the treatment of cancer and for the inhibition of the multiplication of a tumor cell or cancer cell in an animal. Cancer, or a precancerous condition, includes, but is not limited to, a tumor, metastasis, or any disease or disorder characterized by uncontrolled cell growth, can be treated or prevented by administration the drug-ligand or drug-cleavable substrate complexes of the current invention. The complex delivers the drug to a tumor cell or cancer cell.

In one embodiment using a drug-ligand complex, the ligand specifically binds to or associates with a cancer-cell or a tumor-cell-associated antigen. Because of its close proximity to the ligand, the drug can be taken up inside a tumor cell or cancer cell through, for example, receptor-mediated endocytosis. The antigen can be attached to a tumor cell or cancer cell or can be an extracellular matrix protein associated with the tumor cell or cancer cell. Once inside the cell, the linker is hydrolytically cleaved by a tumor-cell or cancer-cell-associated proteases, thereby releasing the drug. The released drug is then free to diffuse and induce cytotoxic activities. In an alternative embodiment, the drug is cleaved from the drug-ligand complex outside the tumor cell or cancer cell, and the drug subsequently penetrates the cell.

The ligand may bind to, for example, a tumor cell or cancer cell, a tumor cell or cancer cell antigen which is on the surface of the tumor cell or cancer cell, or a tumor cell or cancer cell antigen which is an extracellular matrix protein associated with the tumor cell or cancer cell. The ligand can be designed specifically for a particular tumor cell or cancer cell type. Therefore, the type of tumors or cancers that can be effectively treated can be altered by the choice of ligand.

In one embodiment using a drug-cleavable substrate complex, the cleavable substrate is cleaved by an enzyme (or other entity that can cleave the substrate) that is associated with the tumor or other target cell. Once in contact with the enzyme, the cleavable substrate is cleaved by the enzyme, thereby releasing the drug. The released drug is then free to diffuse and induce cytotoxic activities. The cleavable substrate can be cleaved inside or outside of the tumor or target cell. The type of tumors or cancers that

can be effectively treated can be altered by the choice of cleavable substrate and associated enzyme.

Representative examples of precancerous conditions that may be targeted by the conjugates, include, but are not limited to: metaplasia, hyperplasia, dysplasia, colorectal polyps, actinic ketatosis, actinic cheilitis, human papillomaviruses, leukoplakia, lychen planus and Bowen's disease.

Representative examples of cancers or tumors that may be targeted by the conjugates include, but are not limited to: lung cancer, colon cancer, prostate cancer, lymphoma, melanoma, breast cancer, ovarian cancer, testicular cancer, CNS cancer, renal cancer, kidney cancer, pancreatic cancer, stomach cancer, oral cancer, nasal cancer, cervical cancer and leukemias. It will be readily apparent to the ordinarily skilled artisan that the particular ligand or cleavable substrate used in the conjugate can be chosen such that it targets the drug to the tumor tissue to be treated with the drug.

In an embodiment, the present invention provides a method of killing a cell. The method includes administering to the cell an amount of a compound of the invention sufficient to kill said cell. In an exemplary embodiment, the compound is administered to a subject bearing the cell. In a further exemplary embodiment, the administration serves to retard or stop the growth of a tumor that includes the cell (*e.g.*, the cell can be a tumor cell). For the administration to retard the growth, the rate of growth of the cell should be at least 10% less than the rate of growth before administration. Preferably, the rate of growth will be retarded at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or completely stopped.

Effective Dosages

Pharmaceutical compositions suitable for use with the present invention include compositions wherein the active ingredient is contained in a therapeutically effective amount, *i.e.*, in an amount effective to achieve its intended purpose. The actual amount effective for a particular application will depend, *inter alia,* on the condition being treated. Determination of an effective amount is well within the capabilities of those skilled in the art, especially in light of the detailed disclosure herein.

For any compound described herein, the therapeutically effective amount can be initially determined from cell culture assays. Target plasma concentrations will be those concentrations of active compound(s) that are capable of inhibition cell growth or

division. In preferred embodiments, the cellular activity is at least 25% inhibited. Target plasma concentrations of active compound(s) that are capable of inducing at least about 50%, 75%, or even 90% or higher inhibition of cellular activity are presently preferred. The percentage of inhibition of cellular activity in the patient can be monitored to assess the appropriateness of the plasma drug concentration achieved, and the dosage can be adjusted upwards or downwards to achieve the desired percentage of inhibition.

As is well known in the art, therapeutically effective amounts for use in humans can also be determined from animal models. For example, a dose for humans can be formulated to achieve a circulating concentration that has been found to be effective in animals. The dosage in humans can be adjusted by monitoring cellular inhibition and adjusting the dosage upwards or downwards, as described above.

A therapeutically effective dose can also be determined from human data for compounds which are known to exhibit similar pharmacological activities. The applied dose can be adjusted based on the relative bioavailability and potency of the administered compound as compared with the known compound.

Adjusting the dose to achieve maximal efficacy in humans based on the methods described above and other methods as are well-known in the art is well within the capabilities of the ordinarily skilled artisan.

In the case of local administration, the systemic circulating concentration of administered compound will not be of particular importance. In such instances, the compound is administered so as to achieve a concentration at the local area effective to achieve the intended result.

For use in the prophylaxis and/or treatment of diseases related to abnormal cellular proliferation, a circulating concentration of administered compound of about 0.001 μM to 20 μM is preferred, with about 0.01 μM to 5 μM being preferred.

Patient doses for oral administration of the compounds described herein, typically range from about 1 mg/day to about 10,000 mg/day, more typically from about 10 mg/day to about 1,000 mg/day, and most typically from about 50 mg/day to about 500 mg/day. Stated in terms of patient body weight, typical dosages range from about 0.01 to about 150 mg/kg/day, more typically from about 0.1 to about 15 mg/kg/day, and most typically from about 1 to about 10 mg/kg/day, , for example 5 mg/kg/day or 3 mg/kg/day.

In at least some embodiments, patient doses that retard or inhibit tumor growth can be 1 μmol/kg/day or less. For example, the patient doses can be 0.9, 0.6, 0.5, 0.45,

0.3, 0.2, 0.15, or 0.1 μmol/kg/day or less (referring to moles of the drug) of the drug or a drug conjugate, such as an antibody-drug conjugate. Preferably, the drug or drug conjugate growth of the tumor when administered in the daily dosage amount over a period of at least five days. In at least some embodiments, the tumor is a human-type tumor in a SCID mouse. As an example, the SCID mouse can be a CB17.SCID mouse (available from Taconic, Germantown, NY).

For other modes of administration, dosage amount and interval can be adjusted individually to provide plasma levels of the administered compound effective for the particular clinical indication being treated. For example, in one embodiment, a compound according to the invention can be administered in relatively high concentrations multiple times per day. Alternatively, it may be more desirable to administer a compound of the invention at minimal effective concentrations and to use a less frequent administration regimen. This will provide a therapeutic regimen that is commensurate with the severity of the individual's disease.

Utilizing the teachings provided herein, an effective therapeutic treatment regimen can be planned which does not cause substantial toxicity and yet is entirely effective to treat the clinical symptoms demonstrated by the particular patient. This planning should involve the careful choice of active compound by considering factors such as compound potency, relative bioavailability, patient body weight, presence and severity of adverse side effects, preferred mode of administration and the toxicity profile of the selected agent.

The compounds, compositions and methods of the present invention are further illustrated by the examples that follow. These examples are offered to illustrate, but not to limit the claimed invention.

EXAMPLES

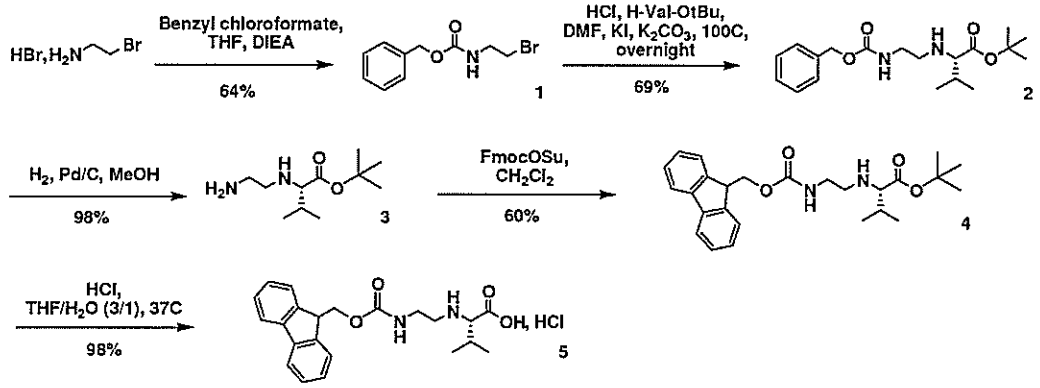
Material and Methods

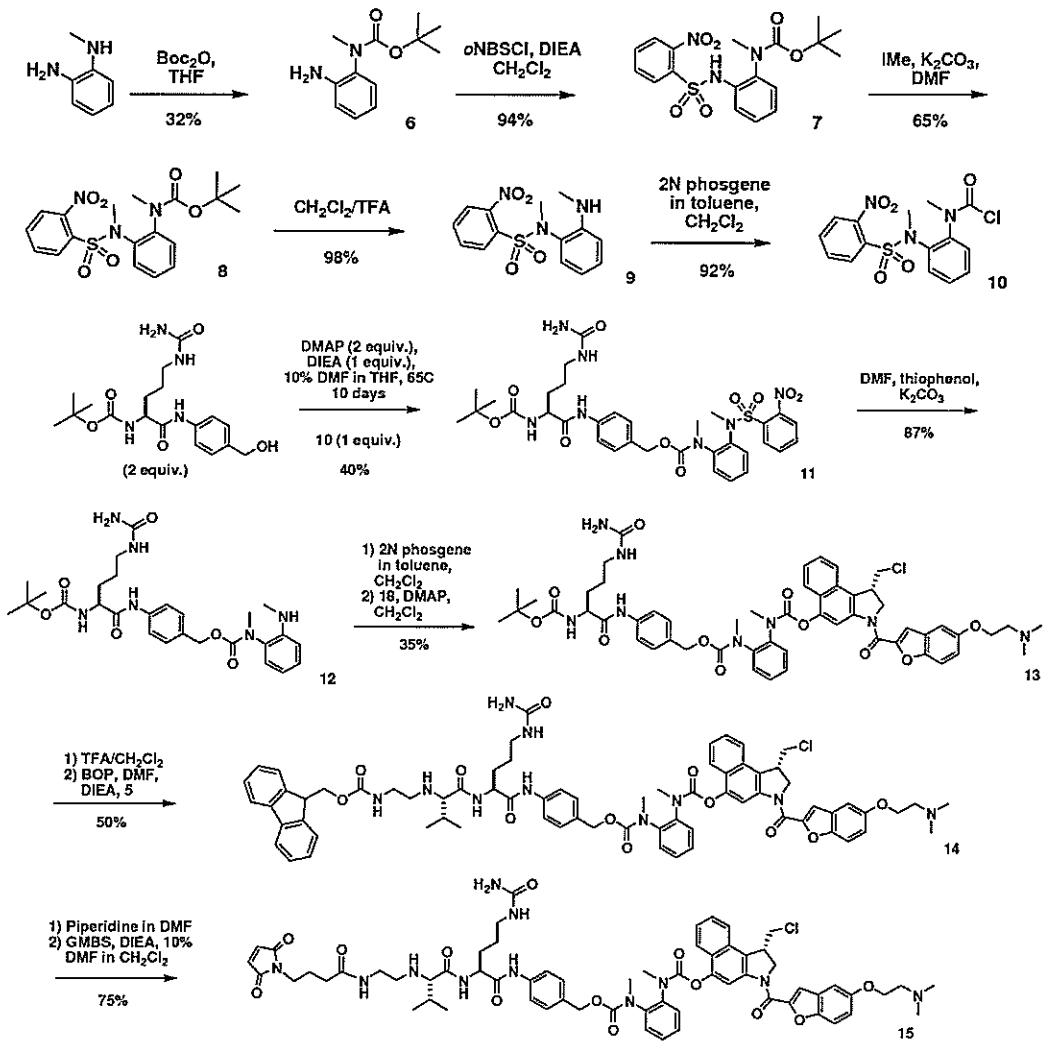
In the examples below, unless otherwise stated, temperatures are given in degrees Celsius (°C); operations were carried out at room or ambient temperature (typically a range of from about 18-25 °C; evaporation of solvent was carried out using a rotary evaporator under reduced pressure (typically, 4.5-30 mmHg) with a bath temperature of up to 60 °C; the course of reactions was typically followed by TLC and reaction times are provided for illustration only; melting points are uncorrected; products exhibited

satisfactory ^{1}H-NMR and/or microanalytical data; yields are provided for illustration only; and the following conventional abbreviations are also used: mp (melting point), L (liter(s)), mL (milliliters), mmol (millimoles), g (grams), mg (milligrams), min (minutes), LC-MS (liquid chromatography-mass spectrometry) and h (hours).

^{1}H-NMR spectra were measured on a Varian Mercury 300 MHz spectrometer and were consistent with the assigned structures. Chemical shifts were reported in parts per million (ppm) downfield from tetramethylsilane. Electrospray mass spectra were recorded on a Perkin Elmer Sciex API 365 mass spectrometer. Elemental analyses were performed by Robertson Microlit Laboratories, Madison, NJ. Silica gel for flash chromatography was E. Merck grade (230-400 mesh). Reverse-Phase analytical HPLC was performed on either a HP 1100 or a Varian ProStar 210 instrument with a Phenomenex Luna 5 µm C-18(2) 150 mm x 4.6 mm column or a Varian Microsorb-MV 0.1 µm C-18 150 mm x 4.6 mm column. A flow rate of 1 mL/min was with either a gradient of 0% to 50% buffer B over 15 minutes or 10% to 100% buffer B over 10 minutes with detection by UV at 254nm. Buffer A, 20 mM ammonium formate + 20% acetonitrile or 0.1% trifluoroacetic acid in acetonitrile; buffer B, 20 mM ammonium formate + 80% acetonitrile or 0.1% aqueous trifluoroacetic acid. Reverse phase preparative HPLC were performed on a Varian ProStar 215 instrument with a Waters Delta Pak 15 µm C-18 300 mm x 7.8 mm column.

EXAMPLE 1





Synthesis of Compound **1**: To a solution of 2-bromoethylamine bromide (5 g, 24.4 mmole) in DMF (50 mL) was added diisopropylethylamine (8.5 mL, 48.8 mmole) and benzyl chlroroformate (3.48 mL, 24.4 mmole). The mixture thus obtained was stirred at room temperature for 2 hours. The reaction mixture was concentrated and the residue was purified by flash chromatography on silica gel with ethyl acetate/hexanes (3/7) as gradient to give Compound **1** as an oil (4 g, 64%). ^{1}H NMR ($CDCl_3$) δ 3.54 (bs, 2H), 3.61 (bs, 2H), 5.12 (s, 2H), 7.36 (m, 5H).

Synthesis of Compound **2**: To a solution of Compound **1** (3.34 g, 12.99 mmole) and valine tert-butyl ester (3.27 g, 15.59 mmole) in DMF (50 mL) was added potassium carbonate (5.39 g, 38.97 mmole) and potassium iodide (2.59 g, 15.59 mmole). The

mixture thus obtained was stirred at 100°C overnight. The reaction mixture was concentrated and the residue was purified by flash chromatography on silica gel with ethyl acetate/hexanes (2/8) as gradient to give Compound **2** as an oil (3.12 g, 69%). ^{1}H NMR ($CDCl_3$) δ 0.92 (m, 6H), 1.46 (s, 9H), 1.86 (m, 1H), 2.53 (m, 1H), 2.80 (m, 2H), 3.18 (m, 1H), 3.31 (m, 1H), 5.10 (s, 2H), 5.25 (bs, 1H), 7.36 (m, 5H); LC-MS (ESI) 296 (M+H-*t*butyl$^+$), 352 (M+H$^+$).

Synthesis of Compound **3**: A solution of Compound **2** (3.4 g, 9.72 mmole) and palladium on charcoal (200 mg) in methanol (30 mL) was placed under hydrogen atmospheric pressure at room temperature. The mixture thus obtained was stirred at room temperature for 2 hours. The palladium was filtrated and the reaction mixture was concentrated to dryness to give Compound **3** as an oil (2.1 g, 98%). ^{1}H NMR (CD_3OD) δ 0.94 (m, 6H), 1.47 (s, 9H), 1.63 (bs, 2H), 1.90 (m, 1H), 2.47 (m, 1H), 2.73 (m, 2H).

Synthesis of Compound **4**: To a solution of Compound **3** (2.1 g, 9.72 mmole) in dichloromethane (30 mL) was added FmocOSu (N-(9-Fluorenylmethoxycarbonyloxy)succinimide (3.28 g, 9.72 mmole) at 0°C. The mixture thus obtained was stirred for 2 hours at 0°C. The mixture was concentrated to dryness and then the residue was purified by flash chromatography on silica gel with 100% dichloromethane, followed by 0.5% methanol in dichloromethane and finally 1% methanol in dichloromethane as gradient to give Compound **4** as colorless oil (2.55 g, 60%). ^{1}H-NMR ($CDCl_3$) δ 1.03 (d, 3H), 1.14 (d, 3H), 1.52 (s, 9H), 2.28 (m, 1H), 3.14 (m, 2H), 3.46 (m , 2H), 3.89 (d, 1H), 4.24 (m, 1H), 4.44 (m, 2H), 7.29 (m, 2H), 7.40 (m, 2H), 7.64 (m, 2H), 7.80 (d, 2H); LC-MS (ESI) 383 (M+H-*t*butyl$^+$), 440 (M+H$^+$), 462 (M+Na$^+$), 478 (M+K$^+$).

Synthesis of Compound **5**: To a solution of Compound **4** (177 mg, 0.4 mmole) in tetrahydrofurane-water (3/1, 8 mL) was bubbled HCl gas for 5 min. The reaction mixture was stirred at 37°C overnight then the mixture was concentrated to dryness to give Compound **5** as solid (168 mg, 98%) which was used in next step without further purification. ^{1}H-NMR ($CDCl_3$) δ 1.04 (d, 3H), 1.14 (d, 3H), 2.32 (m, 1H), 3.18 (m, 2H), 3.46 (m , 2H), 3.95 (d, 1H), 4.22 (m, 1H), 4.42 (m, 2H), 7.29 (m, 2H), 7.39 (m, 2H), 7.64 (m, 2H), 7.79 (d, 2H); LC-MS (ESI) 383 (M+H$^+$), 405 (M+Na$^+$).

Synthesis of Compound **6**: To a solution of N-methyl-1,2-phenylenediamine (2 ml, 17.6 mmole) in THF (15 mL) was added di-*tert*-butyldicarbonate (3.32 g, 15.2

mmole). The mixture thus obtained was stirred at room temperature overnight. The solvent was removed and the residue was purified by flash chromatography on silica gel with 30% ethyl acetate in hexanes as gradient to give Compound **6** as colorless oil (1.12 g, 32%). ^{1}H NMR ($CDCl_3$) δ 1.46 (bs, 9H), 3.15 (s, 3H), 3.73 (bs, 2H), 6.75 (d, 2H), 7.06 (m, 2H).

Synthesis of Compound **7**: To a solution of Compound **6** (777 mg, 3.05 mmole) in dichloromethane (30 mL) were added 2-nitrobenzenesulfonyl chloride (811 mg, 3.66 mmole) and diisopropylethylamine (796 μL, 4.57 mmole) at 0°C. The mixture thus obtained was stirred at room temperature overnight. The solvent was removed and the residue was purified by flash chromatography on silica gel with 10% ethyl acetate in hexanes as gradient to give Compound **7** as yellow solid (1.17 g, 94%). ^{1}H NMR ($CDCl_3$) δ 1.49 (bs, 9H), 2.47 and 2.61 (2bs, 3H), 7.05 (bd, 1H), 7.27 (m, 2H), 7.56-7.92 (m, 5H).

Synthesis of Compound **8**: To a solution of Compound **7** (326 mg, 0.8 mmole) in DMF (5 mL) were added carbonate potassium (164 mg, 1.19 mmole) and methyl iodide (148 μL, 2.39 mmole). The mixture thus obtained was stirred at room temperature overnight. The solvent was removed and the residue was purified by flash chromatography on silica gel with 10% ethyl acetate in hexanes as gradient to give Compound **8** as yellow solid (370 mg, 65%). ^{1}H NMR (CD_3OD) δ 1.35 and 1.52 (2s, 9H), 3.16 and 3.21 (2s, 3H), 3.26 and 3.29 (2s, 3H), 6.93 (d, 1H), 7.20 (m, 1H), 7.6 (m, 2H), 7.68-7.80 (m, 4H); LC-MS (ESI) 444 ($M+Na^+$), 460 ($M+K^+$).

Synthesis of Compound **9**: To a solution of Compound **8** (355 mg, 0.85 mmole) in dichloromethane (5 mL) was added trifluoroacetic acid (5 mL) at room temperature. The reaction mixture was stirred for 30 min, then partitioned between ethyl acetate (100 mL) and saturated sodium ammonium bicarbonate (200 mL). The organic layer was washed with brine (100 mL), dried over Na_2SO_4, and concentrated to give the free amine (Compound **9**) as a white solid (270 mg, 98%).^{1}H NMR ($CDCl_3$) δ 2.68 (s, 3H), 3.30 (s, 3H), 6.56 (m, 2H), 6.88 (m, 1H), 7.20 (m, 1H), 7.48 (m, 1H), 7.57 (m, 1H), 7.65 (m, 2H).

Synthesis of Compound **10**: To a solution of Compound **9** (270 mg, 0.84 mmole) in dichloromethane (10 mL) was added 2N phosgene in toluene (440 μL, 2.5 mmole) at 0°C. The mixture was stirred for 30 min and concentrated to dryness to give Compound **10** as white solid (297, 92%) which was used in next step without further purification. ^{1}H NMR ($CDCl_3$) δ 3.25 and 3.30 (2s, 3H), 3.34 and 3.44 (2s, 3H), 6.98 (m, 1H), 7.25-7.40

(m, 2H), 7.45 (m, 1H), 7.55-7.80 (m, 4H); LC-MS (ESI) 384 ($M+H^+$), 407 ($M+Na^+$), 422 ($M+K^+$).

Synthesis of Compound **11**: To a solution of Compound **10** (120 mg, 0.31 mmole) in solution of 10% DMF in THF (3 mL) were added Boc-Cit-PABOH ((S)-*tert*-butyl 1-(4-(hydroxymethyl)phenylamino)-1-oxo-5-ureidopentan-2-ylcarbamate, 238 mg, 0.62 mmole), N,N-dimethylaminopyridine (76 mg, 0.62 mmole) and diisopropylethylamine (55 μL, 0.31 mmole). The mixture thus obtained was stirred at 65°C for 10 days. The solvent was removed and the residue was purified by flash chromatography on silica gel with 5% methanol in dichloromethane as gradient to give Compound **11** as a solid (90 mg, 40%). ^1H NMR (CD_3OD) δ 1.43 (bs, 9H), 1.56-1.70 (m, 3H), 1.79 (m, 1H), 3.06-3.27 (m, 8H), 4.17 (2m, 1H), 5.04 and 5.17 (2bs, 2H), 6.89 and 6.95 (2d, 1H), 7.15-7.26 (m, 2H), 7.37-7.72 (m, 8H), 7.80 (m, 1H); LC-MS (ESI) 729 ($M+H^+$), 751 ($M+Na^+$), 767 ($M+K^+$).

Synthesis of Compound **12**: To a solution of Compound **11** (84 mg, 0.11 mmole) in DMF (2 mL) were added potassium carbonate (48 mg, 0.33 mmole) and thiophenol (60 μL, 0.55 mmole). The mixture thus obtained was stirred at room temperature for 2 hours. The solvent was removed and the residue was purified by flash chromatography on silica gel with 5% methanol in dichloromethane as gradient to give Compound **12** as a solid (55 mg, 87%). ^1H NMR (CD_3OD) δ 1.44 (bs, 9H), 1.56-1.70 (m, 3H), 1.78 (m, 1H), 2.76 (bs, 3H), 3.05-3.20 (m, 5H), 4.17 (bs, 1H), 4.99 (bs, 2H), 6.62 (m, 2H), 6.93 (m, 1H), 7.15 (m, 2H), 7.40-7.60 (m, 3H); LC-MS (ESI), 566 ($M+Na^+$), 581 ($M+K^+$).

Synthesis of Compound **13**: To a solution of Compound **12** (65 mg, 0.12 mmole) in dichloromethane (3 mL) was added 2N phosgene in toluene (180 μL, 0.36 mmole). The mixture thus obtained was stirred at 0°C for 1 hour. Then the mixture was concentrated to dryness to give an oil (72 mg, 100%) which was used in next step without further purification.

To a solution of the oil (72 mg, 0.12 mmole) in 5% N-methylpyrrolidone in dichloromethane (1 mL) were added Compound **18** (see Example 2 below) (25 mg, 0.06 mmole) and N,N-dimethylaminopyridine (22 mg, 0.18 mmole). The mixture thus obtained was stirred at room temperature overnight. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give Compound **13** as an oil (15 mg, 35%). ^1H NMR (CD_3OD) δ 1.42 (bs, 9H), 1.45-1.80 (m, 4H), 2.97 (bs, 6H), 3.10-3.36 (m, 7H), 3.57 (bs, 3H), 3.98 (bs, 1H), 4.10-4.35 (m, 4H), 4.50-4.75 (m, 3H), 5.15 (bs, 2H),

7.16-7.95 (m, 16H), 8.2 (d, 1H); LC-MS (ESI), 1034 ($M+H^+$), 1056 ($M+Na^+$), 1073 ($M+K^+$).

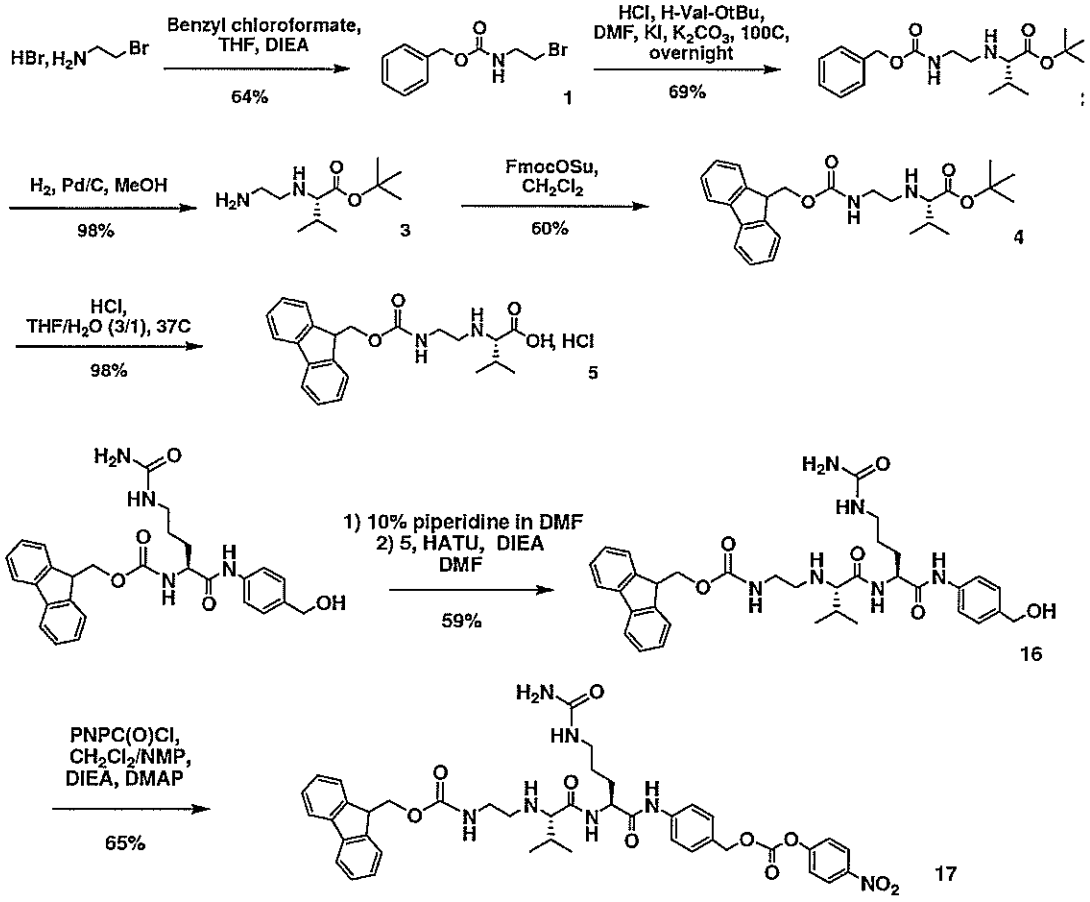
Synthesis of Compound **14**: To a solution of Compound **13** (13 mg, 0.011 mmole) in dichloromethane (0.5 mL) was added trifluoroacetic acid (0.5 mL) at room temperature. The mixture thus obtained was stirred at room temperature for 30 minutes. Then the mixture was concentrated to dryness to give an oil which was used in next step without further purification.

To a solution of the TFA salted amine in dichloromethane (0.5 mL) were added the Compound **5** (5 mg, 0.012 mmole), Benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphoniumhexafluorophosphate (BOP) (6 mg, 0.013 mmole) and diisopropylethylamine (8 μL, 0.044 mmole). The mixture thus obtained was stirred at room temperature for 1 hour. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give Compound **14** as an oil (9 mg, 50%). ^1H NMR (CD_3OD) δ 1.03 and 1.10 (2m, 6H), 1.60 (m, 2H), 1.80 (m, 2H), 2.20 (m, 1H), 2.99 (s, 6H), 3.04-3.50 (m, 11H), 3.60 (bs, 3H), 3.8 (bs, 1H), 4.00 (bs, 1H), 4.20-4.45 (m, 6H), 4.50-4.75 (m, 4H), 5.15 (bs, 2H), 7.20-7.70 (m, 22H), 7.77 (d, 2H), 7.90 (bs, 2H); LC-MS (ESI), 1300 ($M+H^+$).

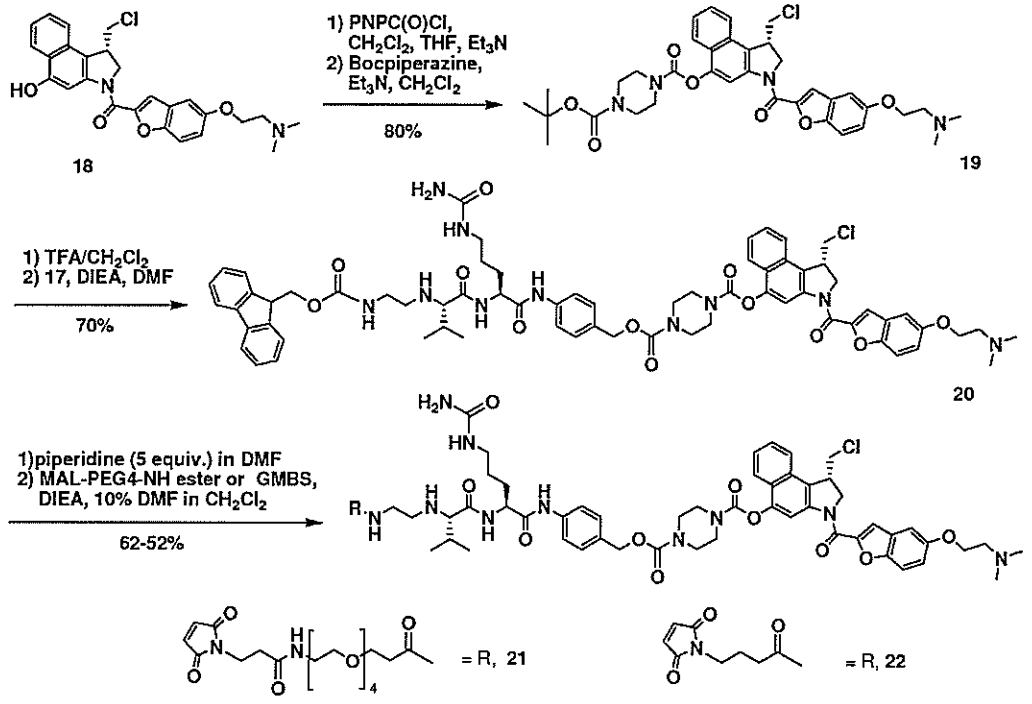
Synthesis of Compound **15**: To a solution of Compound **14** (9 mg, 0.006 mmole) in DMF (0.5 mL) was added piperidine (6 μL, 0.06 mmole) at room temperature. The mixture thus obtained was stirred at room temperature for 1 hour. Then the mixture was concentrated to dryness to give an oil which was used in next step without further purification.

To a solution of the oil in a solution of 10% DMF in dichloromethane (1 mL) were added (N-{y-maleimidobutyrloxy}succinimide ester (GMBS) (3.5 mg, 0.012 mmole) and diisopropylethylamine (2 μL, 0.012 mmole). The mixture thus obtained was stirred at room temperature for 1 hour. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give Compound **15** as an oil (6.5 mg, 75%). ^1H NMR (CD_3OD) δ 1.04-1.13 (m, 6H), 1.65 (m, 2H), 1.75 (m, 1H), 1.90 (m, 3H), 2.23 (m, 3H), 2.98 and 3.01 (2s, 6H), 3.05-3.25 (m, 6H), 3.60 (m, 2H), 3.45-3.55 (m, 6H), 3.64 (t, 2H), 3.80 (m, 2H), 4.05 (m, 1H), 4.35 (bs, 1H), 4.41 (m, 2H), 4.55 (m, 2H), 4.80 (m, 1H), 5.15 (bs, 2H), 6.78 (s, 2H), 7.22 (dd, 2H), 7.35-7.65 (m, 11H), 7.90-8.00 (m, 3H), 7.77 (d, 2H), 7.90 (bs, 2H); LC-MS (ESI), 1243 ($M+H^+$).

EXAMPLE 2


HBr, H2N Br
Benzyl chloroformate, THF, DIEA
64%
1
HCl, H-Val-OtBu, DMF, KI, K2CO3, 100C, overnight
69%
H2, Pd/C, MeOH
98%
3
FmocOSu, CH2Cl2
60%
4
HCl, THF/H2O (3/1), 37C
98%
OH, HCl
5
1) 10% piperidine in DMF
2) 5, HATU, DIEA
DMF
59%
16
PNPC(O)Cl, CH2Cl2/NMP, DIEA, DMAP
65%
NO2
17

5



Synthesis of Compound **16**: To a solution of Fmoc-Cit-PABOH (2 g, 3.98 mmole) in DMF (45 mL) was added piperidine (5 mL) at room temperature. The mixture thus obtained was stirred at room temperature for 1 hour. The mixture was concentrated to dryness and then the residue was purified by flash chromatography on silica gel with 100% dichloromethane, followed by 10% methanol in dichloromethane and finally 30% methanol in dichloromethane as gradient to give H-Cit-PABOH as colorless oil (1 g, 90%).

To a solution of H-Cit-PABOH (80 mg, 0.28 mmole) in DMF (4 mL) were added the compound **5** (100 mg, 0.24 mmole) (see Example 1 for preparation), HATU (100 mg, 0.26 mmole) and diisopropylethylamine (125 µL, 0.84 mmole). The mixture thus obtained was stirred at room temperature for 1 hour. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give the Compound **16** as an oil (119 mg, 66%). ^{1}H NMR (CD_3OD) δ 1.03-1.11 (2d, 6H), 1.58 (m, 2H), 1.77 (m, 1H), 1.88 (m, 1H), 2.23 (m, 1H), 3.05-3.20 (m, 4H), 3.44 (m, 2H), 3.83 (d, 1H), 4.21 (m, 1H), 4.39 (m, 2H), 4.54 (bs, 2H), 4.60 (m, 1H), 7.30 (m, 4H), 7.37 (m, 2H), 7.51 (m, 2H), 7.62 (m, 2H), 7.77 (m, 2H), 8.80 (d, 1H), 10.05 (s, 1H); LC-MS (ESI), 646 ($M+H^+$), 668 ($M+Na^+$), 684 ($M+K^+$).

Synthesis of Compound **17**: To a solution of Compound **16** (190 mg, 0.25 mmole)) in 10% NMP in dichloromethane (4 mL) were diisopropylethylamine (131 μL, 0.75 mmole), N,N-dimethylaminopyridine (15 mg, 0.12 mmole) and 4-nitrophenyl chloroformate (101 mg, 0.5 mmole). The mixture thus obtained was stirred at room temperature for 1 hour. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give Compound **17** as an oil (151 mg, 65%). (CD_3OD) δ 1.04-1.11 (2d, 6H), 1.59 (m, 2H), 1.79 (m, 1H), 1.89 (m, 1H), 2.24 (m, 1H), 3.05-3.20 (m, 4H), 3.44 (m, 2H), 3.83 (d, 1H), 4.20 (m, 1H), 4.39 (m, 2H), 4.60 (m, 1H), 5.22 (bs, 1H), 7.29 (m, 2H), 7.39 (m, 4H), 7.60 (m, 4H), 7.75 (d, 2H), 8.81 (d, 1H), 10.05 (s, 1H); LC-MS (ESI), 811 ($M+H^+$), 833 ($M+Na^+$), 848 ($M+K^+$).

Synthesis of Compound **19**: To a solution of Compound **18** (50 mg, 0.11 mmole)) in 40% THF in dichloromethane (8 mL) were added 4-Nitrophenyl Chloroformate (87 mg, 0.43 mmole) and triethylamine (88 μL, 0.64 mmole) at 0°C. The mixture thus obtained was let warmed to room temperature and stirred overnight. The solvent was evaporated and the residue was isolated by precipitation in ether and followed by filtration to give PNPC-**18** as a yellow solid (60 mg, 90%).

To a solution of the PNPC-**18** (60 mg, 0.095 mmole) in dichloromethane (5 mL) were added the compound bocpiperazine (71 mg, 0.38 mmole) and triethylamine (53 μL, 0.38 mmole). The mixture thus obtained was stirred at room temperature overnight. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give Compound **18** as an oil (77 mg, 90%). LC-MS (ESI), 678 ($M+H^+$), 700 ($M+Na^+$), 716 ($M+K^+$)

Synthesis of Compound **20**: To a solution of Compound **19** (28 mg, 0.035 mmole) in dichloromethane (0.5 mL) was added trifluoroacetic acid (0.5 mL) at room temperature. The mixture thus obtained was stirred at room temperature for 30 minutes. Then the mixture was concentrated to dryness to give an oil which was used in next step without further purification.

To a solution of the oil in DMF (1 mL) were added the compound **17** (28 mg, 0.035 mmole) and diisopropylethylamine (18 μL, 0.105 mmole). The mixture thus obtained was stirred at room temperature overnight. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give Compound **20** as an oil (36 mg, 70%). ^1H NMR (CD_3OD) δ 1.03-1.10 (2d, 6H), 1.58 (m, 2H), 1.77 (m, 1H), 1.87 (m, 1H), 2.23 (m, 1H), 2.97 (bs, 6H), 3.05-3.20 (m, 4H), 3.30-3.85 (m, 14H), 3.97 (m, 1H), 4.19-

4.41 (m, 6H), 4.60 (m, 2H), 4.69 (m, 1H), 5.11 (bs, 2H), 7.16 (dd, 1H), 7.27-7.38 (m, 7H), 7.45 (m, 1H), 7.51-7.63 (m, 7H), 7.76 (d, 2H), 7.85 (d, 2H), 8.24 (bs, 1H), 8.79 (d, 1H), 10.00 (s, 1H); LC-MS (ESI), 1248 ($M+H^+$).

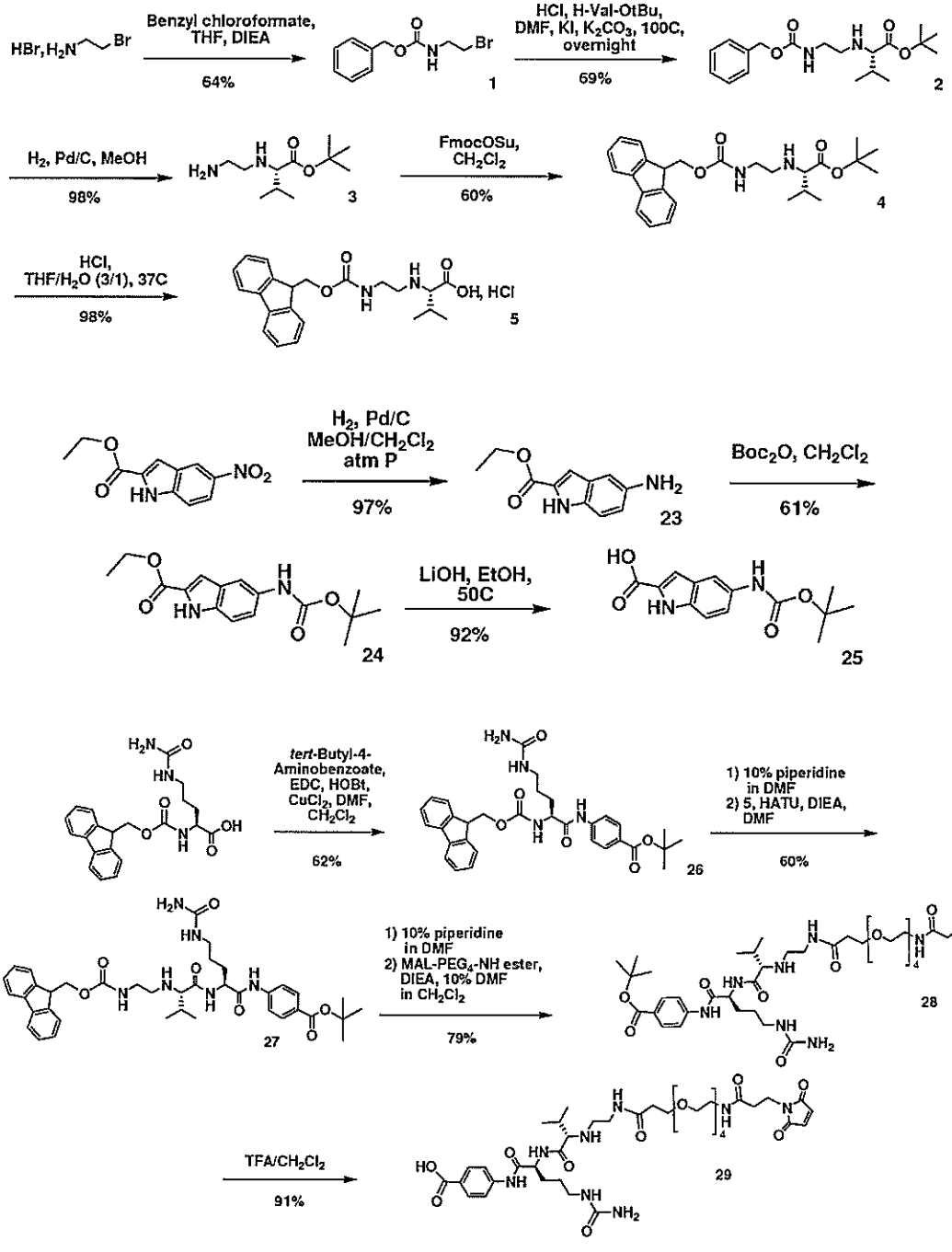
Synthesis of Compound **21**: To a solution of Compound **20** (36 mg, 0.024 mmole) in DMF (1 mL) was added piperidine (12 μL, 0.12 mmole) at room temperature. The mixture thus obtained was stirred at room temperature for 1 hour. Then the mixture was concentrated to dryness to give an oil which was used in next step without further purification.

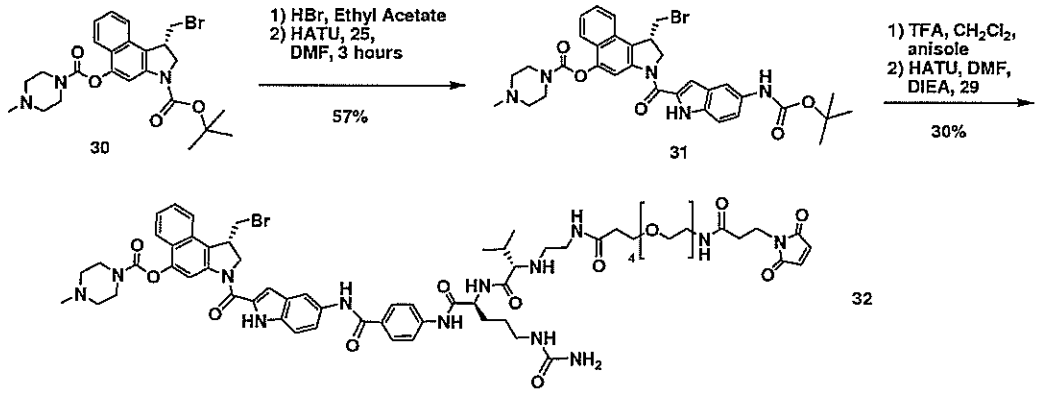
To a solution of the oil in 10% DMF in dichloromethane (1 mL) were added MAL-PEG_4-NH ester (19 mg, 0.037 mmole) and diisopropylethylamine (8.5 μL, 0.048 mmole). The mixture thus obtained was stirred at room temperature for 1 hour. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give Compound **21** as an oil (25 mg, 62%). ^1H NMR (CD_3OD) δ 1.06-1.12 (2d, 6H), 1.59 (m, 2H), 1.78 (m, 1H), 1.89 (m, 1H), 2.24 (m, 1H), 2.44 (t, 2H), 2.50 (t, 2H), 2.99 (bs, 6H), 3.05-3.20 (m, 4H), 3.46-3.85 (m, 34H), 3.99 (m, 1H), 4.25 (m, 1H), 4.35 (m, 2H), 4.59-4.73 (m, 3H), 5.13 (bs, 2H), 6.79 (s, 2H), 7.18 (dd, 1H), 7.31 (d, 1H), 7.36 (m, 2H), 7.47 (m, 1H), 7.58 (m, 5H), 7.89 (m, 2H), 8.24 (bs, 1H), 8.79 (d, 1H); LC-MS (ESI), 1423 ($M+H^+$).

Synthesis of Compound **22**: To a solution of Compound **20** (26 mg, 0.017 mmole) in DMF (1 mL) was added piperidine (9 μL, 0.088 mmole) at room temperature. The mixture thus obtained was stirred at room temperature for 1 hour. Then the mixture was concentrated to dryness to give the an oil which was used in next step without further purification.

To a solution of the oil in 10% DMF in dichloromethane (1 mL) were added GMBS (7.5 mg, 0.025 mmole) and diisopropylethylamine (6 μL, 0.034 mmole). The mixture thus obtained was stirred at room temperature for 1 hour. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give Compound **22** as an oil (13 mg, 52%). δ 1.07-1.13 (2d, 6H), 1.60 (m, 2H), 1.78 (m, 1H), 1.90 (m, 3H), 2.24 (m, 3H), 3.00 (bs, 6H), 3.05-3.24 (m, 4H), 3.42-3.74 (m, 16H), 3.78-3.90 (m, 4H), 4.00 (m, 1H), 4.34 (m, 1H), 4.39 (m, 2H), 4.60 (m, 1H), 4.80 (m, 2H), 5.14 (bs, 2H), 6.81 (s, 2H), 7.22 (dd, 1H), 7.37 (m, 3H), 7.50 (m, 1H), 7.60 (m, 5H), 7.92 (m, 2H), 8.24 (bs, 1H), 8.79 (d, 1H), 10.05 (s, 1H); LC-MS (ESI), 1191 ($M+H^+$).

EXAMPLE 3


HBr, H2N Br
Benzyl chloroformate, THF, DIEA
64%
1
HCl, H-Val-OtBu, DMF, KI, K2CO3, 100C, overnight
69%
2
H2, Pd/C, MeOH
98%
3
FmocOSu, CH2Cl2
60%
4
HCl, THF/H2O (3/1), 37C
98%
OH, HCl
5
H2, Pd/C MeOH/CH2Cl2 atm P
97%
NO2
NH2
23
Boc2O, CH2Cl2
61%
24
LiOH, EtOH, 50C
92%
HO
25
tert-Butyl-4-Aminobenzoate, EDC, HOBt, CuCl2, DMF, CH2Cl2
62%
26
1) 10% piperidine in DMF
2) 5, HATU, DIEA, DMF
60%
27
1) 10% piperidine in DMF
2) MAL-PEG4-NH ester, DIEA, 10% DMF in CH2Cl2
79%
28
TFA/CH2Cl2
91%
29



Synthesis of Compound **23**: A solution of ethyl-5-nitroindole-2 carboxylate (2 g, 8.5 mmole) and palladium on charcoal (200 mg) in 50% methanol in dichloromethane (100 mL) was placed under hydrogen atmospheric pressure at room temperature. The mixture thus obtained was stirred at room temperature for 2 hours. The palladium was filtrated and the reaction mixture was concentrated to dryness to give Compound **23** as colorless oil (1.68 g, 97%). ^{1}H NMR (CD_3OD) δ 1.38 (t, 3H), 4.34 (q, 2H), 6.86 (dd, 1H), 6.95 (d, 1H), 6.98 (d, 1H), 7.25 (d, 1H).

Synthesis of Compound **24**: To a solution of Compound **23** (300 mg, 1.47 mmole) in dichloromethane (5 mL) was added Boc_2O (385 mg, 1.76 mmole). The mixture thus obtained was stirred at room temperature for 2 hours. The reaction mixture was concentrated and the residue was purified by flash chromatography on silica gel with 10% ethyl acetate in hexanes as gradient to give Compound **24** as a white solid (272 mg, 61%). ^{1}H NMR (CD_3OD) δ 1.39 (t, 3H), 1.52 (s, 9H), 4.37 (q, 2H), 7.07 (s, 1H), 7.23 (dd, 1H), 7.34 (d, 1H), 7.68 (bs, 1H).

Synthesis of Compound **25**: A solution of Compound **24** (100 mg, 0.33 mmole) in ethanol (3 mL) was added a solution of LiOH (12 mg, 0.49 mmole) in water (1 mL). The mixture thus obtained was stirred at room temperature for 2 hours at 50°C. The reaction mixture was concentrated to dryness to give an oil. The residue was dissolved in water and acidified to pH 3 with 10% HCl, followed by extraction with EtOAc. The organic solution was dried over Na_2SO_4, filtered and concentrated to dryness to give Compound **25** as colorless oil (85 mg, 92%). ^{1}H NMR (CD_3OD) δ 1.51 (s, 9H), 7.07 (d, 1H), 7.23 (dd, 1H), 7.33 (d, 1H), 7.68 (bs, 1H).

Synthesis of Compound **26**: To a solution of Fmoc-Cit-OH (206 mg, 0.52 mmole) in solution of 30% DMF in dichloromethane (3 mL) were added EDC (120 mg, 0.62 mmole), HOBt (84 mg, 0.62 mmole) and tert-butyl-4-amino benzoate (120 mg, 0.62 mmole) at room temperature. The mixture thus obtained was stirred for 10 minutes then copper chloride (84 mg, 0.62 mmole) was added to the mixture. The mixture was stirred overnight. The mixture was concentrated to dryness and then the residue was purified by flash chromatography on silica gel with 5% methanol in dichloromethane as gradient to give Compound **26** as colorless oil (184 mg, 62%). ^{1}H NMR (CD_3OD) δ 1.53-1.58 (m, 2H), 1.57 (s, 9H), 1.71 (m, 1H), 1.82 (m, 1H), 3.08 (m, 1H), 3.19 (m, 1H), 4.21 (m, 1H), 4.28 (m, 1H), 4.38 (m, 2H), 7.28-7.39 (m, 3H), 7.49 (m, 2H), 7.56-7.86 (m, 5H), 7.89 (m, 2H); LC-MS (ESI), 573 ($M+H^+$), 595 ($M+Na^+$), 611 ($M+K^+$).

Synthesis of Compound **27**: To a solution of Compound **26** (1 g, 1.75 mmole) in DMF (18 mL) was added piperidine (2 mL) at room temperature. The mixture thus obtained was stirred at room temperature for 1 hour. The mixture was concentrated to dryness and then the residue was purified by flash chromatography with 100% dichloromethane, followed by 5% methanol in dichloromethane and finally 20% methanol in dichloromethane as gradient to give a colorless oil (561 mg, 92%).

To a solution of the oil (561 mg, 1.6 mmole) in DMF (10 mL) were added diisopropylethylamine (679 µL, 3.9 mmole), the compound **5** (509 mg, 1.3 mmole) (see Example 1 for preparation) and HATU (494 mg, 1.3 mmole) at room temperature. The mixture thus obtained was stirred at room temperature for 3 hours. The mixture was concentrated to dryness and then the residue was purified by flash chromatography on silica gel with 5% methanol in dichloromethane as gradient to give Compound **27** as colorless oil (691 mg, 65%). ^{1}H NMR (CD_3OD) δ 1.36 (dd, 6H), 1.58-1.62 (m, 2H), 1.6 (s, 9H), 1.71 (m, 1H), 1.82 (m, 1H), 2.00 (m, 1H), 2.65 (m, 2H), 3.2-3.3 (m, 4H), 3.70 (m, 1H), 4.21 (m, 1H), 4.28 (m, 2H), 4.38 (m, 2H), 4.60 (m, 1H), 7.28-7.39 (m, 4H), 7.60-7.70 (m, 4H), 7.8 (d, 2H), 7.89 (d, 2H); LC-MS (ESI), 716 ($M+H^+$), 737 ($M+Na^+$), 753 ($M+K^+$).

Synthesis of Compound **28**: To a solution of Compound **27** (300 mg, 0.45 mmole) in DMF (9 mL) was added piperidine (1 mL) at room temperature. The mixture thus obtained was stirred at room temperature for 1 hour. Then the mixture was concentrated to dryness to give an oil which was crashed out in ether (20 mL). The material was filtered to give a white solid (186 mg, 84%).

To a solution of the free amine (32 mg, 0.065 mmole) in dichloromethane (1 mL) was added MAL-PEG$_4$-NH ester (50 mg, 0.097 mmole). The mixture thus obtained was stirred at room temperature for 4 hours. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give Compound **28** as an oil (47 mg, 95%). ^{1}H NMR (CD_3OD) δ 1.10 and 1.15 (2d, 6H), 1.58-1.62 (m, 2H), 1.6 (s, 9H), 1.75 (m, 1H), 1.90 (m, 1H), 2.25 (m, 1H), 2.45 (t, 2H), 2.5 (t, 2H), 3.10-3.25 (m, 4H), 3.30 (m, 2H), 3.45-3.65 (m, 16H), 3.75 (m, 4H), 3.85 (d, 1H), 4.65 (m, 1H), 6.80 (s, 2H), 7.67 (d, 2H), 7.90 (d, 2H), 8.80 (d, 1H), 10.20 (s, 1H); LC-MS (ESI), 891 ($M+H^+$), 913 ($M+Na^+$), 929 ($M+K^+$).

Synthesis of Compound **29**: To a solution of Compound **28** (47 mg, 0.062 mmole) in dichloromethane (0.5 mL) was added trifluoroacetic acid (0.5 mL) at room temperature. The mixture thus obtained was stirred at room temperature for 30 minutes. Then the mixture was concentrated to dryness to give Compound **29** as an oil which was used in next step without further purification (40 mg, 92%). ^{1}H NMR (CD_3OD) δ 1.10 and 1.15 (2d, 6H), 1.60 (m, 2H), 1.80 (m, 1H), 1.90 (m, 1H), 2.25 (m, 1H), 2.45 (t, 2H), 2.5 (t, 2H), 3.10-3.25 (m, 4H), 3.30 (m, 2H), 3.45-3.65 (m, 16H), 3.75 (m, 4H), 3.85 (d, 1H), 4.65 (m, 1H), 6.80 (s, 2H), 7.67 (d, 2H), 7.95 (d, 2H), 8.80 (d, 1H); LC-MS (ESI), 836 ($M+H^+$), 858 ($M+Na^+$), 874 ($M+K^+$).

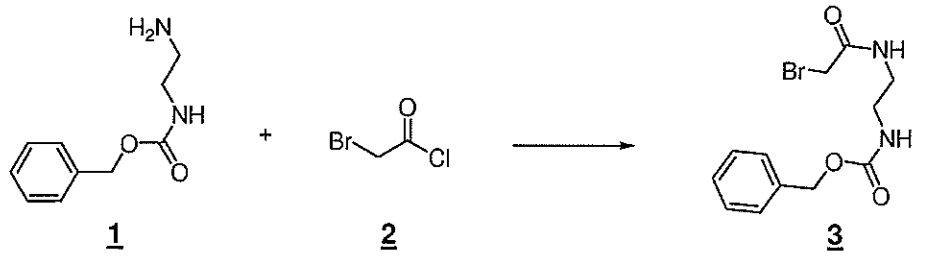
Synthesis of Compound **31**: To a solution of **30** (100 mg, 0.2 mmole) in EtOAc (2 mL) was added a concentrated HBr solution in EtOAc (3 mL) at room temperature. The Boc deprotection was completed after 1 hour. The precipitated material was filtered (quantitative yield). Then the TFA salted amine was dissolved in DMF (3 mL). To this solution were added the compound **25** (55 mg, 0.2 mmole), diisopropylethylamine (173 μL, 1 mmole) and HATU (79 mg, 0.2 mmole). The mixture thus obtained was stirred at room temperature for 3 hours. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give Compound **31** as a white solid (86 mg, 57%). ^{1}H NMR (CD_3OD) δ 1.54 (s, 9H), 2.91 (s, 3H), 3.10-3.60 (m, 8H), 3.72 (m, 1H), 3.97 (m, 1H), 4.30-4.60 (m, 3H), 6.94 (bs, 1H), 7.05 (m, 1H), 7.12 (d, 1H), 7.45 (m, 2H), 7.68 (d, 1H), 7.75 (bs, 1H), 7.86 (d, 1H), 8.23 (bs, 1H); LC-MS (ESI), 562 ($M+H-100^+$), 606 ($M+H-56^+$), 662 ($M+H^+$), 685 ($M+Na^+$), 701 ($M+K^+$).

Synthesis of Compound **32**: To a solution of Compound **31** (30 mg, 0.039 mmole) in dichloromethane (0.5 mL) were added anisole (100 μL) and trifluoroacetic acid (0.4 mL) at room temperature. The mixture thus obtained was stirred at room

temperature for 30 minutes. Then the mixture was concentrated to dryness to give an oil which was used in next step without further purification.

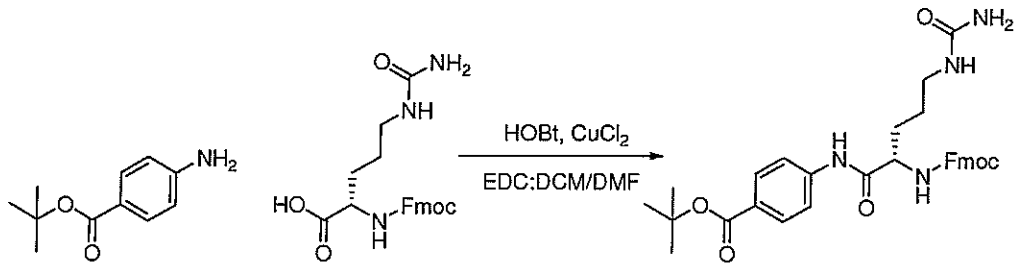
To a solution of the oil in DMF (1 mL) were added the Compound **29** (36 mg, 0.039 mmole), diisopropylethylamine (40 µL, 0.23 mmole) and HATU (15 mg, 0.039 mmole). The mixture thus obtained was stirred at room temperature for 1 hour. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give Compound **32** as an oil (36 mg, 60%). ^{1}H NMR (CD_3OD) δ 1.09 and 1.15 (2d, 6H), 1.62 (m, 2H), 1.81 (m, 1H), 1.93 (m, 1H), 2.27 (m, 1H), 2.45 (t, 2H), 2.51 (t, 2H), 2.98 (s, .3H), 3.13-3.25 (m, 4H), 3.47-3.62 (m, 24H), 3.76 (m, 4H), 3.82 (m, 1H), 3.85 (d, 1H), 4.20 (m, 1H), 4.55-4.70 (m, 4H), 6.79 (s, 2H), 7.06 (s, 1H), 7.36 (bs, 1H), 7.43-7.54 (m, 2H), 7.72-7.81 (m, 3H), 7.91 (m, 3H), 8.05 (s, 1H), 8.25 (bs, 1H), 8.82 (d, 1H), 10.25 (s, 1H); LC-MS (ESI), 691 ($M+2H^+$)/2, 1381 ($M+H^+$), 1419 ($M+K^+$).

EXAMPLE 4



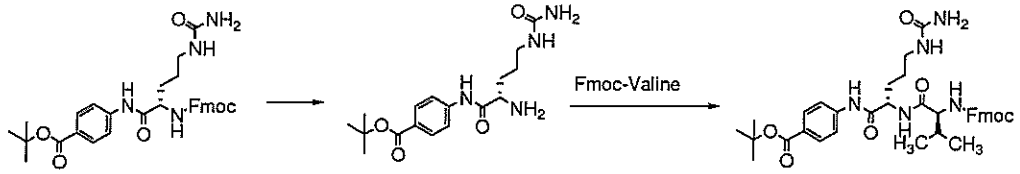
1 2 3

Synthesis of Compound 3: Bromo acetyl chloride **2** (240uL, 2.86 mMoles) was added dropwise to a solution of benzyl 2-aminoethylcarbamate **1** (500 mg, 2.6 mMoles) and TEA (800 uL, 5.7 mMoles) in 10 mL dichloromethane at 0° C. The reaction mixture was allowed to stir for 2 hours and the the temperature gradually rose to room temperature.. The solvents were evaporated followed by aqueous work up and extraction with ethyl acetate. The organic layer was washed with 10 % citric acid, water and saturated sodium bicarbonate and brine and dried over anhydrous sodium sulfate, giving Compound **3** (260 mg, 32 % yield) MS: M^{+1} = 315.3. H^1 NMR (CDCL3): 7.37 ppm (5H), 5.11ppm (2H), 3.83 ppm (2H), 3.40 ppm (4H).



4 5 6

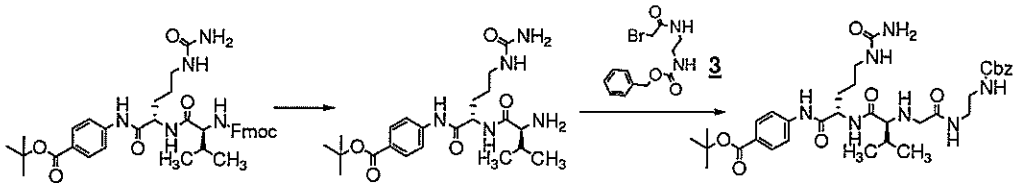
Synthesis of Compound 6: 1.5 grams of Fmoc-Citruline **5** (0.0038 Moles), 0.88 grams of t-butyl-4-amino benzoate **4** (0.0045 Moles), 0.6 g of HOBt 0.0045 Moles), 0.68 g of EDC (0.0043 Moles) and a catalytic amount of copper chloride were allowed to stir overnight in a mixture of DCM/DMF (2:1) 9mL. The solvents were removed and the product purified by silica gel flash column chromatography using 5-10 % MeOH in DCM to give Compound **6** (1.54 g, 71 % yield). MS: $M^{+1} = 573.9$.



6 7 8

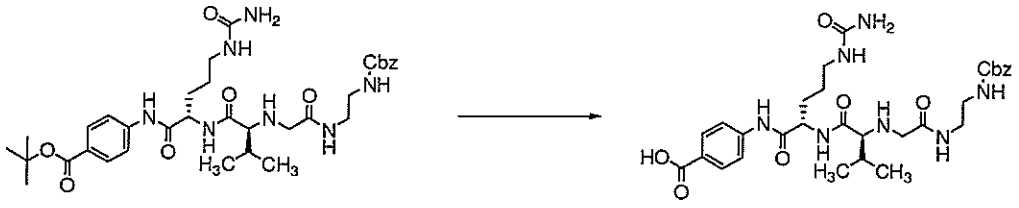
Synthesis of Compound 8: Deprotection of the Fmoc protecting group in Compound **6**, 300 mg, 0.66 mMoles was done using 5 % piperidine in DMF in 20 minutes. The solvents were evaporated and the crude solid rinsed with diethyl ether to give 230 mg of Compound **7** (99% yield). MS: $M^{+1} = 352$

230 mg of Compound **7** (0.65 mMoles) was reacted with Fmoc-Valine 333 mg (0.98 mMoles) and 188 mg of EDC (0.98 mmoles) in DMF and DCM to give 240 mg of Compound **8** (55 % yield) after purification over silica gel 5-10 % MeOH in DCM. MS: $M^{+1} = 673$.



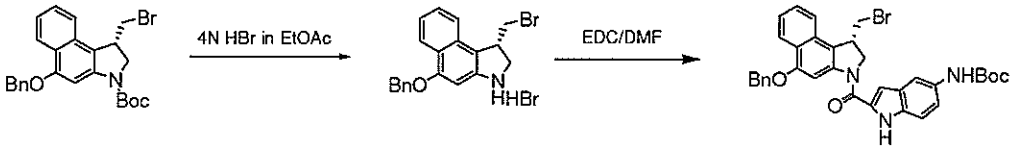
8 9 10

Synthesis of Compound **10**: 500 mg of Compound **8** (0.75 mMoles) was deprotected using piperidine in DMF to give Compound **9** which was rinsed with Diethyl ether after removing solvents. Compound **9** without further purification was reacted with 235 mg of **3** (0.75 mmoles) in the presence of 125 mg of KI (0.75 mMoles) and 313 uL triethyl amine at 40° C for 2 hours. The crude reaction mixture was concentrated and purified by reverse phase HPLC to give 300mg of Compound **10** in 55.8 % yield. MS: $M^{[+1]} = 685$.



10 **11**

Synthesis of Compound **11**: Compound **10** (300 mg) was deprotected with HCL-EA for 3 hours. The solvents were evaporated and the product dried under high vacuum to give Compound **11**. MS: $M^{[+1]} = 628$. . H^1 NMR (DMSO): 10.45 ppm (1H), 8.8 ppm (1H), 8.5 (1H), 7.88 ppm (2H), 7.75 ppm (2H), 7.3 ppm (5H), 6.1 (1H), 5.5 (2H), 4.99 (2H), 4.5 ppm (1H), 3.7-3.4 (3H), 3.2-2.9 (5H), 2.16 (1H), 1.45 (2H). 1.1 (3H), 0.92 ppm (3H)



12 **13** **14**

Synthesis of Compound **14**: A solution of Compound **12** (42 mg, 0.09 mmol) in 4 N HBr in EtOAc (5 ml) was stirred at 25 ^{0}C for 45 min. The solvent was removed and was further dried in high vacuo for 4 h. To the residue in DMF (2mL) was added 5-(amino-*tert*-butoxycarbonyl)-indone-2-carboxylic acid (39.2 mg, 0.14 mmol) and BOP (71mg, 0.16 mmol) followed by adding DIPEA (83uL, 0.48 mmol). The reaction mixture was stirred at 25 ^{0}C for 20 min and passed through a short column of silica gel. The solvent was removed, and the crude product was chromatographed on silica gel eluted with 20% EtOAc in hexane to give Compound **14** (49 mg, 88%). ^{1}HNMR DMSO-d_6) δ11.63 (s, 1H), 9.18 (br s, 1H), 8.19 (d, 1H, J=8.4 Hz), 8.09 (br s, 1H), 7.90 (d, 1H, J=8.4

Hz), 7.79 (br s, 1H), 7.53-7.58 (m, 3H), 7.39-7.43 (m, 3H), 7.28-7.35 (m, 3H), 7.11 (s, 1H), 5.29 (s, 2H), 4.80 (t, 1H, 11.2 Hz), 4.54 (dd, 1H, 8.8 Hz), 4.31 (m, 1H), 3.92 (dd, 1H, J=10.2 Hz), 3.82 (dd, 1H, J=10.7 Hz), 1.47 (s, 9H).



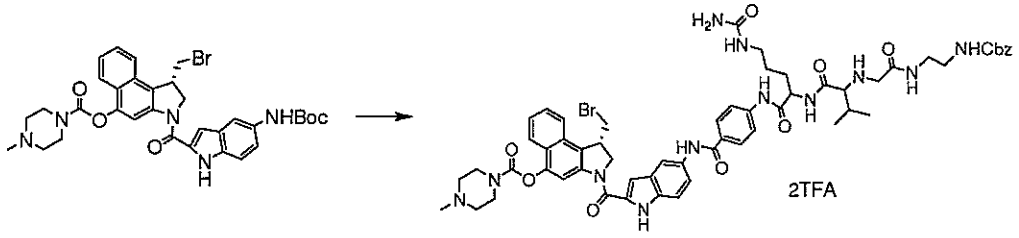
14 **15**

Synthesis of compound **15**: A mixture of Compound **14** (49 mg, 0.08 mmol) and 10% Pd-C (35 mg) in MeOH/CH_2Cl_2 (1/2, 10 ml) was degassed in vacuo for 40 s. The resulting mixture was placed under an atmosphere of hydrogen and stirred at 25 ^{0}C for 7 h. The reaction mixture was filtered through Celite (MeOH-CH_2Cl_2 wash). The solvent was removed in vacuo. Chromatography on silica gel eluted with 2% MeOH in DCM to afford Compound **15** (40.6 mg, 97%). ^{1}NMR DMSO-d_6) δ 11.59(s, 1H), 10.43 (s, 1H), 9.18 (br s, 1H), 8.09 (d, 1H, J=8.2 Hz), 7.93 (br s, 1H), 7.81 (d, 1H, J=8.2 Hz), 7.78 (br s, 1H), 7.49 (t, 1H, J=8.4 Hz), 7.27-7.35 (m, 3H), 7.08 (s, 1H), 4.80 (t, 1H, 11.2 Hz), 4.54 (dd, 1H, 8.8 Hz), 4.31 (m, 1H), 3.92 (dd, 1H, J=10.2 Hz), 3.82 (dd, 1H, J=10.7 Hz), 1.47 (s, 9H).



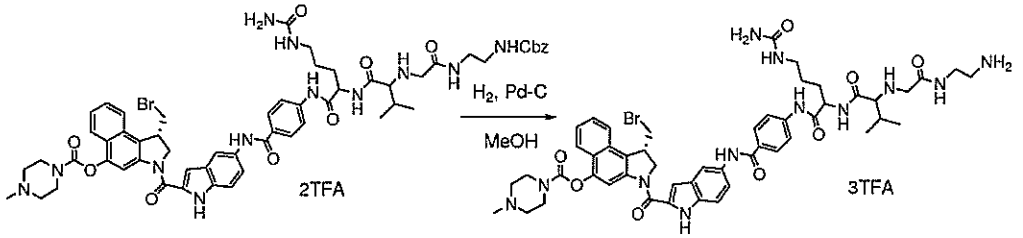
15 **16**

Synthesis of Compound **16**: A solution mixture of Compound **15** (36 mg, 0.07 mmol), 4-methyl-1-piperazinecarbonyl chloride hydrochloride (20 mg, 0.10 mmol), allyl alcohol (0.1 mL) and anhydrous pyridine (63 μml) in CH_2Cl_2 (7mL) was stirred for 16 h at room temperature. Without removing solvent, the crude product was chromatographed on silica gel, eluted with 2%-10% MeOH in CH_2Cl_2 to obtain Compound **16** (32.4 mg, 73%). ^{1}NMR DMSO-d_6) δ 11.59(s, 1H), 9.18 (br s, 1H), 8.09 (d, 1H, J=8.2 Hz), 7.93 (br s, 1H), 7.81 (d, 1H, J=8.2 Hz), 7.78 (br s, 1H), 7.49 (t, 1H, J=8.4 Hz), 7.27-7.35 (m, 3H), 7.08 (s, 1H), 4.80 (t, 1H, 11.2 Hz), 4.54 (dd, 1H, 8.8 Hz), 4.31 (m, 1H), 3.92 (dd, 1H, J=10.2 Hz), 3.82 (dd, 1H, J=10.7 Hz), 3.77 (br s, 2H), 3.47 (br s, 2H), 3.37 (br s, 2H), 2.63 (s, 3H), 2.38 (br s, 2H), 1.47 (s, 9H).



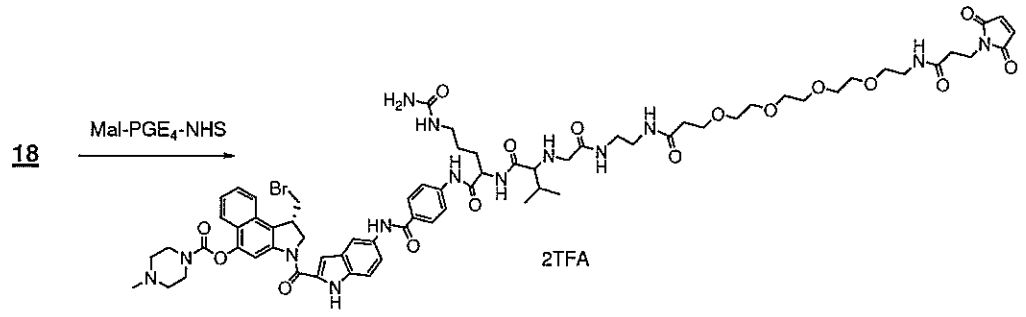
16 **17**

Synthesis of Compound **17**: A solution of Compound **16** (32 mg, 0.05 mmol) in 4 N HBr in EtOAc (4 ml) was stirred at 25 ^{0}C for 45 min. The solvent was removed and was further dried in high vacuo for 4 h. To the residue in DMF (2 mL) was added Compound **11** (48.2 mg, 0.07 mmol) and Benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphoniumhexafluorophosphate (BOP) (34.5 mg, 0.08 mmol) followed by adding DIPEA (68 uL) and the reaction mixture was stirred for 25 min at 25^{0}C. The solvent was removed under vacuo and the product was purified by Prep HPLC (SymmetrPrep C18, 7µm, 19 x 150 mm column), eluted at 10 ml/min (0.01% TFA in water/acetonitrile) with a gradient: 10% acetonitrile in 5 min, 10% to 50% acetonitrile in 15 min, maintaining 50% acetonitrile in 5 min, 50% to 100% acetonitrile in 5 min, to give Compound **17** (42.1 mg, 64%). MS: calcd for $C_{58}H_{67}BrN_{12}O_{10}$ (M+H) m/z 1171.43 found 1172.40.



17 **18**

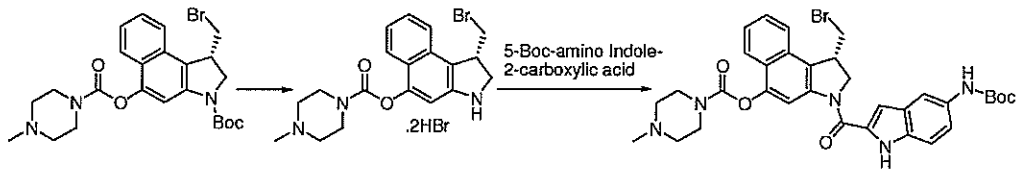
Synthesis of compound **18**: To a solution of Compound **17** (33.6 mg, 0.025 mmol) in MeOH (5mL) was added 10% Pd-C (28 mg), and the mixture was degassed with N_2. The reaction mixture was flushed with H_2 and then stirred under a H_2 atmosphere. Upon complete reaction (40 min), the reaction mixture was filtered and concentrated to afford Compound **18** (31.5 mg, 96%). MS: calcd for $C_{50}H_{61}BrN_{12}O_8$ (M+H) m/z 1037.39 found 1038.20.



19

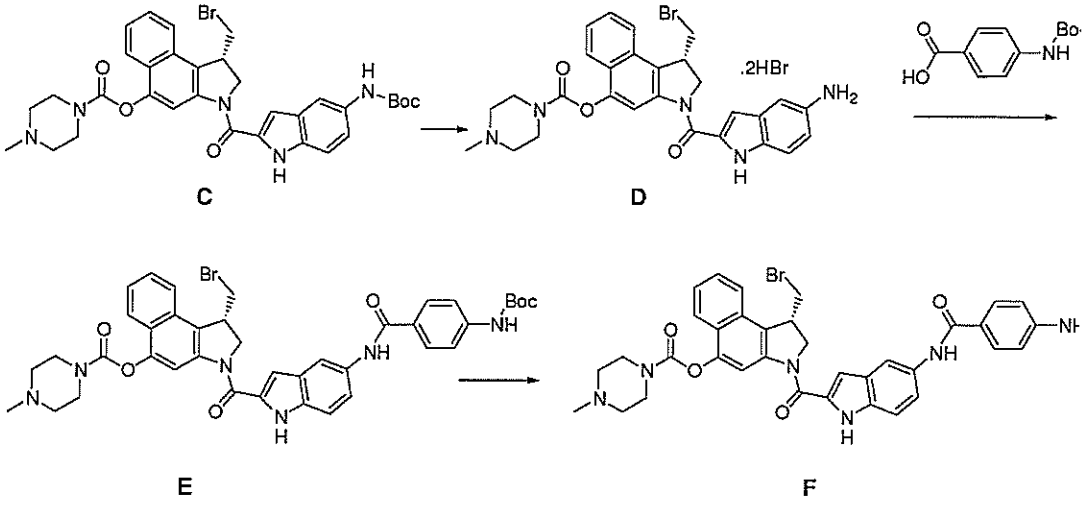
Synthesis of compound **19**: A solution of Compound **18** (31.5 mg, 0.024 mmol) in DMF (2mL) was added Mal-PEG4-NHS ester (42 mg, 0.08 mmol) in DCM (0.5mL). The reaction mixture was stirred for 1 hr at 25°C. The final product was purified by Prep HPLC (SymmetrPrep C18, 7μm, 19 x 150 mm column), eluted at 10 ml/min (0.01% TFA in water/acetonitrile) with a gradient: 10% acetonitrile in 5 min, 10% to 50% acetonitrile in 15 min, maintaining 50% acetonitrile in 5 min, 50% to 100% acetonitrile in 5 min, to obtain Compound **19** (29.7 mg, 77%). MS: calcd for $C_{68}H_{87}BrN_{14}O_{16}$ (M+H) m/z 1435.56 found 1437.00.

EXAMPLE 5



A B C

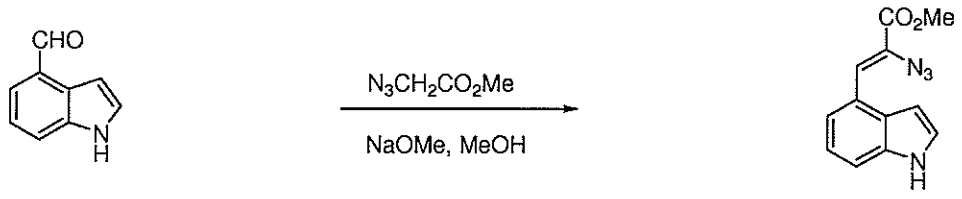
54 mg (0.107 mMoles) of compound **A** was stirred in HBr-ethyl acetate for 30 minutes the solvent was evaporated to give compound **B**, which was dried under Hi vacuum. 33.3 mg (0.053 mMoles) of **B** were reacted with 5-Boc-amino indole-2-carboxylic acid 29.6 mg (0.107 mMoles) in the presence of EDC 22 .6 mg (0.118 mMoles) in 1.5 mL of DMF fro 2 hours to give **C**. Compound C was purified by silica gel Column chromatography 5-10 % MeOH/DCM to give 21 mg (60 % yield) of compound **C**. MS $M^{[+1]} = 662$, $M^{[+Na]} = 685$, $M^{[+K]} = 701$.



21 mg (0.032 mMoles) of Compound **C** was treated with a solution of HBr-Ethyl acetate for 30 minutes the solvent was evaporated and the residue dried under Hi vacuum and further reacted with 11.4 mg (0.032 mMoles) of Boc-4-amino benzoic acid in the presence of 18.3 mg (0.053 mMoles) of HATU, 10 uL triethylamine (0.63 mMoles) in 2 mL of anhydrous DMF fro 4 hours at room temperature. The solvent was evaporated and purified by reverse phase HPLC to give 14 mgs (0.018 mMoles) in 56 % yield of compound **E**. $M^{[+1]} = 782$.

Boc deprotection of compound **E** was carried out in HBr-Ethyl acetate solution and purification by reverse phase HPLC gave 12 mg of compound **F**. 98 % yield. $M^{[+1]} = 681.8$

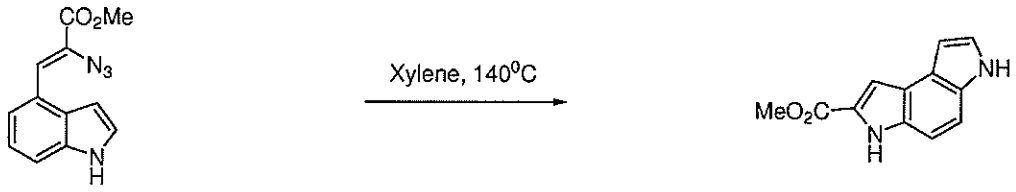
EXAMPLE 6



1 **2**

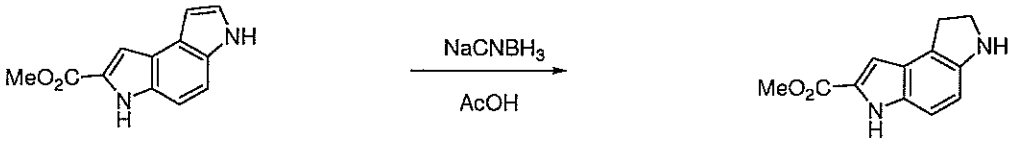
Compound 2. To a solution of indole-4-carboxaldehyde (**1**, 583 mg, 4 mmol) and methyl azidoacetate (460 mg, 40 mmol) in dry methanol (20 mL) was added sodium methoxide in methanol dropwise (6.9 mL of 25% NaOMe, 32 mmol) at -25°C (dry ice/CCl_4) under N_2. The reaction mixture was warmed to 0°C and was stirred 3.5 h. The reaction mixture was poured into water (120 mL) and was extracted with EtOAc (2 x 60

mL). The combined extracts were washed with saturated aqueous NaCl (60 mL) and were dried ($MgSO_4$). The solvent was removed in vacuo to give **2** (890 mg, 91%). ^{1}HNMR ($CDCl_3$) δ 8.28 (br s, 1H), 8.06 (d, 1H, J=7.6 Hz), 7.45 (s, 1H), 7.42 (d, 1H, J=7.6 Hz), 7.30 (t, 1H, J=3.2 Hz), 7.26 (s, 1H), 6.73 (br s, 1H), 3.96 (s, 3H).



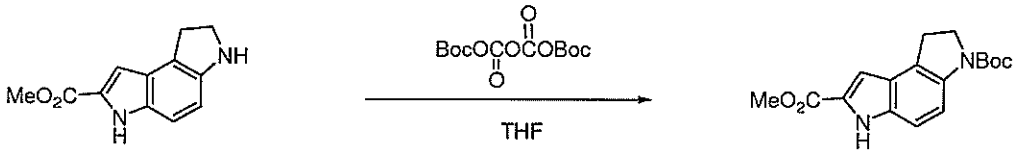
2 **3**

Compound 3. A suspension of methyl 2-azido-3-(1*H*-indol-4-yl)acrylate (**2**, 890 mg, 3.68 mmol) in dry xylenes (100 mL) was refluxed under N_2 for 1h. The solvent was removed in vacuo and the residue passed through a column of silica gel (30% EtOAc-hexane) to afford **3** (663 mg, 85%). ^{1}HNMR ($CDCl_3$) δ 8.97 (br s, 1H), 8.36 (br s, 1H), 7.47 (d, 1H, J=2 Hz), 7.40 (d, 1H, J=9.2 Hz), 7.26 (t, 1H, J=2.8 Hz), 7.22 (d, 1H, J=9.2 Hz), 6.82 (t, 1H, J=2.4 Hz), 3.95 (s, 3H).



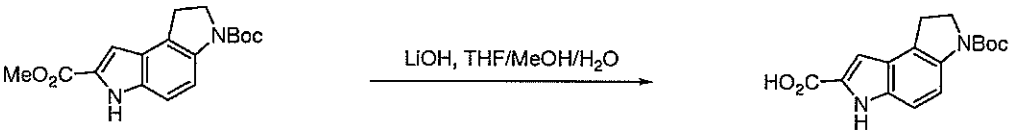
3 **4**

Compound 4. A solution of methyl pyrrolo[3,2-*e*]indole-2-carboxylate (**3**, 663 mg, 3.1 mmol) in glacial acetic acid (9 mL) under N_2 at 15°C was added sodium cyanoborohydride (600 mg, 9.5 mmol), and the reaction mixture was stirred for 2.5 h (13-18°C). The reaction mixture was poured into water (60 mL) and was made pH 8-9 by the careful addition of solid sodium carbonate. The aqueous mixture was extracted with EtOAc (3 x 60 mL), and the combined extracts were dried ($MgSO_4$). The solvent was removed in vacuo. Flash chromatography (silica gel, 20% EtOAc-hexane) afforded **4** (562 mg, 84%). ^{1}HNMR ($CDCl_3$) δ 8.85 (br s, 1H), 7.15 (d, 1H, J=8.4 Hz), 7.04 (s, 1H), 6.89 (d, 1H, J=8.4 Hz), 3.94 (s, 3H), 3.68 (t, 2H, J=8.4 Hz), 3.24 (t, 2H, J=8.4 Hz).



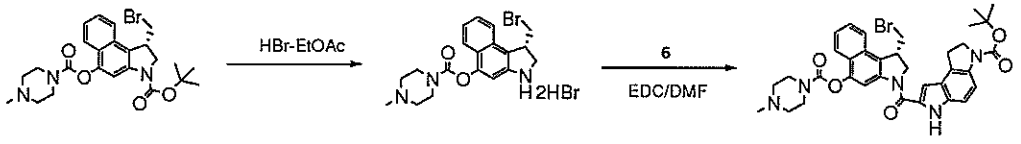
4 **5**

Compound 5. A solution of methyl 1,2-dihydro-3*H*-pyrrolo[3,2-*e*]indole-7-carboxylate (**4**, 450 mg, 1.42 mmol) in dry THF (8 mL) was treated with di-*tert*-butyl dicarbonate (621 g, 2.8 mmol) at 25°C under N_2. The reaction mixture was stirred 16 h, then the solvent was removed in vacuo. Flash chromatography (silica gel, 30% EtOAc-hexane) gave **5** (551 mg, 84%).^{1}HNMR ($CDCl_3$) δ 8.78 (br s, 1H), 7.26 (s, 1H), 7.25 (d, 1H, J=8.5 Hz), 7.07 (s, 1H), 4.12 (br s, 2H), 3.94 (s, 3H), 3.27 (t, 2H, J=8.8 Hz), 1.57 (s, 9H).



5 **6**

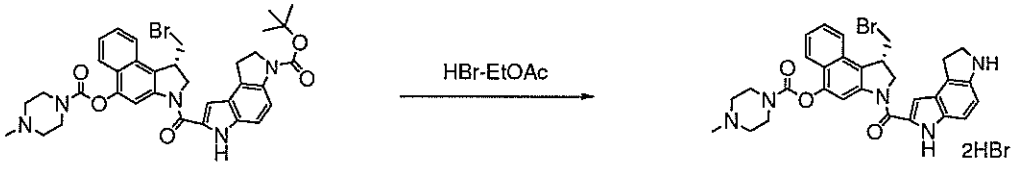
Compound 6. An aqueous solution of LiOH (2.2 mL of 4.0 M solution, 8.7 mmol) was added to a slurry of methyl 3-(*tert*-Butyloxycarbonyl)-1,2-dihydro-3*H*-pyrrolo[3,2-*e*]indole-7-carboxylate (**5**, 551 mg, 1.74 mmol) in 60 mL of THF/MeOH/H_2O (3:2:1), and the reaction mixture was stirred at 25°C for 14 h. The reaction mixture was diluted with water (30mL) and was then adjusted pH to 4 with 2N HCl, producing a white precipitate. The solid was collected by filtering and was washed with water (10 mL). Drying the solid in high vacuo afforded 6 (524 mg, 99%). ^{1}HNMR (DMSO-d_6) δ 12.93 (br s, 1H), 11.69 (s, 1H), 7.80 (br s, 1H), 7.20 (d, 1H, J=8.8 Hz), 6.91 (s, 1H), 3.96 (t, 2H, J=8.8 Hz), 3.18 (t, 2H, J=8.8 Hz), 1.48 (s, 9H).



7 **8** **9**

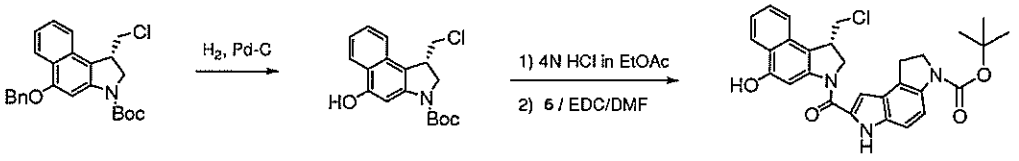
Compound 9. A solution of **7** (48 mg, 0.1 mmol) in 4 N HBr in EtOAc (5 ml) was stirred at 25 °C under nitrogen for 45 min. The solvent was removed and was further dried in high vacuo for 4 h. To the residue was added 3-(tert-butyloxycarbonyl)-1,2-

dihydro-3*H*-pyrrolo[3,2-*e*]indole-7-carboxylic acid (**6**, 36.24 mg, 0.12 mmol). A solution of EDC (22.9 mg, 0.12 mmol) in DMF (3 ml) was added and the reaction mixture was stirred at 25 ^{0}C for 6 h. The solvent was removed. The crude product was chromatographed on silica gel eluted with 3-10% MeOH in CH_2Cl_2 to give **9** (26 mg, 40%). ^{1}HNMR (DMSO-d_6) δ11.69 (s, 1H), 8.17 (s, 1H), 8.01 (d, 1H, J=8.4 Hz), 7.81 (d, 2H, J=8.8 Hz), 7.59 (t, 1H, J=7.6 Hz), 7.49 (t, 1H, J=7.6 Hz), 7.29 (d, 1H, J=9.2 Hz), 7.07 (s, 1H), 4.87 (t, 1H, 10 Hz), 4.54 (d, 1H, 8.8 Hz), 4.43 (br s, 1H), 3.89-4.03 (m, 4H), 3.76 (br s, 2H), 3.46 (br s, 2H). 3.26 (m, 2H), 2.46 (br s, 2H), 2.38 (br s, 2H), 2.24 (s, 3H), 1.49 (s, 9H).



9 **10**

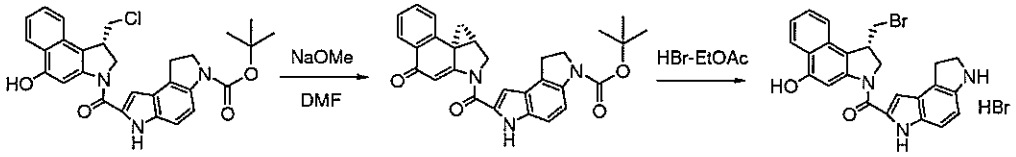
Compound 10. A solution of **9** (26 mg, 0.04 mmol) in 4 N HBr in EtOAc (5 ml) was stirred at 25 ^{0}C under nitrogen for 45 min. The solvent was removed and was further dried in vacuo for 14 h to afford **10** (27.7 mg, 98%). ^{1}HNMR (DMSO-d_6) δ12.09 (s, 1H), 8.25 (s, 1H), 8.03 (d, 1H, J=8.4 Hz), 7.92 (d, 1H, J=8.8 Hz), 7.63 (t, 1H, J=7.6 Hz), 7.49-7.55 (m, 2H), 7.34 (d, 1H, J=8.8 Hz), 7.32 (s, 1H), 4.91 (t, 1H, 10.8 Hz), 4.54 (d, 1H, 10.8 Hz), 4.47 (br s, 1H), 3.84-3.99 (m, 4H), 3.27- 3.50 (m, 10H), 2.88 (s, 3H).



11 **12** **13**

Compound 12 A solution of **11** (20 mg, 0.05 mmol) and 10% Pd-C (15 mg) in MeOH/CH_2Cl_2 (1/2, 10 ml) was degassed in vacuo for 40 s. The resulting mixture was placed under an atmosphere of hydrogen and stirred at 25 ^{0}C for 7 h. The reaction mixture was filtered through Celite (CH_2Cl_2 wash). The solvent was removed in vacuo. Chromatography on silica gel eluted with EtOAc/Hex (2/8) afforded **12** (15.4 mg, 98%). ^{1}NMR (DMSO-d_6) δ 10.36 (s, 1H), 8.04 (d, 1H, J=8.2 Hz), 7.72 (d, 1H, J=8.2 Hz), 7.61 (br s, 1H), 7.45 (t, 1H, J=8.4 Hz), 7.261 (t, 1H, J=8.4 Hz), 4.06 (m, 4H), 3.73 (br s, 1H), 1.52 (s, 9H).

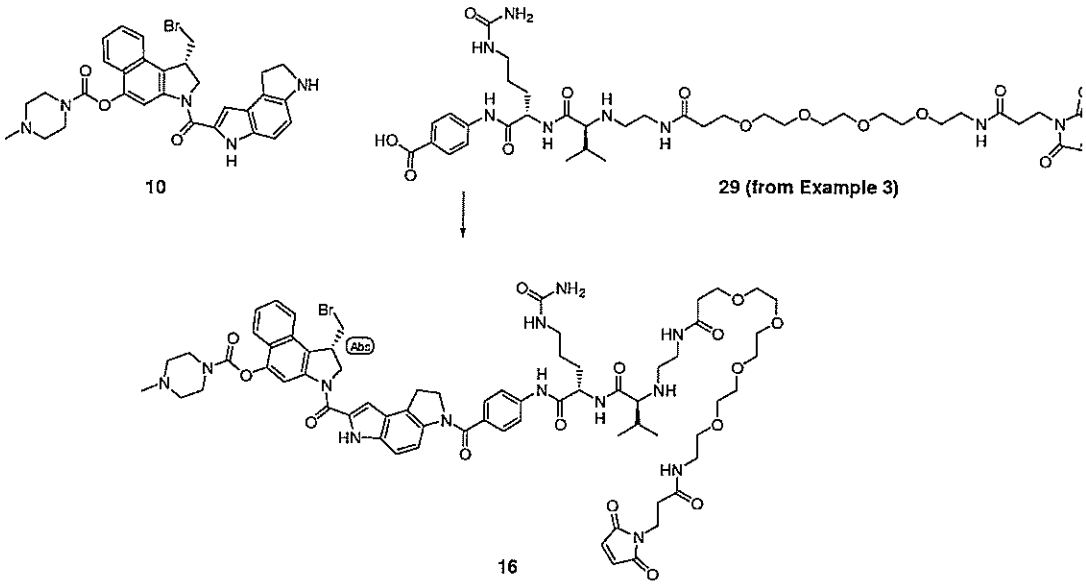
Compound 13 A solution of **12** (14 mg, 0.04 mmol) in 4 N HCl in EtOAc (3 ml) was stirred at 25 ^{0}C under nitrogen for 30-45 min. The solvent was removed and was further dried in high vacuo for 14 h. To the residue was added 3-(tert-butyloxycarbonyl)-1,2-dihydro-3*H*-pyrrolo[3,2-*e*]indole-7-carboxylic acid (**6**, 15.1 mg, 0.05 mmol). A solution of EDC (9.6 mg, 0.05 mmol) in DMF (2 ml) was added and the reaction mixture was stirred at 25 ^{0}C for 15 h. The solvent was removed. The crude product was chromatographed on silica gel eluted with 3-10% MeOH in CH_2Cl_2 to give **13** (18.6 mg, 86%). ^{1}HNMR ($CDCl_3$) δ 9.45 (s, 1H), 8.31 (s, 1H), 8.29 (d, 1H, J=8.4 Hz), 8.02 (s, 2H), 7.63 (d, 1H, J=8.4 Hz), 7.54 (t, 1H, J=6.8 Hz), 7.43 (t, 1H, J=6.8 Hz), 7.29 (d, 1H, J=8.0 Hz), 6.89 (s, 1H), 4.75 (d, 1H, 10.8 Hz), 4.64 (t, 1H, 8.8 Hz), 4.12 (br s, 2H), 4.03 (t, 1H, J=8.8 Hz), 3.93 (dd, 1H, J=11.2 Hz), 3.42 (t, 1H, J=10.8 Hz), 3.22-3.31 (m, 2H), 1.61 (s, 9H).



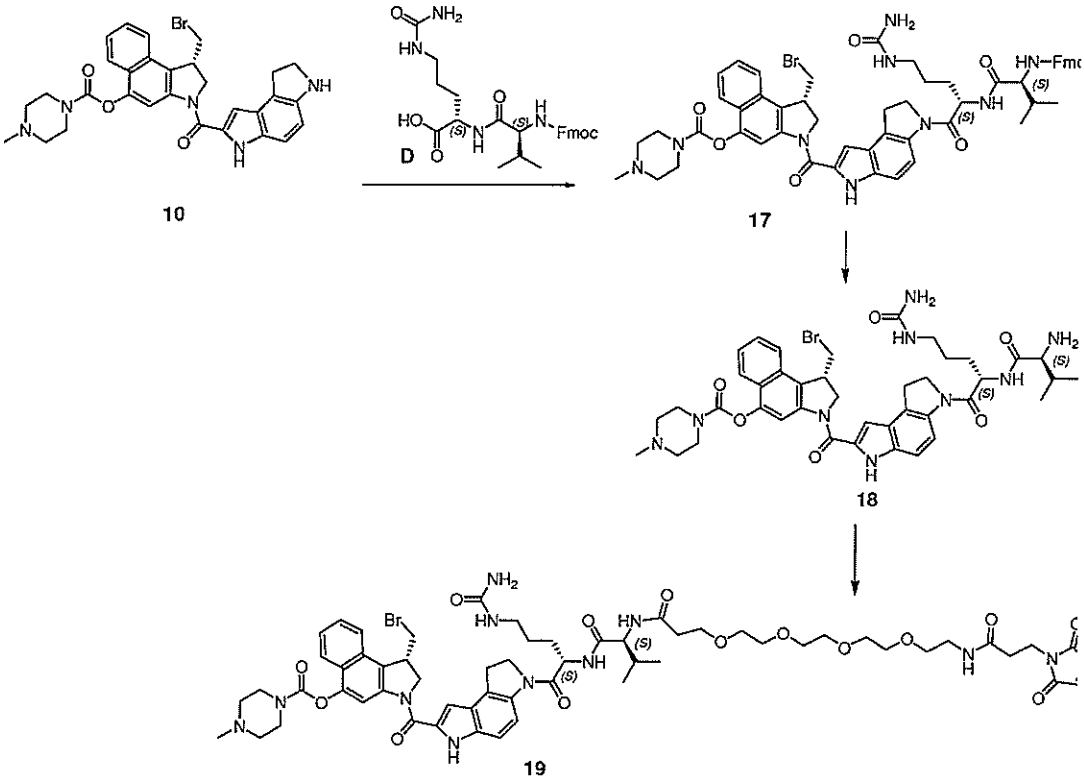
13 **14** **15**

Compound 14. To a solution of **13** (19 mg, 0.04 mmol) in DMF (3 ml) was added sodium methoxide (0.5 M in MeOH, 79 uL, 0.04 mmol) at 0-5 ^{0}C and was stirred for 5 min. To the reaction mixture was added water (20 mL), and the aqueous mixture was extracted with EtOAc (2 x 10 mL). The combined extracts were dried ($MgSO_4$). The solvent was removed in vacuo, and the crude product was chromatographed on silica gel eluted with 10% MeOH in CH_2Cl_2 to give **14** (15.3 mg, 87%). ^{1}NMR ($CDCl_3$) δ 9.24 (br s, 1H), 8.25 (dd, 1H, J=9.2, 1.6 Hz), 8.02 (s, 1H), 7.54 (tt, 1H, J=7.6, 1.6 Hz), 7.43 (tt, 1H, J=7.6, 1.6 Hz), 7.28 (d, 1H, J=9.2 Hz), 7.20 (s, 1H), 6.94 (d, 1H, J=7.6 Hz), 6.88 (s, 1H), 4.49 (m, 2H), 4.12 (br s, 2H), 3.26 (m, 2H), 2.92 (m, 1H), 1.76 (dd, 2H, J=7.6 Hz), 1.61 (s, 9H).

Compound 15. A solution of **14** (6.4 mg, 0.013 mmol) in 4 N HBr in EtOAc (3 ml) was stirred at 25 ^{0}C under nitrogen for 30 min. The solvent was removed in vacuo and was further dried in high vacuo for 14 h to give **15** (7.1 mg, 99%). ^{1}HNMR (DMSO-d_6) δ12.16 (s, 1H), 10.45 (s, 1H), 8.11 (d, 1H, J=8.4 Hz), 7.94 (br s, 1H), 7.83 (d, 1H, J=8.0 Hz), 7.51 (m, 2H), 7.34 (m, 2H), 7.27 (s, 1H), 4.81 (t, 1H, 10.8 Hz), 4.48 (d, 1H, 10.8 Hz), 4.28 (br s, 1H), 3.77-3.91 (m, 4H), 3.41- 3.48 (m, 2H).

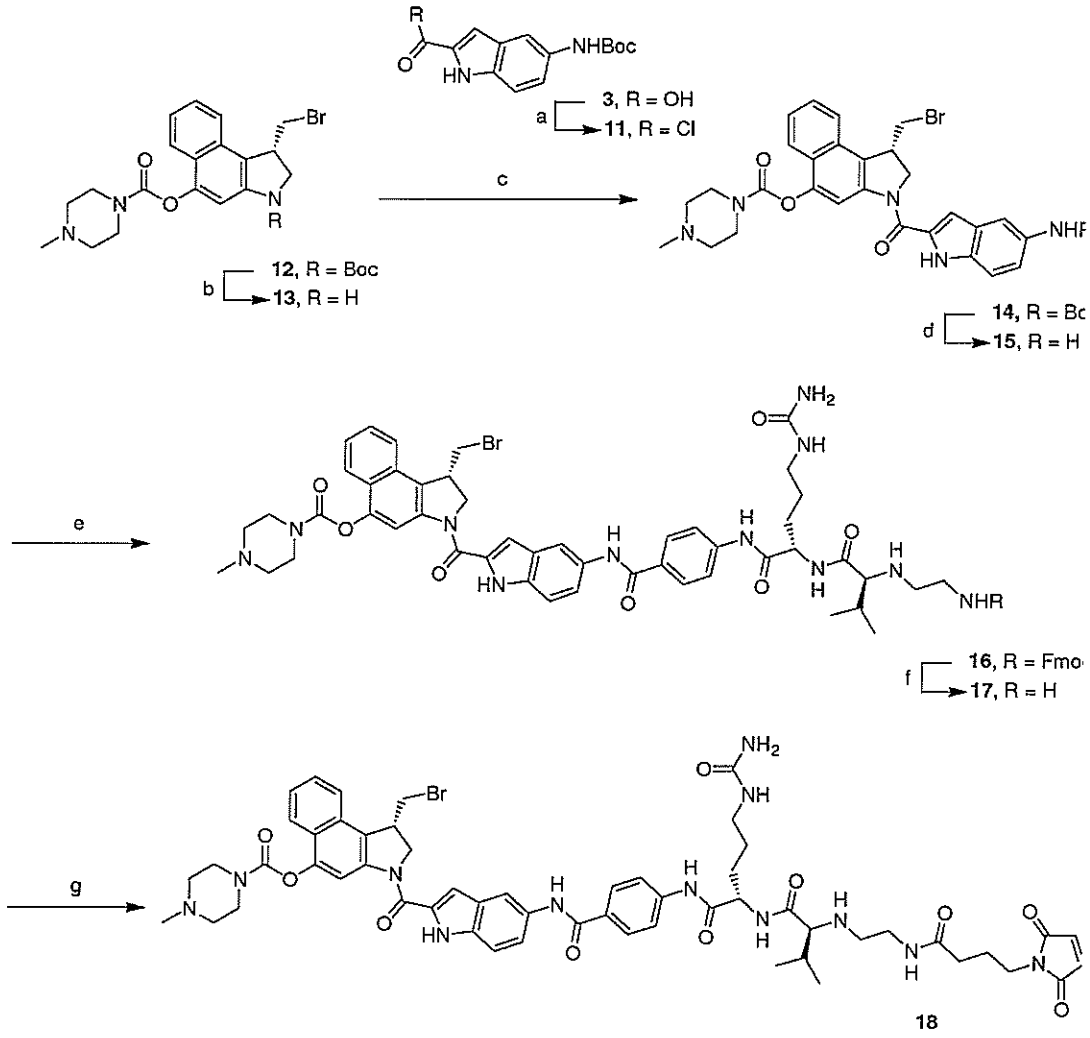


Compound 16: Compound **10** (0.033 mMoles, 2HBr salt) was reacted with Compound **29** of Example 3 (45 mg, 0.054 mMoles) in 2.5 mL DMF in the presence of HATU (20 mg, 0.054 mMoles) and TEA (15-20 µL) for 50 minutes. The solvents were evaporated and the Crude was purified by Reverse phase HPLC to give 10 mg of Compound **16** (21 % yield). MS: 1405.6, 1427.8 and 1444.6.



Compound 19. Compound **10** (25mg, 0.033 mMoles, 2HBr Salt)) was reacted with compound **D** of Example 5 (21.5 mg, 0.043 mMoles) in 2.5 mL DMF in the presence of HATU (16.5 mg, 0.0433 mMoles) and TEA (10-15 μL) for 45 minutes. The solvent was evaporated and crude purified by reverse phase HPLC to give 25 mg of Compound **17**. (71 % yield). MS: 1067.0. Compound **17** (0.0187 mMoles) was deprotected with 5 % piperidine in DMF (3 mL) in 45 minutes. The solvent was evaporated and the residue washed with diethyl ether to give **18** (MS: 845.2). To a Solution of 0.00935 mMoles of **18** in 3 mL DMF was added Mal-dPEG4-NHS ester (10 mg, 0.019 mMoles) in DCM 1 mL, followed by TEA (5 μL). After 30 minutes the solvents were evaporated and the crude purified by Reverse Phase HPLC to give 5.2 mg of pure Compound **19** (MS: 1243.2, 1266.8 and 1281.2)

Example 7



a $SOCl_2$, CH_2Cl_2 100% **b** TFA, CH_2Cl_2 100% **c** $_{11}$, pyridine, CH_2Cl_2 67% **d** TFA, anisole, CH_2Cl_2 100% **e** $_{10}$, HATU DMF, DIEA $_{69}$% **f** piperidine, DMF 98% **g** $_{20}$% DMF in CH_2Cl_2, DIEA, N-succinimidyl-4-maleimidobutyrate 53%

Compound 11: To a solution of **3** (236 mg, 0.86 mmol) in solution of toluene (10 mL) was added 1-methyl-2-pyrrolidone (502 µL, 5.13 mmol). The mixture thus obtained was cooled to 0°C. Then thionyl chloride (502 µL, 2.58 mmol) in toluene (4 mL) was added and the resulting solution was stirred at 0°C for 10 minutes. The solvent was removed and the product was used immediately without further purification for the following step (252 mg, 100%).

Compound 13: To a solution of **12** (137 mg, 0.27 mmol) in dichloromethane (3 mL) was added TFA (6 mL). The resulting solution was stirred for 20 minutes. The solvent was co evaporated twice with toluene *in vacuo*. The residue (171 mg, 100%) was used without further purification immediately for the next step.

Compound 14: To a solution of **13** (171 mg, 0.27 mmol) in dichloromethane (6 mL) was added pyridine (0.6 ml) and **11** (159 mg, 0.54 mmol) at 0°C. The mixture thus obtained was stirred at 0°C for 1 hour. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give compound **14** as a white solid (152 mg, 72%). ^{1}H NMR (CD_3OD) δ 1.54 (s, 9H), 2.91 (s, 3H), 3.00-3.60 (m, 8H), 3.72 (m, 1H), 3.99 (m, 1H), 4.31-4.50 (m, 3H), 6.94 (s, 1H), 7.05 (m, 1H), 7.13 (d, 1H), 7.45 (m, 2H), 7.68 (d, 1H), 7.75 (s, 1H), 7.86 (d, 1H), 8.23 (s, 1H); LC-MS (ES^+) 563 $(M+H\text{-}Boc)^+$, 607 $(M+H\text{-}56)^+$, 663 $(M+H)^+$, 686 $(M+Na)^+$, 701 $(M+K)^+$.

Compound 15: To a solution of **14** (198 mg, 0.25 mmol) in dichloromethane (2 mL) was added anisole (0.2 mL) and TFA (0.8 mL). The resulting solution was stirred for 20 minutes. The solvent was evaporated *in vacuo*. The residue (201 mg, 100%) was used without further purification for the next step.

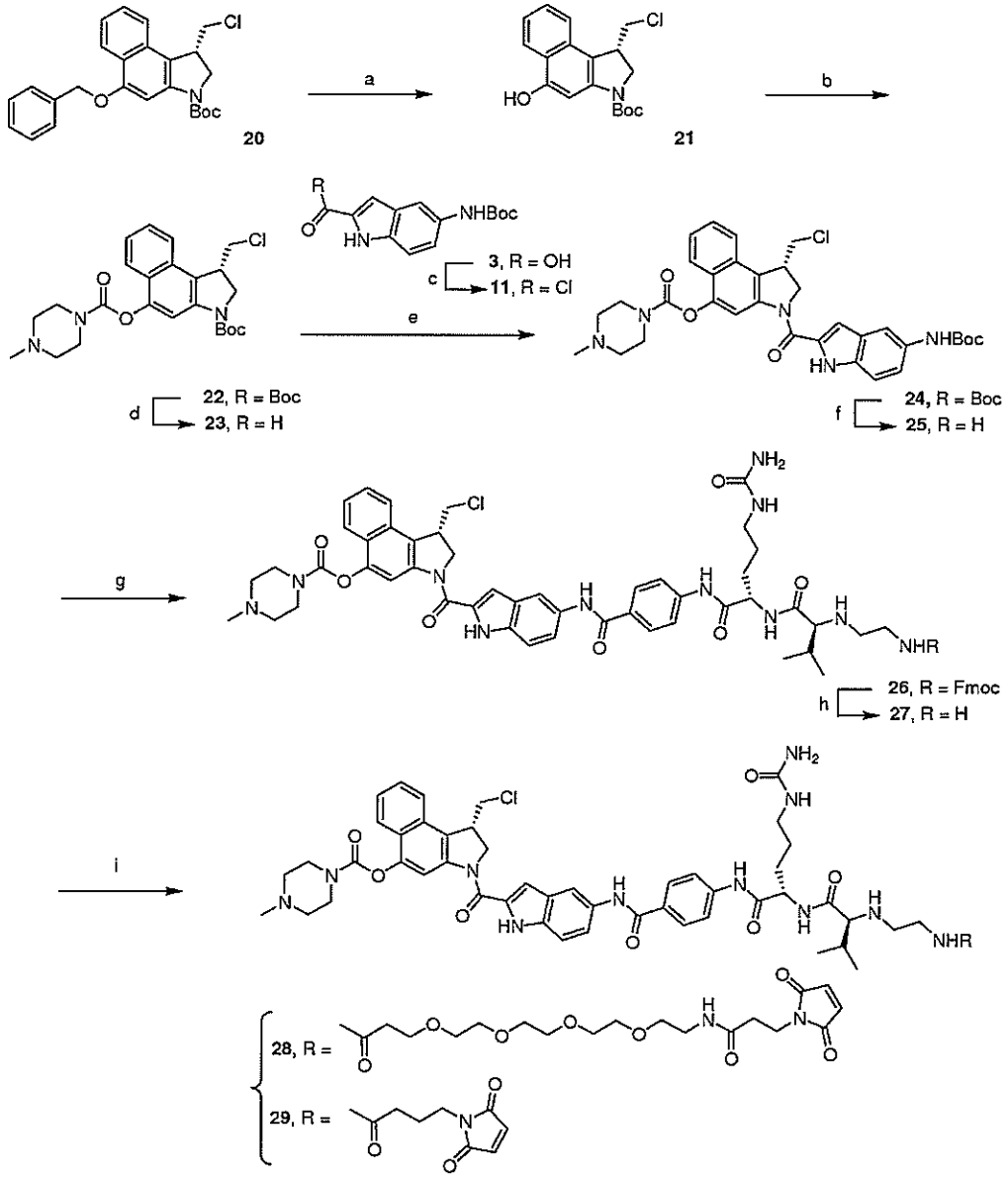
Compound 16: To a solution of **15** (143 mg, 0.25 mmol) in DMF (3 mL) were added **10** (197 mg, 0.25 mmol), diisopropylethylamine (218 μL, 1.25 mmol) and HATU (95 mg, 0.25 mmol) at room temperature. The mixture thus obtained was stirred for 2 hours. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give Compound **16** as a white solid (252 mg, 69%). ^{1}H NMR (CD_3OD) δ 1.06 (d, 3H), 1.14 (d, 3H), 1.60 (m, 2H), 1.81 (m, 1H), 1.90 (m, 1H), 2.25 (m, 1H), 2.91 (s, 3H), 3.05-3.25 (m, 6H), 3.40-3.70 (m, 8H), 3.70 (d, 1H), 3.85 (d, 1H), 4.00 (m, 1H), 4.19 (t, 1H), 4.30-4.50 (m, 5H), 4.65 (m, 1H), 7.02 (s, 1H), 7.28-7.50 (m, 8H), 7.60 (m, 2H), 7.70 (d, 2H), 7.76 (d, 2H), 7.87 (m, 3H), 8.03 (s, 1H), 8.25 (bs, 1H); LC-MS (ES^+) 1203 $(M+H)^+$, 1227 $(M+Na)^+$, 1243 $(M+K)^+$.

Compound 17: See the preparation of **28** in Example 3 for general Fmoc deprotection procedure. Deprotection of **16** (235 mg, 0.16 mmol) gave 161 mg of **17** (98%). The residue was used without further purification for the next step. LC-MS (ES^+) 983 $(M+H)^+$, 1005 $(M+Na)^+$, 1021 $(M+K)^+$.

Compound 18: To a solution of **17** (48 mg, 0.049 mmol) in 20% DMF in dichloromethane (1 mL) were added diisopropylethylamine (34 μL, 0.19 mmol) and N-succinimidyl-4-maleimidobutyrate (21 mg, 0.073 mmol) at room temperature. The

mixture thus obtained was stirred for 2 hours. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give compound **18** as a white solid (36 mg, 53%). ^{1}H NMR (CD_3OD) δ 1.10 (d, 3H), 1.15 (d, 3H), 1.61 (m, 2H), 1.80 (m, 1H), 1.91 (m, 3H), 2.25 (m, 3H), 3.01 (s, 3H), 3.12-3.24 (m, 6H), 3.47-3.65 (m, 10H), 3.84 (d, 1H), 3.90 (dd, 1H), 4.34 (m, 1H), 4.62-4.80 (m, 4H), 6.82 (s, 2H), 7.17 (s, 1H), 7.46 (d, 2H), 7.50 (m, 1H), 7.60 (m, 1H), 7.76 (d, 2H), 7.93 (m, 4H), 8.07 (s, 1H), 8.25 (bs, 1H); LC-MS (ES^+) 574 $(M+2H/2)^+$, 1148 $(M+H)^+$, 1168 $(M+Na)^+$, 1186 $(M+K)^+$.

Example 8



a H_2, Pd/C, methanol, CH_2Cl_2 98% **b** allyl alcohol, pyridine, CH_2Cl_2, 4-methylpiperazine-1-carbonyl chloride 72% **c** $SOCl_2$, CH_2Cl_2 100% **d** TFA, CH_2Cl_2 100% **e 11**, pyridine, CH_2Cl_2 67% **f** TFA, anisole, CH_2Cl_2 100% **g 10**, HATU, DMF, DIEA 69% **h** piperidine, DMF 98% **i** 20% DMF in CH_2Cl_2, DIEA, MAL-dPEG$_4$-NHS ester (**28**, 60%) or N-succinimidyl-4-maleimidobutyrate (**29**, 51%)

Compound 21: A solution of **20** (520 mg, 1.22 mmol) and palladium on charcoal (100 mg) in methanol and diclhoromethane (2 and 4 mL) was placed under hydrogen atmospheric pressure at room temperature. The mixture thus obtained was stirred at room temperature overnight. The palladium was filtrated and the resulting solution was

concentrated to dryness to give a grey solid (400 mg, 98%). ^{1}H NMR (CD_3OD) δ 1.59 (bs, 9H), 3.52 (m, 1H), 3.89-4.00 (m, 2H), 4.07 (m, 1H), 4.18 (m, 1H), 7.26 (m, 1H), 7.45 (m, 1H), 7.55 (bs, 1H), 7.65 (d, 1H), 8.13 (d, 1H).

Compound 22: To a solution of **21** (400 mg, 1.20 mmol) in dichloromethane (10 mL) were added allyl alcohol (1 mL), pyridine (1 mL, 1.2 mmol) and 4-methyl-1-piperazine carbonyl chloride (364 mg, 1.8 mmol). The mixture thus obtained was stirred at room temperature overnight. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give compound **22** as a white solid (509 mg, 72%). ^{1}HNMR (DMSO-d_6) 1.50 (s, 9H), 2.21 (s, 3H), 2.36 (s, 2H), 2.42 (s, 2H), 3.42 (s, 2H), 3.78 (s, 2H), 3.90 (m, 1H), 3.98 (m, 1H), 4.08 (m, 2H), 4.14 (m, 1H), 4.22 (m, 1H), 7.42 (t, 1H), 7.58 (t, 1H), 7.82 (d, 1H), 7.88 (m, 1H), 7.92 (d, 1H); LC-MS (ES^+), 460 $(M+H)^+$, 483 $(M+Na)^+$, 499 $(M+K)^+$.

Compound 23: To a solution of **22** (250 mg, 0.43 mmol) in dichloromethane (3 mL) was added TFA (6 mL). The resulting solution was stirred for 20 minutes. The solvent was co evaporated twice with toluene *in vacuo*. The residue (256 mg, 100%) was used without further purification immediately for the next step. LC-MS (ES^+) 360 $(M+H)^+$, 383 $(M+Na)^+$, 399 $(M+K)^+$.

Compound 24: To a solution of **23** (256 mg, 0.43 mmol) in dichloromethane (8 mL) was added pyridine (1.6 mL) and **11** (252 mg, 0.86 mmol) (see Example 7) at 0°C. The mixture thus obtained was stirred at 0°C for 1 hour. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give compound **24** as a white solid (213 mg, 67%). ^{1}H NMR (CD_3OD) δ 1.54 (s, 9H), 2.87 (s, 3H), 3.00-3.60 (m, 8H), 3.79 (m, 1H), 3.89 (m, 1H), 4.31-4.48 (m, 3H), 6.91 (s, 1H), 7.08 (m, 2H), 7.43 (m, 2H), 7.65 (d, 1H), 7.74 (s, 1H), 7.84 (d, 1H), 8.23 (s, 1H); LC-MS (ES^+) 518 $(M+H\text{-}Boc)^+$, 562 $(M+H\text{-}56)^+$, 619 $(M+H)^+$, 641 $(M+Na)^+$, 657 $(M+K)^+$.

Compound 25: To a solution of **24** (50 mg, 0.08 mmol) in dichloromethane (2 mL) was added anisole (0.2 mL) and TFA (0.8 mL). The resulting solution was stirred for 20 minutes. The solvent was evaporated *in vacuo*. The residue (60 mg, 100%) was used without further purification for the next step. ^{1}H NMR (CD_3OD) δ 3.01 (s, 3H), 3.40-3.60 (bs, 4H), 3.79 (m, 2H), 4.02 (m, 2H), 4.32 (m, 2H), 4.73-4.90 (m, 3H), 7.26 (m, 2H), 7.51 (m, 1H), 7.63 (m, 2H), 7.77 (d, 1H), 7.94 (d, 1H), 8.30 (s, 1H).

Compound 26: To a solution of **25** (60 mg, 0.08 mmol) in DMF (2 mL) were added **10** (53 mg, 0.08 mmol), diisopropylethylamine (59 μL, 0.4 mmol) and HATU (26

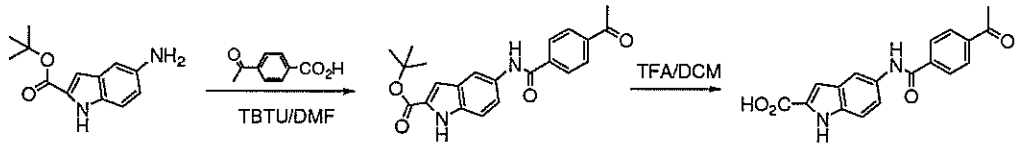
mg, 0.08 mmol) at room temperature. The mixture thus obtained was stirred for 2 hours. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give compound **26** as a white solid (66 mg, 69%). ^{1}H NMR (CD_3OD) δ 1.06 (d, 3H), 1.14 (d, 3H), 1.60 (m, 2H), 1.82 (m, 1H), 1.95 (m, 1H), 2.25 (m, 1H), 2.95 (s, 3H), 3.05-3.25 (m, 6H), 3.40-3.70 (m, 8H), 3.85 (d, 1H), 3.90 (m, 1H), 4.06 (m, 1H), 4.20 (m, 1H), 4.30-4.70 (m, 6H), 7.02 (s, 1H), 7.28-7.50 (m, 8H), 7.60 (m, 2H), 7.70 (d, 2H), 7.76 (m, 2H), 7.87 (m, 3H), 8.03 (s, 1H), 8.25 (bs, 1H); LC-MS (ES^+) 580 $(M+2H/2)^+$, 1159 $(M+H)^+$.

Compound 27: See the preparation of **28** in Example 3 for general Fmoc deprotection procedure. Deprotection of **26** (66 mg, 0.05 mmol) gave 44 mg of **27** (98%). The residue was used without further purification for the next step. LC-MS (ES^+) 937 $(M+H)^+$, 959 $(M+Na)^+$.

Compound 28: To a solution of **27** (23 mg, 0.024 mmol) in 20% DMF in dichloromethane (1 mL) were added diisopropylethylamine (16 μL, 0.09 mmol) and MAL-dPEG$_4$-NHS ester (18 mg, 0.036 mmol) at room temperature. The mixture thus obtained was stirred for 2 hours. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give compound **28** as a white solid (23 mg, 60%). ^{1}H NMR (CD_3OD) δ 1.09 (d, 3H), 1.15 (d, 3H), 1.60 (m, 2H), 1.82 (m, 1H), 1.95 (m, 1H), 2.30 (m, 1H), 2.45 (t, 2H), 2.51 (t, 2H), 2.97 (s, 3H), 3.10-3.25 (m, 6H), 3.46-3.70 (m, 24H), 3.75 (t, 4H), 3.85 (d, 1H), 3.90 (m, 1h), 4.10 (m, 1H), 4.50-4.70 (m, 4H), 6.78 (s, 2H), 7.04 (s, 1H), 7.34 (m, 2H), 7.45 (m, 1H), 7.50 (m, 1H), 7.71-7.79 (m, 2H), 7.90 (m, 3H), 8.04 (s, 1H), 8.25 (bs, 1H); LC-MS (ES^+) 668 $(M+2H/2)^+$.

Compound 29: To a solution of **27** (31 mg, 0.033 mmol) in 20% DMF in dichloromethane (1 mL) were added diisopropylethylamine (22 μL, 0.13 mmol) and N-succinimidyl-4-maleimidobutyrate (14 mg, 0.049 mmol) at room temperature. The mixture thus obtained was stirred for 2 hours. The solvent was evaporated and the residue was purified by semi-preparative HPLC to give compound **29** as a white solid (32 mg, 51%). ^{1}H NMR (CD_3OD) δ 1.09 (d, 3H), 1.15 (d, 3H), 1.62 (m, 2H), 1.84 (m, 1H), 1.92 (m, 3H), 2.25 (m, 3H), 2.97 (s, 3H), 3.12-3.25 (m, 6H), 3.39-3.68 (m, 10H), 3.85 (d, 1H), 3.93 (dd, 1H), 4.08 (m, 1H), 4.50-4.67 (m, 4H), 6.80 (s, 2H), 7.03 (s, 1H), 7.34 (m, 2H), 7.44 (m, 1H), 7.50 (m, 1H), 7.72 (d, 2H), 7.77 (d, 1H), 7.90 (m, 3H), 8.04 (s, 1H), 8.25 (bs, 1H); LC-MS (ES^+) 552 $(M+2H/2)^+$.

Example 9



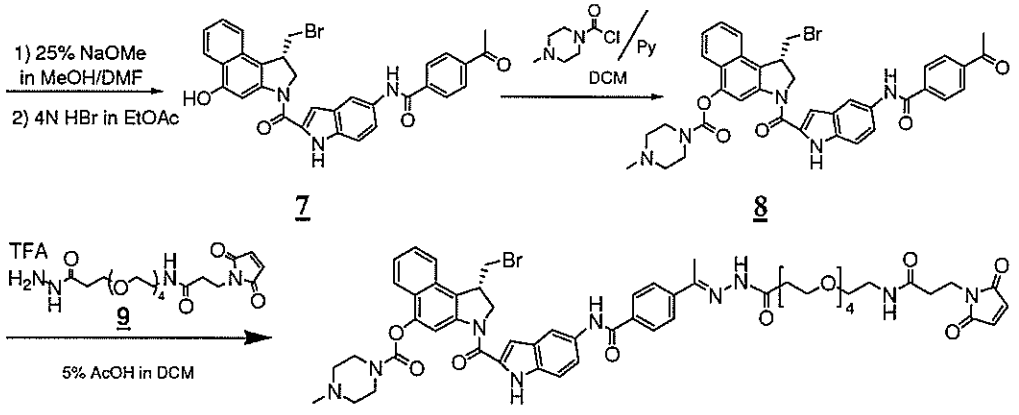
1 2 3

***tert*-butyl 5-(4-acetylbenzamido)-1*H*-indole-2-carboxylate (2).** A solution of *tert*-butyl 5-amino-1*H*-indole-2-carboxylate (220 mg, 0.94 mmol), 4-acetylbenzoic acid (155 mg, 0.94 mmol) and TBTU (303 mg, 0.94 mmol) in DMF (6 mL) at 4 ^{0}C was treated with diisopropylethylamine (329 uL, 1.89 mmol) and stirred 40 min at 0~5 ^{0}C. The solvent was removed under vacuo. The crude product was chromatographed on silica gel eluted with 1% MeOH in CH_2Cl_2 to give **2** (350 mg, 98%). ^{1}HNMR DMSO-d_6) δ11.66 (bs, 1H), 10.34 (s, 1H), 8.09 (d, 1H, J=8.8 Hz), 8.07 (s, 4H), 7.55 (d, 1H, J=8.8 Hz), 7.41 (d, 1H, J=8.8 Hz), 7.03 (s, 1H), 2.63 (s, 3H), 1.56 (s, 9H).

5-(4-acetylbenzamindo)-1*H*-indole-2-carboxylic acid (3). A solution of **2** (350 mg, 0.92 mmol) in methylene chloride (4 mL) was treated with TFA (4 mL) and stirred 15 min at room temperature. The solvent was rmoved and the residue was further dried under high vacuo to give 3 in almost quantitative yield. ^{1}HNMR DMSO-d_6) δ 12.92 (bs, 1H), 11.74 (bs, 1H), 10.33 (s, 1H), 8.04 (d, 1H, J=8.8 Hz), 8.06 (s, 4H), 7.52 (d, 1H, J=8.8 Hz), 7.38 (d, 1H, J=8.8 Hz), 7.06 (s, 1H), 2.63 (s, 3H).



4 5 6



7 8

***seco*-CBI-Boc (5).** A solution of **4** (100 mg, 0.24 mmol) and 10% Pd-C (35 mg) in MeOH/CH_2Cl_2 (1/2, 10 ml) was degassed in vacuo for 40 s. The resulting mixture was placed under an atmosphere of hydrogen and stirred at 25 ^{0}C for 7 h. The reaction mixture was filtered through Celite (CH_2Cl_2 wash). The solvent was removed in vacuo. Chromatography on silica gel eluted with EtOAc/Hex (2/8) afforded **5** (77 mg, 98%). ^{1}NMR DMSO-d_6) δ 10.36 (s, 1H), 8.04 (d, 1H, J=8.2 Hz), 7.72 (d, 1H, J=8.2 Hz), 7.61 (br s, 1H), 7.45 (t, 1H, J=8.4 Hz), 7.261 (t, 1H, J=8.4 Hz), 4.06 (m, 4H), 3.73 (m, 1H), 1.52 (s, 9H).

***seco*-CBI (Cl)-Indole (6).** A solution of **5** (35 mg, 0.1 mmol) in 4 N HCl in EtOAc (5 ml) was stirred at 25 ^{0}C under nitrogen for 30-45 min. The solvent was removed and was further dried in high vacuo for 4 h. To the residue was added 5-(4-acetylbenzamindo)-1*H*-indole-2-carboxylic acid (**3**, 117 mg, 0.44 mmol) and EDC in DMF (5 mL) and the reaction mixture was stirred 5 h at room temperature. The solvent was removed in high vacuo. Chromatography on silica gel eluted with 2-8 % MeOH-CH_2Cl_2 to obtain 6 (118.3 mg, 50%). ^{1}HNMR DMSO-d_6) δ11.73 (s, 1H), 10.43 (s, 1H), 10.36 (s, 1H), 8.18 (s, 1H), 8.08 (s, 5H), 7.92 (s, 1H), 7.83 (d, 1H, J=8.4 Hz), 7.55 (t, 1H, J=8.4 Hz), 7.51 (t, J=8.4 Hz, 1H), 7. 45 (d, J=8.4 Hz, 1H), 7.19 (s, 1H), 4.80 (t, J=10.8 Hz, 1H), 4.55 (d, 1H, J=10.8Hz), 4.22 (m, 1H), 4.0 (dd, 1H, J=10.8 Hz), 3.86 (dd, J=11 Hz, 1H), 2.63 (s, 3H).

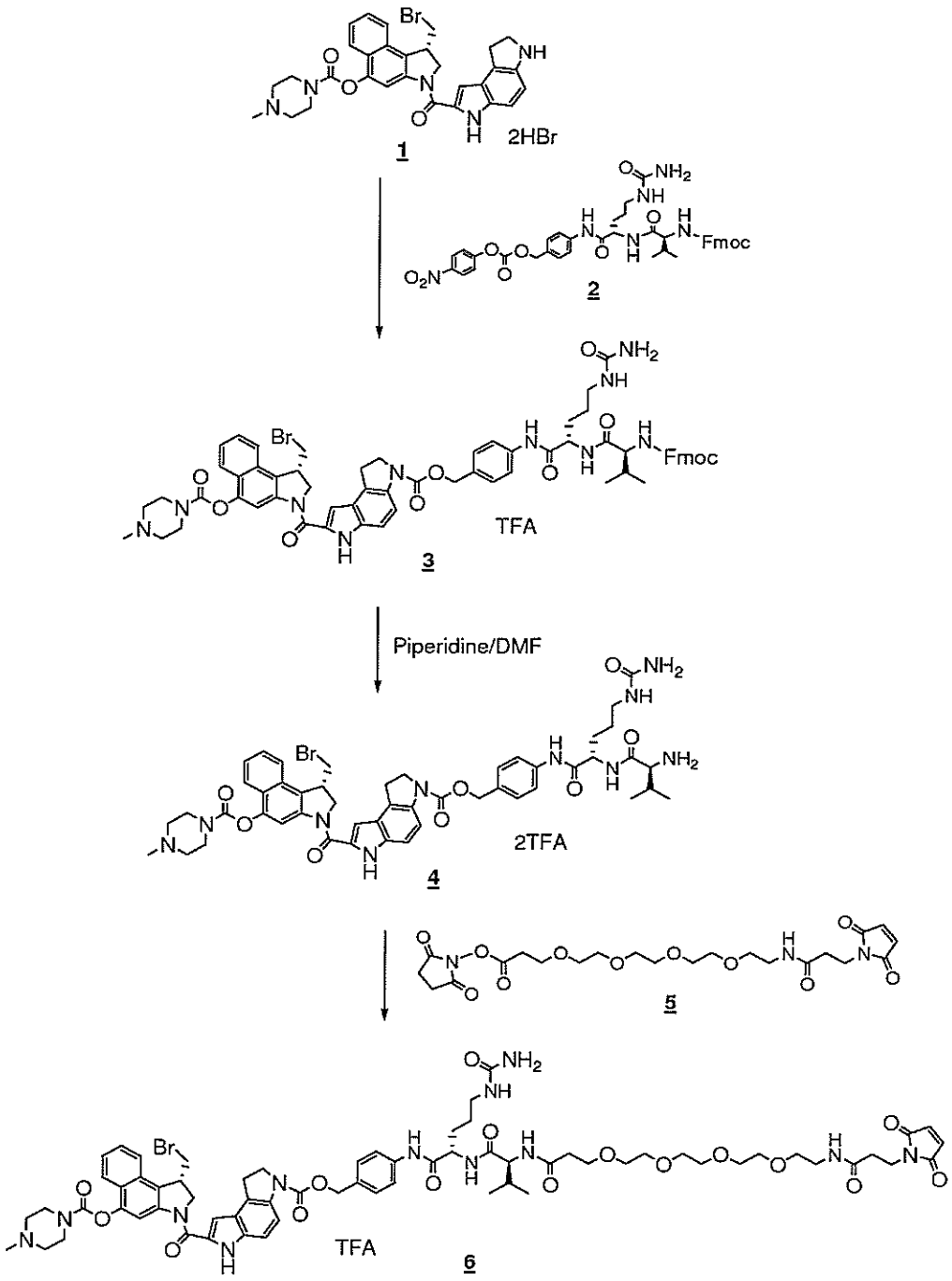
***seco*-CBI (Br)-Indole (7).** To a solution of **6** (35.5 mg, 0.066 mmol) in anhydrous DMF (1 mL) was added 60% NaH (5.3 mg, 0.13 mmol) at 0^{0}C and was stirred for 15-30 min. Solvent was removed under high vacuo. The crude product was chromatographed on silica gel eluted with 3% MeOH in CH_2Cl_2 to give cyclopropane (31 mg, 94%). The resulting cyclopropane was treated with 4N HBr in ethyl acetate (3 mL) and the reaction mixture was stirred 15 min. The solvent was removed under vacuo. Chromatography on silica gel eluted with 2-8 % MeOH- CH_2Cl_2 to obtain **7** (34.5 mg, 96%). ^{1}HNMR DMSO-d_6) δ11.73 (s, 1H), 10.43 (s, 1H), 10.36 (s, 1H), 8.18 (s, 1H), 8.08 (s, 5H), 7.92 (s, 1H), 7.83 (d, 1H, J=8.4 Hz), 7.55 (t, 1H, J=8.4 Hz), 7.51 (t, J=8.4 Hz, 1H), 7. 45 (d, J=8.4 Hz, 1H), 7.19 (s, 1H), 4.80 (t, J=10.8 Hz, 1H), 4.55 (d, 1H, J=10.8Hz), 4.26 (m, 1H), 4.0 (dd, 1H, J=10.8 Hz), 3.86 (dd, J=11 Hz, 1H), 2.63 (s, 3H).

Carbamate (8). A solution of **7** (34 mg, 0.06 mmol), 4-Methyl-1-piperazine-carbonyl chloride hydrochloride (15.9 mg, 0.08 mmol), allyl alcohol (80 uL) and

anhydrous pyridine (80 uL) in CH_2Cl_2 (5 ml) was stirred 16 h at room temperature. Purification of the crude product was performed on silica gel eluted with 2% to 10% MeOH in CH_2Cl_2 to obtain **8** (41.3 mg, 78%). ^{1}HNMR DMSO-d_6) δ11.73 (s, 1H), 10.38 (s, 1H), 8.18 (bs, 2H), 8.08 (s, 4H), 8.02 (d, 1H, J=8.4 Hz), 7.83 (d, J=8.4 Hz, 1H), 7.60 (t, 1H, J=8.4 Hz), 7. 56 (d, J=8.4 Hz, 1H), 7.50 (t, J=8.4 Hz, 1H), 7.47 (d, 1H, J=8.4 Hz), 7.24 (s, 1H), 4.89 (t, J=10.8 Hz, 1H), 4.58 (d, 1H, J=10.8Hz), 4.45 (m, 1H), 3.97 (dd, 1H, J=10.8 Hz), 3.91 (dd, J=11 Hz, 1H), 3.77 (bs, 2H), 3.47 (bs, 2H), 2.63 (s, 3H), 2.47 (bs, 2H), 2.40 (bs, 2H), 2.25 (s, 3H).

Compound 10: To a solution of **8** (11.35 mg, 0.016 mmol) and **9** (17 mg, 0.032 mmol) in 5% acetic acid in dry methylene chloride (2 ml, pre-dried over molecule sieves) was stirred 10-15 min at 25 ^{0}C. The solvent was removed and was further dried in high vacuo overnight. The formation of hydrazone was checked by analytical HPLC[2)] (Nova Pak C18, 5μm, 3.9 x 150 mm column), eluted at 1 ml/min (acetonitrile/20 mM of ammonium formate, pH 7) with a gradient: 10% to 100% ammonium formate in 10 min (Retention time: 6.93 min). The hydrazone **10** was finally purified by Prep HPLC (SymmetrPrep C18, 7μm, 19 x 150 mm column), eluted at 10 ml/min (acetonitrile /20 mM of ammonium formate, pH 7) with a gradient: 10% to 70% acetonitrile in 20 min, maintaining 70% acetonitrile in 3 min, 70% to 100% acetonitrile in 7 min (Retention time: 26.1 min), to give **10** (14.7 mg, 82%). MS: calcd for $C_{55}H_{62}BrN_9O_{12}$ (M+H) m/z 1121.04 found 1121.1.

Example 10



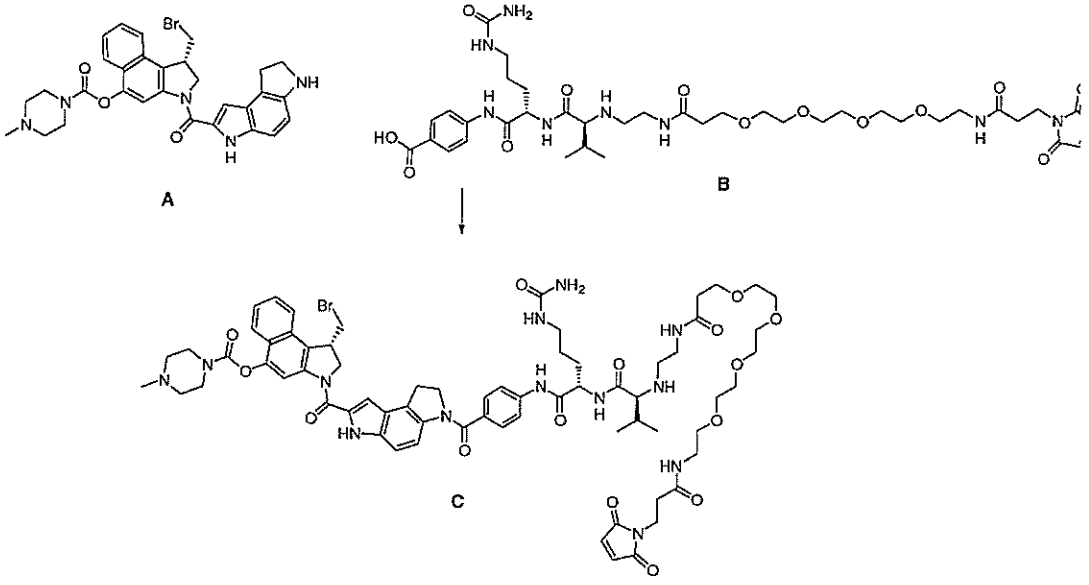
Compound (3). A solution of HBr salt **1** (12 mg, 0.016 mmol) (see compound 10 of Example 6) and **2** (25 mg, 0.032 mmol) in DMF (1.5 mL) was treated with diisopropylethylamine (11uL, 0.064 mmol) and was stirred at 25 ^{0}C under nitrogen for 48

h. The solvent was removed and crude product was purified by Prep HPLC to afforded **3** (6.4 mg, 30%). MS: calcd for $C_{64}H_{67}BrN_{10}O_{10}$ (M+H) m/z 1216.18 found 1216.40.

Compound (4). To a solution of TFA salt **3** (6.4 mg, 4.9 umol) in DMF (1 mL) was added with piperidine (50uL) and the reaction mixture was stirred at 25 ^{0}C under nitrogen for 15 min. The solvent was removed and crude product was purified by Prep HPLC to afforded **4** (3.8 mg, 66%). MS: calcd for $C_{64}H_{67}BrN_{10}O_{10}$ (M+H) m/z 994.94 found 994.80.

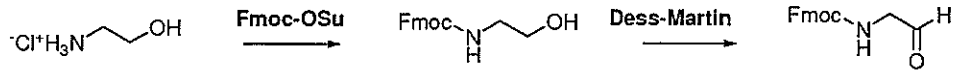
Compound (6). A solution of TFA salt **4** (3.8 mg, 3.2 umol) in DMF (1 mL) was added with **5** (3.3 mg, 6.4 umol) and piperidine (10uL) and the reaction mixture was stirred at 25 ^{0}C under nitrogen for 30 min. The solvent was removed and crude product was purified by Prep HPLC to yield **6** (3.4 mg, 71%). MS: calcd for $C_{67}H_{83}BrN_{12}O_{16}$ (M+H) m/z 1393.35 found 1393.60.

Example 11



Compound **A** (0.033 mMoles, 2HBr salt) (see compound 10 of Example 6) was reacted with Compound **B** (45 mg, 0.054 mMoles) in 2.5 mL DMF in the presence of HATU (20 mg, 0.054 mMoles) and TEA (15-20 μL) for 50 minutes. The solvents were evaporated and the Crude was purified by Reverse phase HPLC to give 10 mg of Compound **C** (21 % yield). MS: 1405.6, 1427.8 and 1444.6.

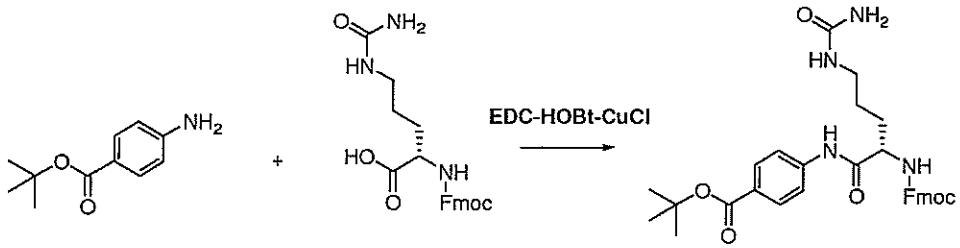
Example 12:



1 2 3

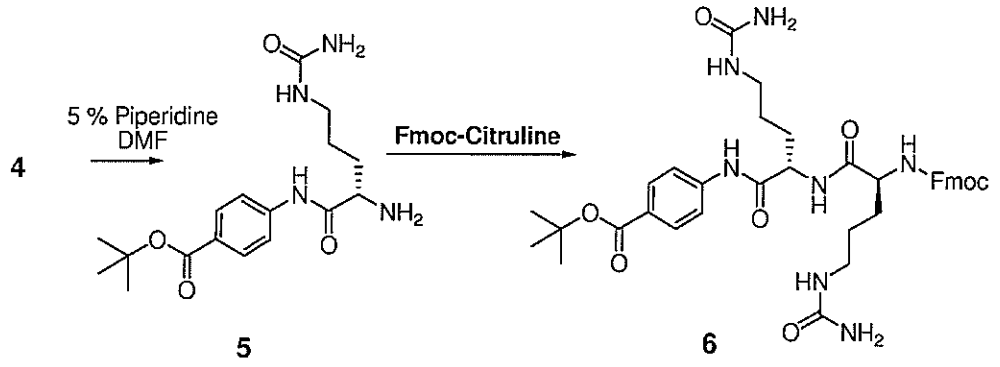
Synthesis of 2: 1.44 Grams (0.015 Moles) of the HCl salt of 2-aminoethanol (**1**), 5.0 grams (0.018 Moles) of Fmoc-succinimide (**2**) were dissolved in 20 mL of acetonitrile and 20 mL of 10% aqueous potassium carbonate solution in a round bottom flask and stirred at room temperature for 30 minutes. The reaction was quenched with a 10 % citric acid solution and concentrated to remove the acetonitrile. The aqueous layer was extracted 3 times with 30 mL of ethyl acetate and the organic layer was washed with brine twice with 20 mL. Although the compound was pure enough it was purified over silica gel with 2-5 % MeOH in DCM to give 2.5 grams of **2** in 64 % yield.

Synthesis of 3: 1.3 grams (4.58 mMoles) of **2** was allowed to stir in 50 mL of dichloromethane in the presence of 2.5 grams (5.6 mMoles) of Dess-Martin reagent in a round bottom flask for 2 hours. The crude reaction mixture was purified over silica gel eluting with 20-50% of ethyl acetate. To give 1.06 grams of the aldehyde **3** in 82 % yield. $MS^{+1} = 281.8$



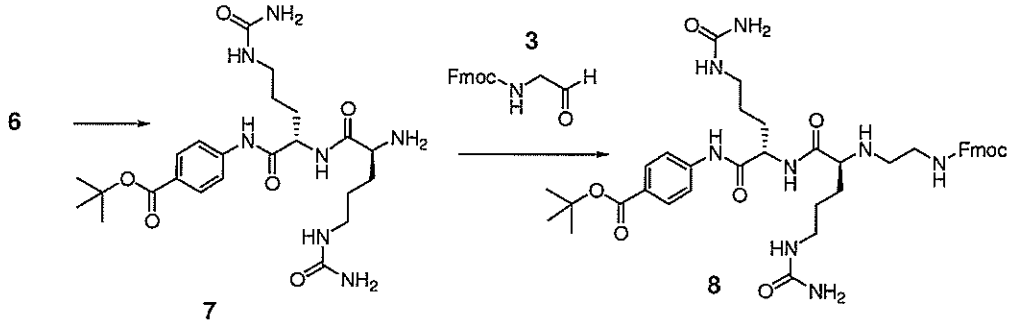
tert-butyl 4-aminobenzoate Fmoc Citruline 4

Synthesis of 4: 1.5 grams (3.77 mMoles) of Fmoc-citruline were dissolved in 3 mL of DMF in a round bottom flask to which was added 0.87 grams (4.5 mMoles) of EDC, 0.61 grams (4.5 mMoles) of HOBt. 6 mL of DCM were then added followed by the addition 0.88 grams (4.5 mMoles) of *t*-butyl-4-aminobenzoate and a catalytic amount of copper chloride. The reaction mixture was allowed to stir overnight The solvents were evaporated and the crude product purified over silica gel with 5 to 10 % MeOH in DCM to give 2 grams of **4** in 92 % yield. $M^{+1} = 574$



Synthesis of 5 and 6: 205 mg (0.36 mMoles) of compound **4** were treated with 3 ml of a solution of 2.5 % piperidine in DMF for 30 minutes. The reaction mixture was concentrated to dryness and rinsed with hexanes (20-30 mL) twice and then with diethyl ether (20-30 mL) twice. The free amine **5** was dried under high vacuum and reacted in the next step without any further purification. $M^{+1} = 352.2$

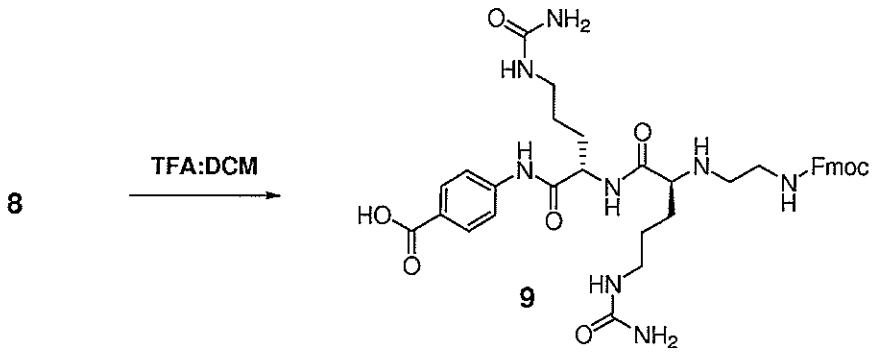
167 mg (0.42 mMoles) of Fmoc-Citruline were dissolved in 6 mL of DMF. 160 mg (0.42 mMoles) of HATU were added. Compound **5** (0.35 mMoles) prepared above was dissolved in 2 mL of DMF and added to the above solution of Fmoc-Citruline and HATU. 146 uL (1.05 mMoles) of triethylamine were added to the reaction mixture and allowed to stir for 1.5 hours. The solvent was evaporated and the crude purified by Reverse Phase HPLC to give 199 mg of compound **6** in 78 % yield. $M^{+1} = 731.2$, $M^{+Na} = 753.4$ and M^{+K} 769.0



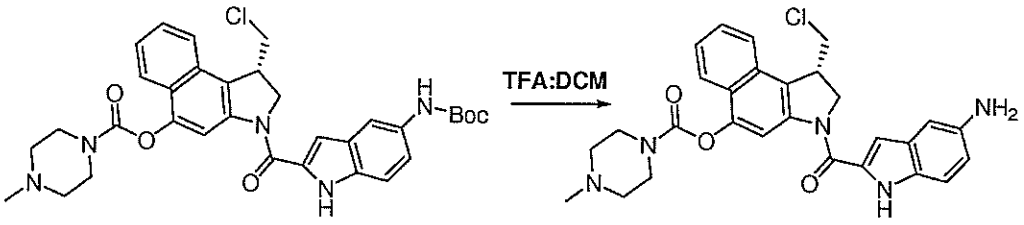
Synthesis of 7 and 8: 280 mg (0.38 mMoles) of compound **6** was deprotected in 3 mL of 2.5 % piperidine similar to the synthesis of compound **5** to give **7**. $M^{+1} = 509$

Compound **8** was synthesized by reductive-amination of compound **3** with **7** prepared above. 0.38 mMoles of compound **7** were dissolved in 6 mL of methanol in a round bottom flask and cooled to 0°C over an ice bath followed by the addition of 250 mg (3.0 mMoles) of sodium acetate. Compound **3** 120 mg (0.43 mMoles) was added to the

above flask and the resulting reaction mixture was stirred at 0°C for 30 minutes. 68 mg (1.0 mMoles) sodium cyanoborohydride was then added. The reaction mixture was allowed to stir at ice bath temperature for 20 minutes and then at room temperature for 1 hour. The reaction mixture was concentrated and purified by reverse phase HPLC to give 150 mg of desired product **8** in 51 % yield. M^{+1} = 774.4

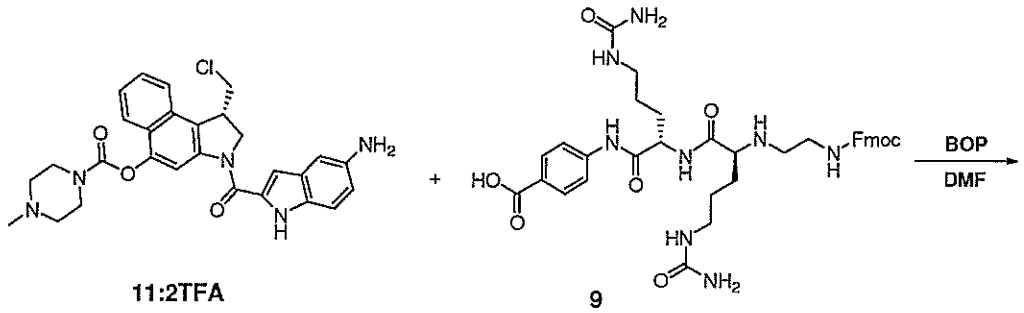


Synthesis of Acid 9: 33 mg (0.045 mMoles) of **8** was treated with 3 mL of a 2:1 mixture of DCM:TFA for 25 minutes. The solvent was evaporated the residue was titrated with diethyl ether two times and the ether layer discarded. The resulting acid **9** was dried under high vacuum for several hours and carried on to the next step. MS^{+1}=718



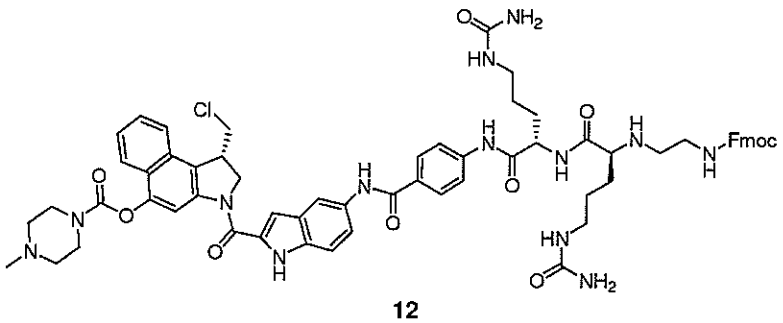
10 **11:2TFA**

Synthesis of 11: 20 mg of **10** (0.032 mMoles) was treated with 3 mL of a 2:1 mixture of DCM:TFA for 5 minutes. The solvent was evaporated the residue was titrated with diethyl ether two time and the ether layer discarded. The resulting TFA salt of amine **11** was dried under high vacuum for several hours and carried on to the next step. MS^{+1}=519



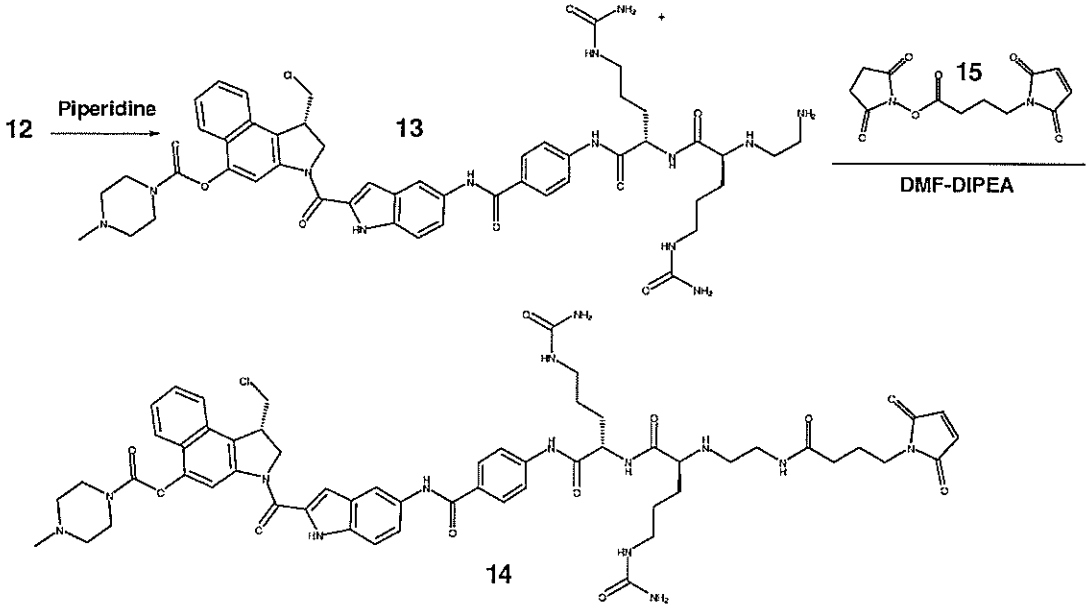
11:2TFA

9



12

Synthesis of 12: Amine **11:2TFA** prepared above was dissolved in 3 mL of DMF and transferred to a flask containing acid **9** followed by the addition of 20 mg (0.045 mMoles) of BOP and 100 uL of diisopropyl amine and stirred at room temperature for 2 hours. The crude reaction mixture was concentrated and purified by reverse phase HPLC to give 35 mg of desired compound **12** in 89 % yield. MS^{+1}=1217.6, *m/2z* = 609.2

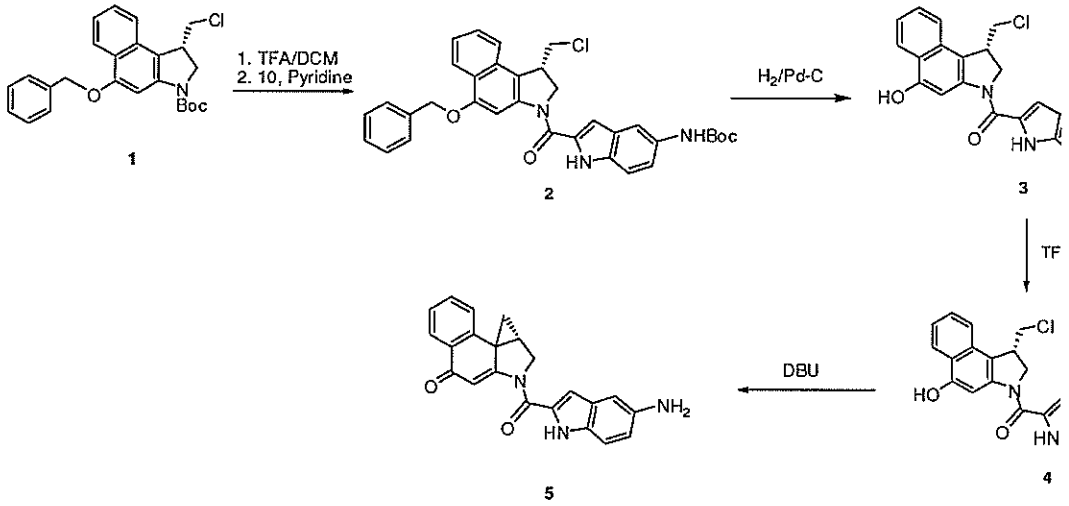


14

Synthesis of 13 and final compound 14: 35 mg (0.029 mMoles) of **12** was treated with a 4 mL DMF containing 100 uL of piperidine for 15 minutes. The solvent was evaporated and the resulting amine **13** was titrated with 10 mL hexanes followed by 10 mL diethyl ether (2x) dried under hi vacuum and carried on to the next step with any further purification. M^{+1} = 996.0, *m/2z* = 498

0.029 mMoles of **13** prepared above was dissolved in 2 mL DMF followed by the addition 17 mg (0.06 mMoles) of commercially available **15** and 32 uL of diisopropyl amine. The resulting reaction mixture was stirred at room temperature for 1 hour. The solvents were evaporated and the crude reaction mixture purified by reverse phase HPLC using acetonitrile and water with 0.1% TFA to give 21 mg of 14 (MED-2500) as its TFA salt in 52 % yield. MS: M^{+1} = 1159.47, *m/2z* = 580.2.

Example 13:



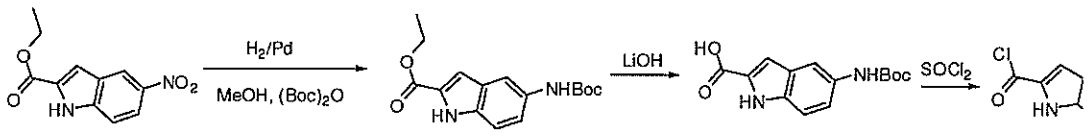
Synthesis of compound 2: To a solution of **1** (350 mg, 0.82 mmol) in dichloromethane (3 mL) was added TFA (6 mL). The resulting solution was stirred for 20 minutes. The solvent was coevaporated with toluene (twice) *in vacuo*. The residue was used without further purification for the next step. To a solution of this Boc deprotected material (0.82 mmol) in dichloromethane (8 mL) was added pyridine (2 ml) and **10** (0.98 mmol, see below) at 0°C. The mixture thus obtained was stirred at 0°C for 1 hour. The solvent was evaporated and the residue was purified by flash chromatography on silica gel and eluted with 5% methanol in dichloromethane to give compound **2** as white foam (417 mg, 90%). ^{1}H NMR (CD_3OD) δ 1.55 (s, 9H), 3.46 (t, 1H), 3.90 (dd, 1H), 4.15 (t,

1H), 4.65 (t, 1H), 4.8 (d, 1H), 5.27 (m, 2H), 6.55 (bs, 1H), 7.03 (d, 1H), 7.18 (dd, 1H), 7.34-7.41 (m, 5H), 7.43-7.56 (m, 3H), 7.70 (d, 1H), 7.85 (bs, 1H), 8.20 (s, 1H), 8.34 (d, 2H), 9.65 (bs, 1H); LC-MS (ES^+) 582 $(M+H)^+$; 604 $(M+Na)^+$.

Synthesis of compound 3: A solution of **2** (315 mg, 0.6 mmol) and palladium on charcoal (100 mg) in methanol / dichloromethane (6 mL) was placed under hydrogen atmospheric pressure at room temperature. The mixture thus obtained was stirred at room temperature overnight. The palladium was filtrated and the reaction mixture was concentrated to dryness to give compound **3** as white foam (175 mg, 66%). ^1H NMR (CD_3OD) δ 1.52 (s, 9H), 3.46 (t, 1H), 3.90 (dd, 1H), 4.00 (t, 1H), 4.50-4.60 (m, 2H), 6.98 (s, 1H), 7.20 (d, 1H), 7.30-7.45 (m, 4H), 7.60 (d, 1H), 7.70 (d, 1H), 7.85 (bs, 1H), 8.20 (d, 1H); LC-MS (ES^+) 436 $(M+H-56)^+$; 493 $(M+H)^+$.

Synthesis of compound 4: To a solution of **3** (350 mg) in dichloromethane (3 mL) was added TFA (3 mL). The resulting solution was stirred for 20 minutes. The solvent was coevaporated with toluene (twice) *in vacuo* to yield compound **4**. The solid was used without further purification for the next step.

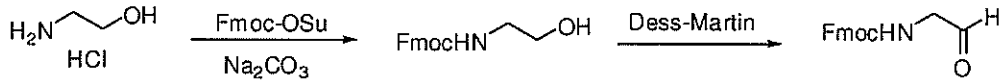
Synthesis of compound 5: To a suspension of **4** (300 mg, 0.59 mmol) in acetonitrile (10 mL) was added DBU (440 μL, 2.96 mmol). The resulting solution was stirred for 30 minutes. The solvent was evaporated *in vacuo*. The reaction mixture was dissolved in dichloromethane (50 mL) and washed with saturated sodium bicarbonate. The organic layer was washed with brine and dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to yield compound **5** as a yellow solid (200 mg, 90%). ^1H NMR (CD_3OD) δ 3.55 (m, 1H), 3.75 (m, 2H), 4.45 (m, 2H), 5.49 (s, 1H), 6.85 (dd, 1H), 6.97-7.01 (m, 2H), 7.15 (d, 1H), 7.25 (d, 1H), 7.40 (t, 1H), 7.60 (t, 1H), 8.10 (d, 1H); LC-MS (ES^+) 356 $(M+H)^+$; 379 $(M+Na)^+$; 394 $(M+K)^+$.



8 9 1(

Synthesis of Compound 10: To a suspension of ethyl 5-nitroindole-2-carboxylate (Acros, 10 g, 42.7 mmol), di-*tert*-butyl dicarbonate (10.24 g, 46.97 mmol) in anhydrous methanol (Acros, 100 mL), palladium (Aldrich, 10% on activated carbon, 400 mg) was

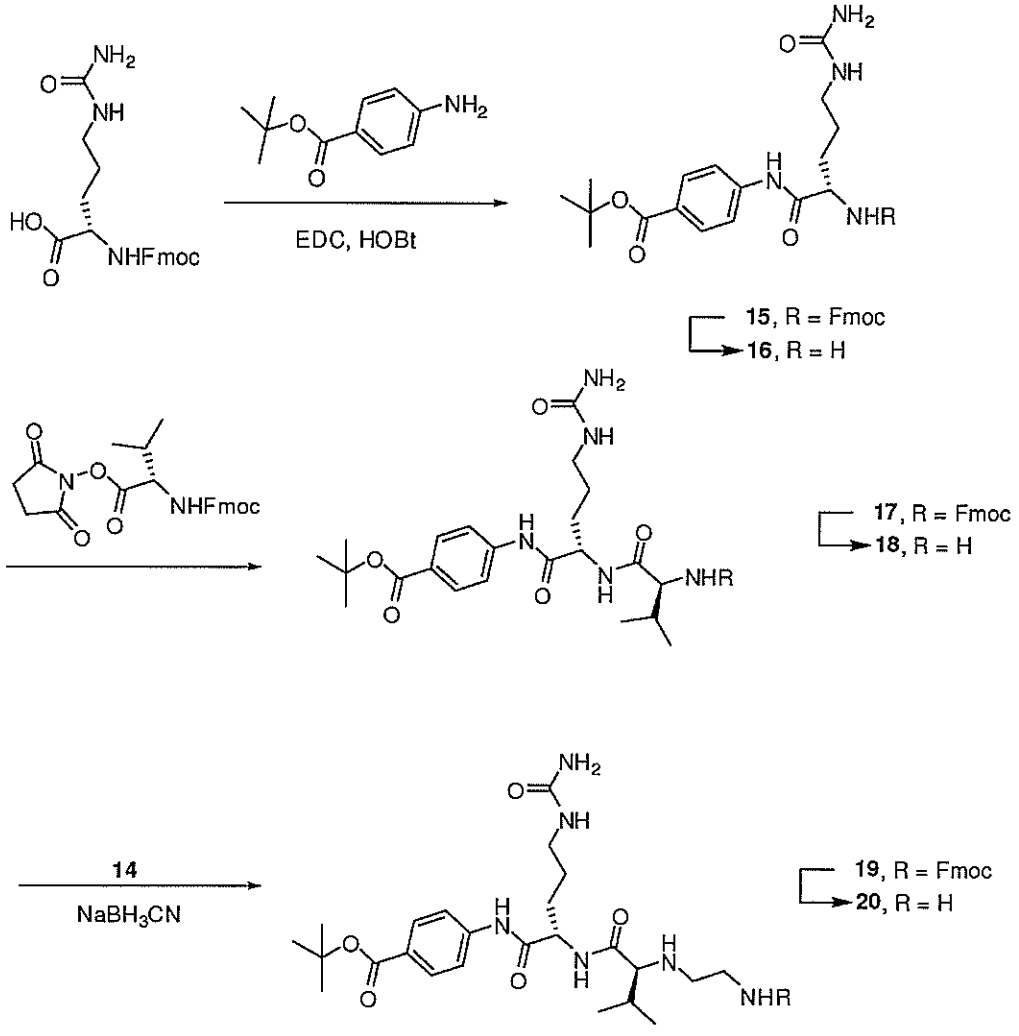
added. The reaction mixture was stirred at room temperature under H_2 balloon for 3 hr. The reaction was completed when H_2 is no longer being consumed. The reaction mixture was filtered and the filtrate was concentrated and dried under high vacuum to yield yellow residue **8** without further purification. To a solution of yellow residue **8** in methanol (150 mL), a solution of LiOH in water (150 mL, 0.57M) was added. The reaction mixture was heated at 60 °C for overnight. The hydrolysis was completed based on HPLC analysis. The reaction mixture was diluted with water (300 mL) and concentrated under vacuum to remove methanol. The resulted solution was extracted with EtOAc (2X150 mL). The aqueous layer was acidified to pH = 4 by adding $KHSO_4$ solution. The resulted mixture was again extracted with EtOAc (3X150 mL). The organic layers were combined, dried over anhydrous $MgSO_4$ and concentrated under vacuum to yield brown solid **9** (9.7 g, 82%). **9** is used in next step without further purification. To a solution of **9** (2.16 g, 7.84 mmol) in anhydrous DCM (Acros, 60 mL) and 1-methyl-2-pyrrolidone (Fluka, 5.03 mL, 52.32 mmol), $SOCl_2$ (Aldrich, 1.9 mL, 26.16 mmol) was added. The reaction mixture was stirred at 0 °C for 0.5 hr and at room temperature for 0.5 hr. The reaction mixture was concentrated and dried on high vacuum for overnight to yeield **10**. Small amount of **10** was treated with methanol and triethylamine to form the methyl ester, which was used for HPLC analysis to check the completion of the formation of acetyl chloride.

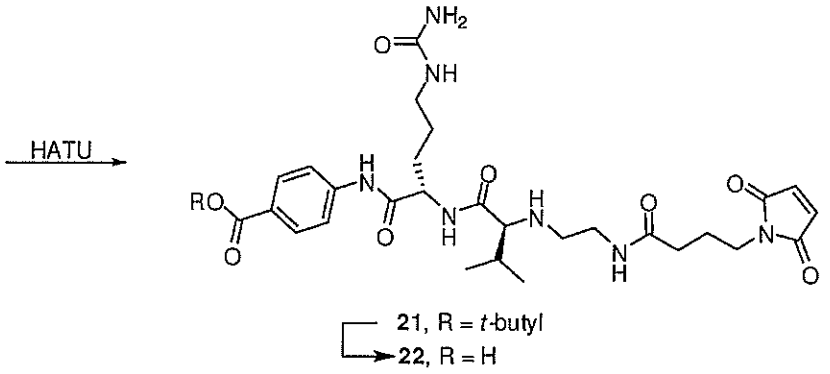


13 **14**

Synthesis of Compound 14: To a suspension of 2-aminoethanol hydrochloride (Aldrich, 5 g, 51.25 mmol) in acetonitrile (80 mL), and Na_2CO_3 (Aldrich, 10%, 80 mL), Fmoc-OSu (BaChem, 17.28 g, 51.25 mmol) was added. The reaction mixture was stirred at room temperature for 2 hr and HPLC analysis showed the reaction completed. The reaction mixture was diluted with water (500 mL) and crude product was collected by filtration. The solid was dissolved in EtOAc (500 mL) and the resulted organic layer was washed with HCl solution (1%, 2x150 mL), sat. $NaHCO_3$ solution (2x150 mL) and brine (1x150 mL). The organic layer was concentrated to yield white solid **13** (13.9 g, 95%), which was used for next step without further purification. To a solution of **13** (13.9 g,

49.12 mmol) in anhydrous DCM (ACROS, 150 mL), Dess-martin periodinane (27.08 g, 63.85 mmol) was added. The reaction mixture was stirred at room temperature for 4 hr. HPLC analysis showed the reaction was completed. The reaction mixture was loaded on flash column chromatograph and purified with 10-50% EtOAc in hexane to yield white solid **14** (12.1 g).



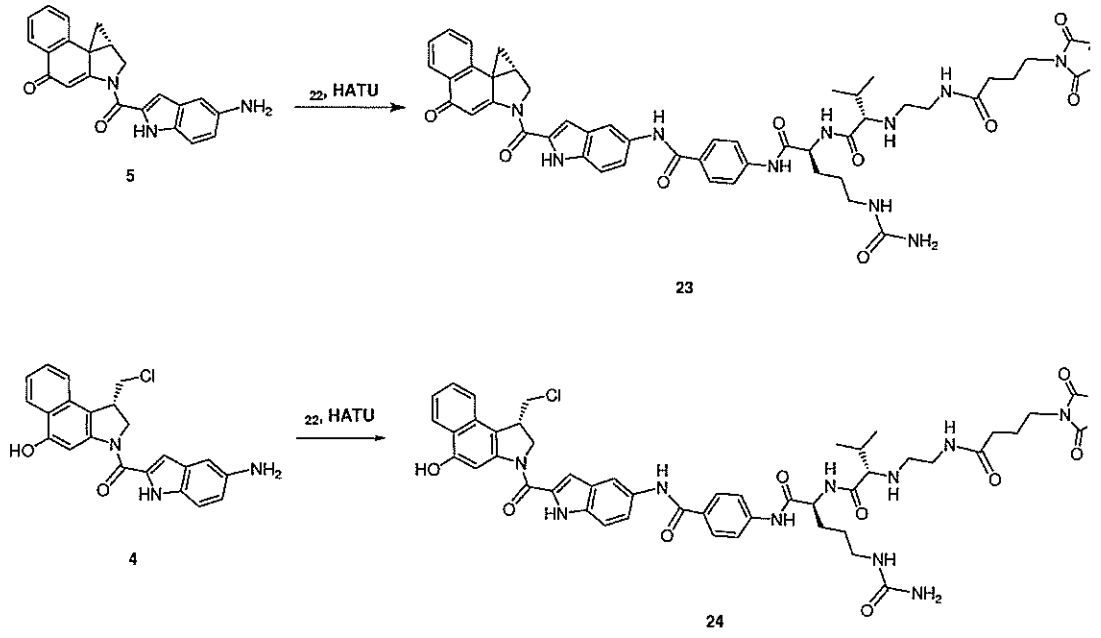


Synthesis of compound 22: To a solution of N_{α}-Fmoc-L-Citruline (Fluka, 10 g, 25.16 mmol), *tert*-butyl 4-aminobenzoate (Fluka, 5.84 g, 30.2 mmol), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (Fluka, 5.79 g, 30.2 mmol), 1-hydroxybenzotriazole (Chem-Impex, 4.08 g, 30.2 mmol) in anhydrous DMF (Acros, 100 mL), $CuCl_2$ (Aldrich, 4.05 g, 30.2 mmol) was added. The reaction mixture was stirred at room temperature for overnight. HPLC analysis showed the reaction was completed. The reaction mixture was concentrated and worked up with EtOAc and water. The organic layer was washed with brine and concentrated to yield brown residue **15** as crude product. To a solution of above mentioned crude product **15** in anhydrous DMF (Acros, 140 mL), piperidine (Aldrich, 7 mL) was added. The reaction mixture was stirred at room temperature for 15 min. HPLC analysis showed the reaction was completed. The reaction mixture was concentrated and the residue was purified on 120 g CombiFlash column with 0-20% methanol in DCM. The desired product was eluted at 14% methanol in DCM. The fractions of desired product were combined and concentrated under high vacuum to yield brown solid **16** (7.34 g, 83.3%). To a solution of **16** (6.6 g, 18.86 mmol) in anhydrous DMF (Acros, 100 mL) and 1-methyl-2-pyrrolidone (Fluka, 25 mL, 188.6 mmol), Fmoc-Val-OSu (BaChem, 8.23 g, 18.86 mmol) was added. The reaction mixture was stirred at room temperature for 2 hr. HPLC analysis showed the reaction was completed. To the reaction mixture, piperidine (Aldrich, 7 mL) was added. The reaction mixture was stirred at room temperature for 0.5 hr. HPLC analysis showed the Fmoc protecting group was completed removed. The reaction mixture was concentrated and the residue was purified on 120 g CombiFlash column with 0-20% methanol in DCM. The fractions of desired product was combined and concentrated

under high vacuum to yield yellow solid **18** (7.4 g, 87%). To a solution of **18** (7.4 g, 16.48 mmol) and **14** (4.63 g, 16.48 mmol) in anhydrous methanol (Aldrich, 175 mmol), sodium acetate (Aldrich, 20 g) was added. The reaction mixture was stirred at 0 °C for 1.5 hr. To this reaction mixture, $NaBH_3CN$ (Aldrich, 1.55 g, 24.72 mmol) was added. The reaction mixture was stirred at room temperature for overnight. HPLC analysis showed the reaction was completed. The reaction mixture was concentrated and then worked up with EtOAc and water. The organic layers were combined, washed with brine and concentrated. The residue was purified on 120 g CombiFlash column with 0-10% methanol in DCM. The fractions of desired product were combined and concentrated under high vacuum to yield brown solid **19** (7.1 g, 60%).

To a solution of **19** (6.7 g, 9.38 mmol) in anhydrous DMF (Acros, 120 mL), piepridine (Aldrich, 6 mL) was added. The reaction mixture was stirred at room temperature for 0.5 hr. HPLC analysis showed the reaction was completed. The reaction mixture was concentrated and the residue was lyophilized with acetonitrile and water to yield yellow solid **20** without purification.

To a solution of compound **20** (assume 8.40 mmol), malimido butyric acid (Aldrich, 1.85 g, 10.08 mmol), O-(7-azabenzotriazo-1-yl)-*N*, *N*, *N*', *N*'-tetramethyluronium (Aldrich, 3.83 g, 10.08 mmol) in anhydrous DMF (Acros, 50 mL), *N*, *N*-diisopropylethylamine (Aldrich) were added to adjust pH value of the reaction mixture over 8. The reaction mixture was stirred at room temperature for 0.5 hr. HPLC analysis showed the reaction was completed. The reaction mixture was concentrated and the residue was purified on 120 g CombiFlash column with 10-20% methanol in DCM. The desired product was eluted out at 14-16% methanol in DCM. The fractions of desired product were combined and concentrated under high vacuum to yield white solid **21** (4.7 g, 85%). To a solution of **21** (4.7 g, 7.17 mmol) in anhydrous DCM (Acros, 30 mL), TFA (Fisher, 30 mL) was added. The reaction mixture was stirred at room temperature for 0.5 hr. HPLC analysis showed the reaction was completed. The reaction mixture was concentrated and the lyophilized with acetonitrile and water to yield white solid **22**(4.9 g, TFA salt).

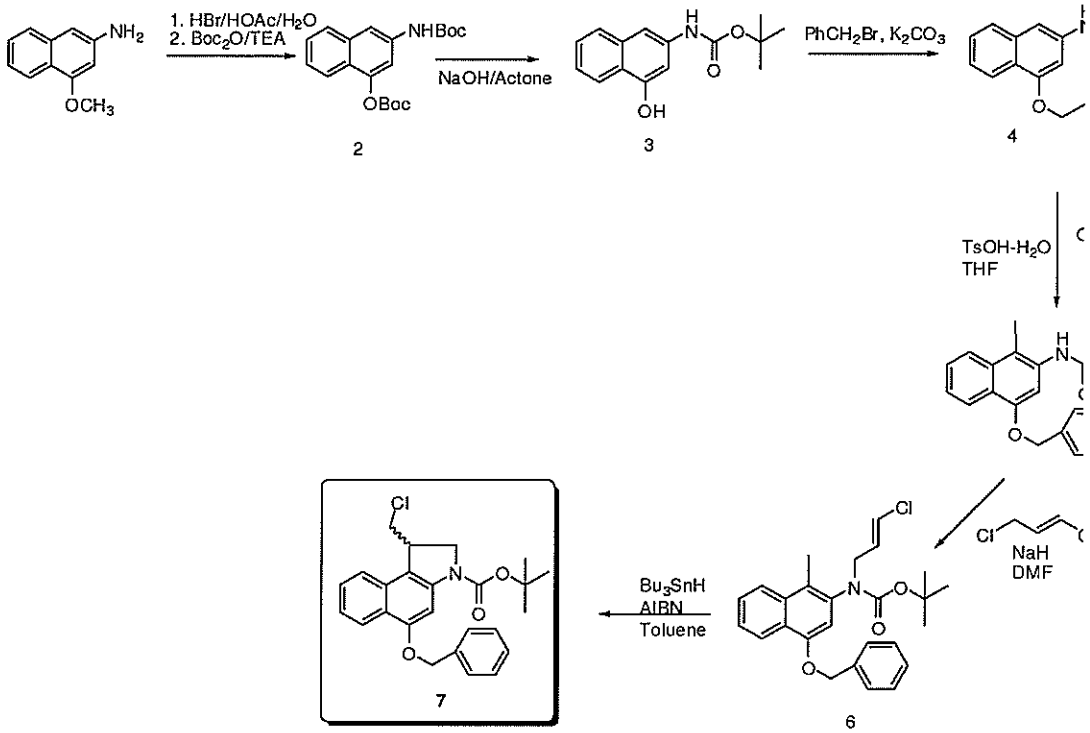


Synthesis of Compounds 24: To a solution of **22** (50 mg, 0.083 mmol) in anhydrous DMF (Acros, 1 mL), O-(7-azabenzotriazo-1-yl)-*N*, *N*, *N'*, *N'*-tetramethyluronium (Aldrich, 28.4 mg, 0.075 mmol) was added. The pH value of reaction mixture was adjusted to over 8 by adding DIEA (Alrdich). The reaction mixture was stirred at room temperature for 0.5 hr and HPLC analysis showed the reaction was completed. **4** (68.2 mg, 0.14 mmol) was added and pH value of reaction mixture was again adjusted to over 8 by adding DIEA. The reaction mixture was stirred at room temperature for another 0.5 hr and HPLC analysis showed the coupling was completed. The reaction mixture was concentrated under high vacuum and the residue was stirred with PBS buffer (Mediatech, Inc, pH=7.4, 10 mL) for 0.5 hr. The suspension was filtered and the solid collected was dissolved in methanol. The resulted solution was concentrated with silica gel and the slurry was purified on 4 g CombiFlash column with 10-20% methanol in DCM. The right fractions was concentrated and lyophilized in acetonitrile and water to yield white powder Compound **24** (45 mg, 45%) ^{1}H NMR (CD_3OD) δ 1.10 (d, 3H), 1.15 (d, 3H), 1.62 (m, 2H), 1.89-1.94 (m, 4H), 2.25 (m, 3H), 3.80 (m, 2H), 3.50 (t, 2H), 3.60 (m, 3H), 3.84 (d, 1H), 4.00 (dd, 1H), 4.20 (m, 1H), 4.65 (m, 1H), 4.72 (m, 2H), 6.83 (s, 2H), 7.18 (s, 1H), 7.37 (t, 2H), 7.50 (m, 3H), 7.75-7.81

(m, 3H), 7.97 (d, 2H), 8.06 (s, 1H), 8.20 (d, 1H), 8.83 (d, 1H); LC-MS (ES^+) 976 $(M+H)^+$, 999 $(M+Na)^+$.

Synthesis of Compound 23: To a solution of **22** (500 mg, 0.7 mmol) in DMF (6 mL) were added diisopropylethylamine (250 µL, 1.4 mmol) and HATU (279 mg, 0.73 mmol) at room temperature. The mixture thus obtained was stirred for 30 minutes. The solvent was evaporated and the residue was purified by flash chromatography on silica gel and eluted with 15% methanol in dichloromethane to give the desired compound. This solid (81 mg) was added slowly to a solution of **5** (40 mg, 0.11 mmol) in 20% 1-methyl-2-pyrrolidone in dichloromethane (2 mL) at room temperature. The mixture thus obtained was stirred for 2 hours. The desired compound precipitated by addition of 6 ml of dichloromethane. The yellow solid was filtrated, dried and could be used without further purification for the next step (86 mg, 82%). Otherwise the residue was purified by semi-preparative HPLC (20 mM ammonium formate buffer solution, pH=7 and acetonitrile) to give Compound **23** as a white solid. ^1H NMR (CD_3OD) δ 1.02 (d, 6H), 1.62 (m, 2H), 1.70 (t, 1H), 1.75-1.95 (m, 5H), 2.05 (m, 1H), 2.25 (t, 3H), 3.80 (m, 2H), 3.10-3.30 (m, 4H), 3.35 (m, 2H), 3.55 (t, 2H), 4.55 (2s, 1H), 4.60-4.70 (m, 2H), 6.77 (s, 2H), 7.07 (s, 1H), 7.15-7.20 (m, 2H), 7.40-7.50 (m, 3H), 7.60 (m, 1H), 7.75 (d, 2H), 7.95 (d, 1H), 8.10 (s, 1H), 8.15 (d, 1H); LC-MS (ES^+) 940 $(M+H)^+$, 962 $(M+Na)^+$.

Example 14:



Synthesis of compound 3: A solution of 4-methoxy-2-naphthylamine (230 mg, 1.33 mmol) in glacial acetic acid (9.6 mL) and hydrobromic acid in water (16 mL, 48%) was refluxed under N_2 for 4 h. A small amount of sample (0.1 mL) was diluted with ethyl acetate (0.5 mL), and then water (0.5 mL) and triethylamine (0.1 mL) were added. TLC (20:1 DCM/methanol) of the organic layer showed no starting material and a new much lower spot (R_f=0.1). The solvent was removed under reduced pressure and the product was dried under vaccum to yield the intermediate 4-hydroxy-2-naphthylamine which was used for the next step without any purification. To a solution of 4-hydroxy-2-naphthylamine in dioxane (10 mL) was added TEA (1 mL) and di-*tert*-butyl dicarbonate (1.149 g, 5.27 mmol). The reaction mixture was refluxed under N_2 for 4 h. TLC (4:1 hexane/ethyl acetate) showed no starting material and a new higher spot (R_f=0.55). The reaction mixture was diluted with ethyl acetate (50 mL) and washed with water. The aqueous layer was extracted with ethyl acetate (2 x 50 mL) and the organics were combined and washed with brine. The organics were dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to yield N-(tert-butyloxycarnonyl)-4-O-(tert-butyloxycarbonyl)-2-naphthylamine (**2**, 80% yield) as oil. To a solution of compound **2** in acetone (10 mL) was added NaOH solution in water (10 mL, 1 M). The

reaction mixture was stirred at room temperature for overnight. TLC (4:1 Hexane/Ethyl acetate) showed no starting material and a new lower spot. The reaction mixture was extracted with ethyl acetate (50 mL) and washed with water. The aqueous layer was extracted with ethyl acetate (2 x 50 mL) and the organics were washed with brine, dried over anhydrous Na_2SO_4 and concentrated. The residue was purified on 10 g silica gel column with 10-20% ethyl acetate in hexane to yield compound **3** (181 mg, 53%) as an oil.

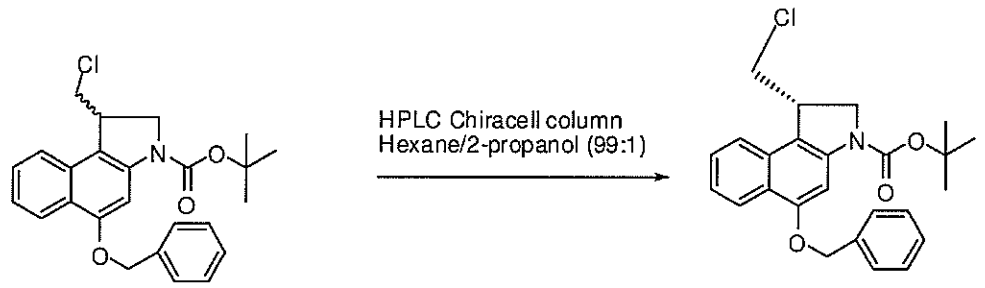
Synthesis of compound 4: A solution of compound **3** (5g, 19.3 mmol) in anhydrous DMF (50 ml) under nitrogen atmosphere was treated with benzyl bromide (4 g, 23.1 moles), potassium carbonate (3.7 g, 27 moles) and tetrabutylammonium iodide(70 mg, 0.01 mmoles). The reaction mixture was stirred at room temperature for 8 h. The reaction mixture was concentrated under reduced pressure. Chromatographic separation (4x 10 cm SiO_2, 10-20% EtOAc-hexane gradient elution) provided pure compound **4** (5.48 g, 83%) as a cream powder. ^{1}H NMR ($CDCl_3$, 400 MHz, ppm) 8.22 (d, 1H J=8.1 Hz, C5-H), 7.68 (d, 1H, J=8.2 Hz, C8-H), 7.3-7.5 (m, 8H, C1-H, C6-H, C7-H, $CH_2C_6H_5$), 7.06 (d, 1H, J=1.1 Hz, C3-H), 6.62 (br s, 1H, NH), 5.23 (S, 2H, $OCH_2(C_6H_5)$, 1.55 (s, 9H, $OC(CH_3)_3$).

Synthesis of compound 5: To a 1000 ml round bottom flask equipped with a stir bar and a rubber septum was combined compound **4** (13 gm, 0.0372 moles) and THF (300 ml).The clear yellow solution was cooled to -20° C with a dry ice bath under a nitrogen atmosphere. *p*-Toluenesulfonic acid (0.10 gm, 0.0005 moles) was added to the reaction and the solution was stirred for 10 minutes. *N*-Iodosuccinimide (10 gm, 0.0446 moles) was dissolved in THF (50 ml) and added to the reaction by cannula (approximately 1 hr). The solution was stirred in the ice bath for 2 hr and turned brownish. Removed ice bath and let warm to room temperature under nitrogen for 1.5 hr. TLC (2:1 Hexane/DCM) showed no starting material and a new higher spot. Reaction was quenched with saturated $NaHCO_3$ (200 ml) and a white solid formed. After stirring the solution for 10 minutes, EtOAc (200 ml) and water (100 ml) were added to the reaction. The aqueous layer was extracted with EtOAc (2 x 100 ml) and the organics were combined and extracted with brine (100 ml). The organics were dried over $MgSO_4$, filtered, and concentrated under vacuum to a dark red-brown solid. The solid was purified by column chromatrography using 2:1 Hexane/DCM as eluant to yield compound **5** (14 gm, 79%) as a brown solid.

Synthesis of compound 6: To a 250 ml round bottom flask equipped with stir bar and nitrogen inlet were combined compound **5** (8.4 gm, 0.0177 moles) and anhydrous DMF (125 ml). The yellow-orange solution was cooled to 0° C with an ice/salt bath under a nitrogen atmosphere. NaH (60%, 2.22 gm, 0.0554 moles) was added to the reaction in one portion. The solution turned cloudy and a gas was formed. The reaction was stirred in the ice bath for 15 minutes and then the ice bath was removed and the solution was stirred for another 15 minutes. *cis/trans*-1,3-Dichloropropene (5.3 ml, 0.0571 moles) was added to the reaction by syringe. The reaction was stirred under nitrogen at room temperature for 3 hr and turned a cloudy brown. TLC (4:1 Hexane/EtOAc) showed no starting material and a new lower spot. The reaction was quenched with water (250 ml). The aqueous layer was extracted with Ethyl acetate (3 x 100 ml) and the organics were washed with brine (2 x 50 ml). The organics were dried over $MgSO_4$, filtered and concentrated under vacuum to a brown oil. The product was purified by column chromatography using 1:1 DCM/Hexane as eluant to yield *(E/Z)-tert*-Butyl 4-(benzyloxy)-1-iodonaphthalene-2-yl(3-chloroallyl)carbamate (**6**) (9 gm, 93%) as a yellow oil.

Synthesis of compound 7: To a 500 ml three necked round bottom flask equipped with stir bar, temperature probe, reflux condenser and nitrogen inlet were combined compound **6** (9 gm, 0.0164 moles),toluene (200 ml), 2,2'-azobis(2-methylpropionitrile) (0.15 gm, 0.0009 moles) and tributyltin hydride (1.5 ml, 0.0056 moles) (by syringe). Nitrogen was bubbled through the solution for 15 minutes and then the reaction was heated to 80° C under nitrogen. After heating at 80° C for 15 minutes, tributyltin hydride (1.5 ml, 0.0056 moles) was added to the reaction by syringe. After heating for another 15 minute tributyltin hydride (1.5 ml, 0.0056 moles) was added to the reaction by syringe. After a further 15 minutes, tributyltin hydride (1.0 ml, 0.0037 moles) was added to the reaction by syringe. The total amount of tributyltin hydride added was 5.5 ml, 0.0204 moles. The reaction was heated at 80° C for 30 minutes and then allowed to cool to room temperature. TLC (10% EtOAc/Hexane) showed no starting material and a new higher spot. The solution was concentrated under vacuum to a yellow oil. The oil was purified by column chromatography using as eluant 100% Hexane to 5% EtOAc/Hexane to 10% EtOAc/Hexane to give a pale yellow solid. The solid was recrystallized from hexane (100 ml, 45 C for 30 min, cooled in fridge for 2 hr, collected

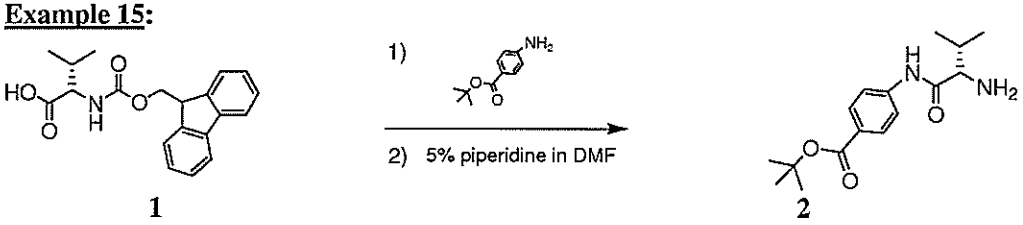
by filtration, dried under vacuum) to yield compound **7** (4.16 gm, 60% yield) as a white solid.



7 **1**

The racemic compound **7** was dissolved in DCM (50mg, 1 ml). The solution was then diluted with hexane (9 ml). The solution was then loaded onto a Chiralcel OD prep column (10 micron, 20 x 250 mm) and separated using hexane/isopropanol (99:1, 15 ml/min). The analytical column (Chiralcel OD, 0.46 x 25 cm, 20 microns) gives a retention time of 10.6 min for **1** (99:1 hexane/IPA, 1 ml/min, 15 minute run). NMR(1H, $CDCl_3$, 400 MHz): d 1.61 (9H, s, C-$(CH_3)_3$); 3.44 (1H, t, J = 25 Hz, CH-CH_2-N); 3.9-4.0 (2H, m, Cl-CH_2-CH); 4.12 (1H, t, J = 26 Hz, CH-CH_2-N); 4.25 (1H, m, CH_2-CH-CH_2); 5.26 (2H, s, O-CH_2-C_6H_5); 7.2-7.5 (8H, m, O-CH_2-C_6H_5, $C_{10}H_5$); 7.63 (1H, d, J = 21 Hz, $C_{10}H_5$); 8.3 (1H, d, J = 21 Hz, $C_{10}H_5$)

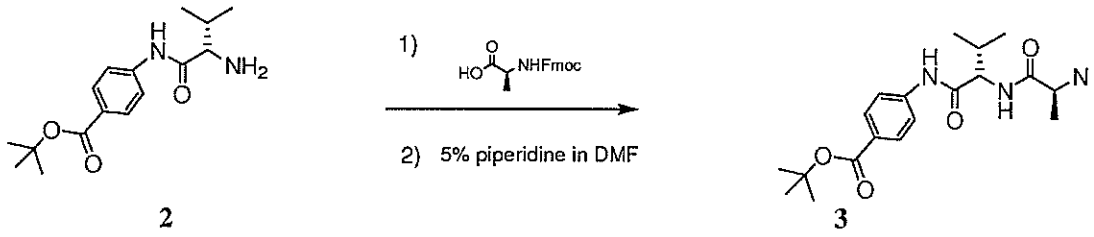
Example 15:



1 **2**

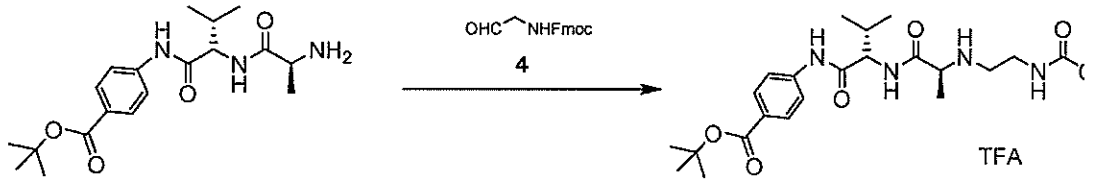
Synthesis of Compound 2: To a solution of Fmoc-Val-OH (200 mg, 0.59 mmole) and tert-butyl-4-amino benzoate (114 mg, 0.59 mmole) in solution of 5% DMF in DCM (5 mL) was added HATU (224 mg, 0.59 mmole) followed by adding diisoproopylethylamine (410 uL, 2.36 mmole) at room temperature. The reaction mixture was stirred for 30 minutes. The solvent evaporated and the residue was purified by flash column chromatography on silica gel eluted with 5% methanol in dichloromethane. The resulting product was dissolved in 5% piperidine in DMF and was stirred at room temperature for 10 minutes. Solvent was removed in vacuo. Crude product was purified

by semi-preparative HPLC to obtain **2** (149 mg, 87%). ^{1}H NMR (DMSO-d_6) δ 8.2 (br, s, 1H), 7.91(d, 2H), 7.75 (d, 2H), 3.54 (m, 1H), 2.33 (m, 1H), 2.12 (br, 2H), 1.47 (s, 9H), 1.10 (m, 3H), 0.94 (m, 3H). $C_{16}H_{24}N_2O_3$ (MS: 292.18), found M+1=293.31.



2 **3**

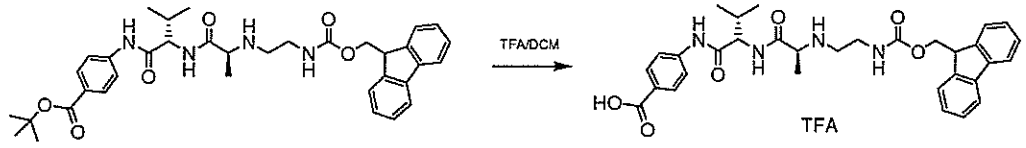
Synthesis of Compound 3: A solution of **2** (149 mg, 0.51 mmole), Fmoc-Ala-OH (159 mg, 0.51 mmole), HATU (194 mg, 0.51 mmole), and diisoproopylethylamine (355 uL, 2.04 mmole) in solution of 5% DMF in DCM (4 mL) was stirred for 30 minutes. The solvent evaporated in vacuo and the residue was purified by flash column chromatography on silica gel eluted with 5% methanol in dichloromethane. The resulting product was directly submitted for deprotection of Fmoc protecting group which was done in 5% piperidine in DMF at room temperature for 10 minutes. Solvent was removed in vacuo. Crude product was purified by semi-preparative HPLC to obtain **3** (146 mg, 79%). ^{1}H NMR (DMSO-d_6) δ 8.2 (br, s, 1H), 8.09 (br, 1H), 7.91(d, 2H), 7.75 (d, 2H), 3.75 (m, 1H), 3.54 (m, 1H), 2.33 (m, 1H), 2.25 (br, 2H), 1.47 (s, 9H), 1.29 (d, 3H), 1.12 (m, 3H), 0.96 (m, 3H). $C_{19}H_{29}N_3O_4$ (MS: 363.22), found M+1=264.01.



3 **5**

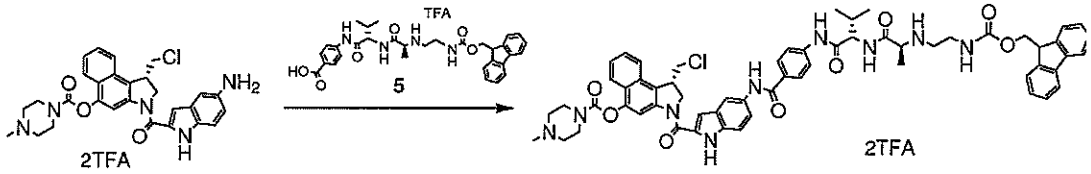
Synthesis of Compound 5: To a solution of compound **3** (146 mg, 0.4 mmole) and sodium acetate (98.4 mg, 1.2 mmole) in dry methanol (5 mL) was added compound **4** (135 mg, 0.48 mmole) at 0-5 ^{0}C. The reaction mixture was kept stirring for 20 minutes. Sodium cyanobrorhydride (76 mg, 1.2 mmole) was added to above solution. The resulting reaction mixture was stirred for another 10 minutes and was allowed to warm to room temperature for 1 hour. The reaction solution was then concentrated and purified by semi-preparative HPLC to obtain **5** (230 mg, 77%). ^{1}H NMR (DMSO-d_6) δ 8.2 (br, s, 1H), 8.09

(br, 1H), 7.91(d, 2H), 7.84 (d, 2H), 7.75 (d, 2H), 7.55 (d, 2H), 7.38 (m, 2H), 7.28 (m, 2H), 4.73 (d, 2H), 4.45 (m, 1H), 3.75 (m, 1H), 3.54 (m, 1H), 3.08 (t, 2H), 2.81 (t, 2H), 2.33 (m, 1H), 2.20-2.25 (br, 2H), 1.47 (s, 9H), 1.29 (d, 3H), 1.13 (m, 3H), 0.92 (m, 3H). $C_{36}H_{44}N_4O_6$ (MS: 628.33), found M+1=629.45.



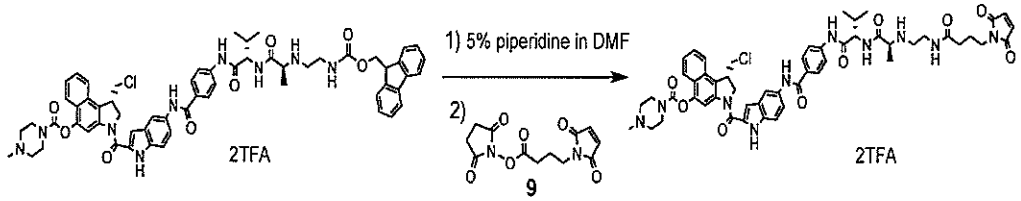
5 **6**

Synthesis of Compound 5: A solution of **5** (72 mg, 0.11 mmole) was combined with TFA/DCM (1:2, 2 mL) for 15 minutes. The solvents were evaporated in vacuo, further dried in high vacuo and was ready for the next reaction (76.8 mg, 98.4%). ^1H NMR (DMSO-d_6) δ 11.55 (br, s, 1H), 8.2 (br, s, 1H), 8.09 (br, 1H), 7.91(d, 2H), 7.84 (d, 2H), 7.75 (d, 2H), 7.55 (d, 2H), 7.38 (m, 2H), 7.28 (m, 2H), 4.73 (d, 2H), 4.45 (m, 1H), 3.75 (m, 1H), 3.54 (m, 1H), 3.08 (t, 2H), 2.81 (t, 2H), 2.33 (m, 1H), 2.20-2.25 (br, 2H), 1.29 (d, 3H), 1.11 (m, 3H), 0.95 (m, 3H). $C_{32}H_{36}N_4O_6$ (MS: 572.26), found M+1=573.38.



7 **8**

Synthesis of Compound 8: To a solution of compound **7** (25 mg, 0.033 mmole), **5** and HATU (13 mg,0.033 mmole) in DMF (1.5 mL) was added diisoproopylethylamine (23 uL, 0.13 mmole) at room temperature. The reaction mixture was stirred for 30 minutes. Solvent was removed in vacuo. Crude product was directly submitted for purification by semi-preparative HPLC to gain **8** (33.3 mg, 76%). $C_{60}H_{62}ClN_9O_8$ (MS: 1077.44), found M+1=1078.31.

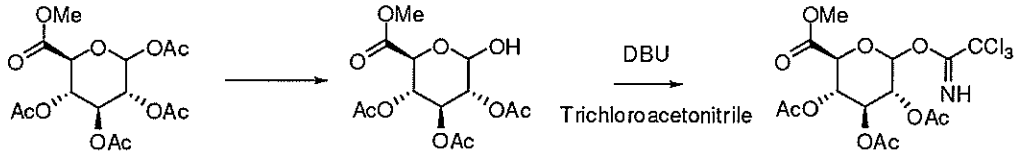


8 **10**

Synthesis of Compound 10: Compound **8** (33 mg, 0.025 mmole) was deprotected using 5% piperidine in DMF (1 mL) at room temperature for 10 minutes. The reaction mixture was concentrated in vacuo and the crude product was rinsed with ether

(5 mL x 2) without further purification. The resulting product was reacted with **9** (11 mg, 0.038 mmole) in the presence of diisoproopylethylamine (6.6 uL, 0.038 mmole) and DMF (1 mL) for 3 hours. The reaction mixture was concentrated and purified by semi-preparative HPLC to gain **10** (24 mg, 75%). $C_{53}H_{59}ClN_{10}O_9$ (MS: 1014.42), found M+1=1015.02.

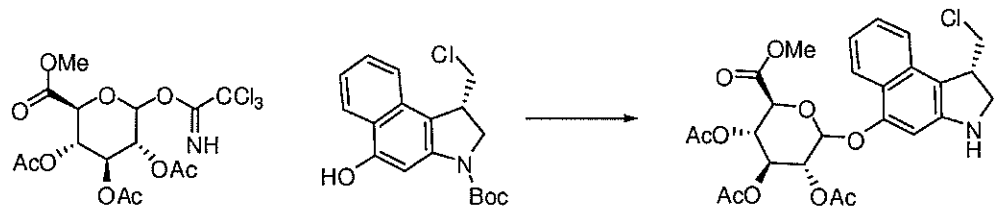
Example 16:



1 **2** **3**

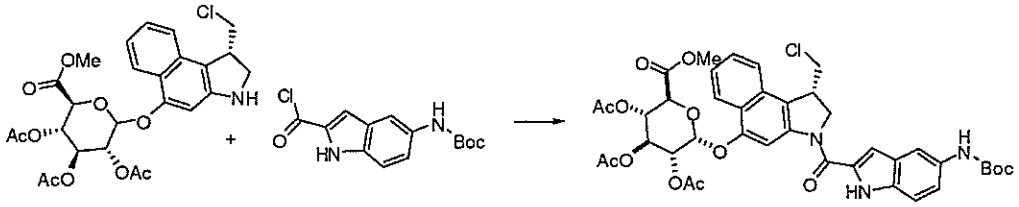
Synthesis of Compound 2: 1 gram (2.66 mMoles) of methyl 1,2,3,4-tetra-O-acetyl-β-D-glucuronate compound (**1**) was dissolved in 1 mL DMF and 4 mL THF in a round bottom flask. 400 µL of Benzyl amine was added to the flask and the reaction mixture was stirred overnight. The Solvent was evaporated the product was purified over silica gel with 10% to 50% Ethyl acetate in Hexanes. This gave 600 mg of (**2**) as light green oil. 67% yield. **NMR:** H1 NMR (400MHz, DMSO-d_6): δ7.5 (d, 1H), δ5.3 (m,1H), δ5.28(m, 1H), δ4.9 (t,1H), δ4.74 (m, 1H), δ4.38 (d, 1H), δ3.6(s,3H), δ1.99-1.96 (3s, 9H)

Synthesis of Compound 3: 600 mg (1.79 mMoles) of compound (**2**) was dissolved in dry dichloromethane in a round bottom flask. 66.5 µL (0.45 mMoles) of DBU was added followed by the addition of 1.26 mL (12.7 mMoles) of trichloroacetonitrile. The reaction mixture was allowed to stir for 1-2 hours. The reaction was monitored by TLC with phosphomolybdic acid stain reagent. After the reaction was complete the solvent was evaporated and product was purified over silica gel. The silica column was packed with 0.1% TEA in hexanes. The product was eluted with 5% to 50% ethlyl acetate in hexanes to give 600 mg (70% yield) of compound (**3**). $M^{+1} = 477.0$ $M^{+[Na]} = 499.0$. H1 NMR (400MHz, DMSO-d_6): δ10.08 (s, 1H), δ6.48 (broad, 1H), δ5.43 (t, 1H), δ5.20 (m, 2H), δ4.32 (d, 1H), δ3.63 (s, 3H, Me), δ1.98-1.96 (9H, 3 x AcO). C13 NMR (100 MHZ, DMSO-d_6): δ170.2, 170.1, 169.8, 167.4, 158.2, 92.3, 90.5, 70.5, 69.5, 68.9, 53.48, 21.0, 20.8, 20.8,



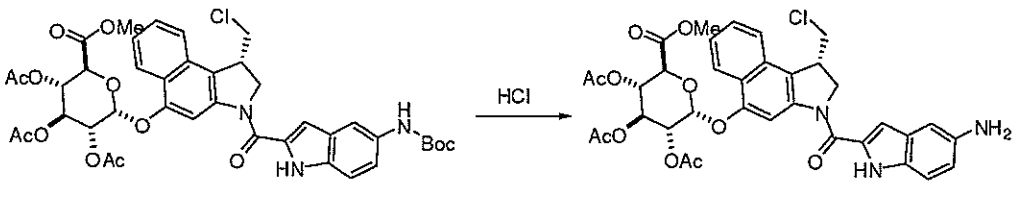
3 4 5

Synthesis of Compound 5: 344 mg (0.72 mMoles) of compound **3** and 200 mg (0.6 mMoles) of compound **4** were dissolved in anhydrous DCM in a round bottom flask containing dry molecular sieves. The flask was placed over a NaCl/ice bath at -10 ºC. 30 µL (0.24 mMoles) of boron trifluoro diethyl etherate was added to the reaction mixture at -10 ºC. The temperature was gradually allowed to rise to -5 ºC and stirred at this temperature for 1 hours and then at 0 ºC for half an hour. 3 equivalents 226 µL (1.8 mMoles) of boron trifluoro etherate were added at 0 ºC and the temperature was allowed to rise to room temperature. Stirring was continued for 2 hours. The solvent was evaporated and product was purified by reverse phase HPLC to give 250 mg (0.45 mMoles) of compound (**5**) (75% yield base on compound **4**). $M^{+1} = 550.0$



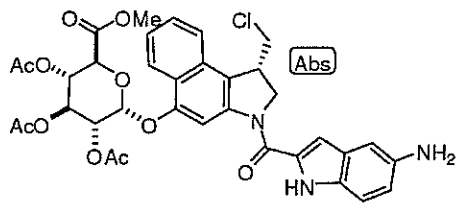
5 6 7

Synthesis of Compound 7: To a solution of 20 mg (0.07 mMoles) compound **6** in 2 mL of anhydrous DCM at 0 ºC was added a solution of 32 mg (0.048 mMoles) compound 5 in 2 mL of anhydrous DCM followed by addition of 400 µL of pyridine at 0 ºC. the reaction mixture was stirred at this temperature for 15 minutes. The product was purified by reverse phase HPLC to give 33 mg of compound 7 (84 % yield). $M^{+1} = 808$ and $M^{+[Na]} = 830$

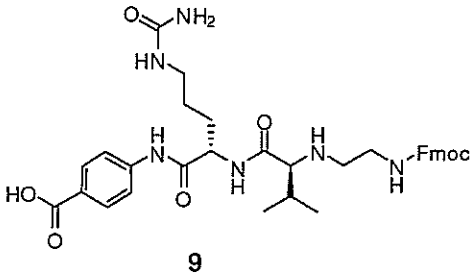


7 8

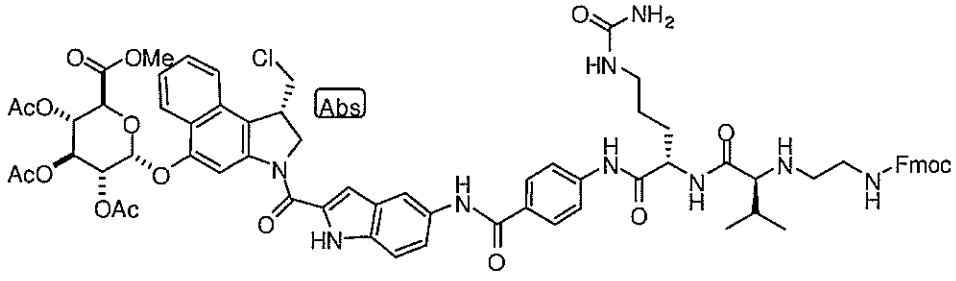
Synthesis of Compound 8: Compound **8** was prepared either as it HCl salt with 4 molar HCl solution in dioxane or its TFA salt with 33% TFA solution in DCM. The reaction was monitored by HPLC until complete. The solvent was evaporated and the product dried overnight under Hi vacuum and used in the next step as such. M^{+1} = 708.2



8

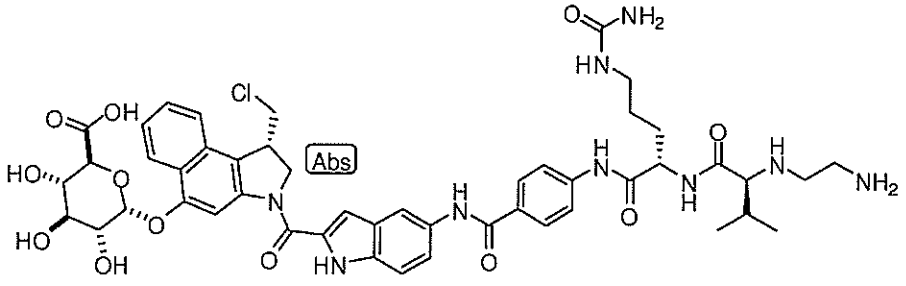


9



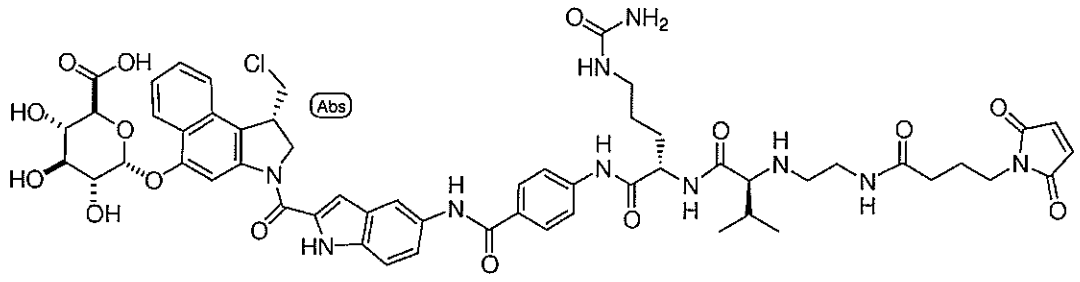
10

Synthesis of Compound 10: 31 mg (0.04mMoles) of Compound **9** in 2 mL DMF was preactivated with 15.5 mg (0.04mMoles) HATU and DIPEA (enough to ensure the pH was 7.0, 21 µL, 0.12mMoles) for 20 minutes in a round bottom flask. A solution of 30 mg (0.036 mMoles) of TFA salt of Compound **8** in 1 mL of DMF was then added to the above flask followed by the addition of 14 µL (0.08mMoles) of DIPEA. The reaction mixture was allowed to stir for 3 hours followed by evaporation of the solvent and purification by reverse phase HPLC to give 31 mg of the TFA salt of desired compound **10** in 59 % yield. M^{+1} = 1348.8



11

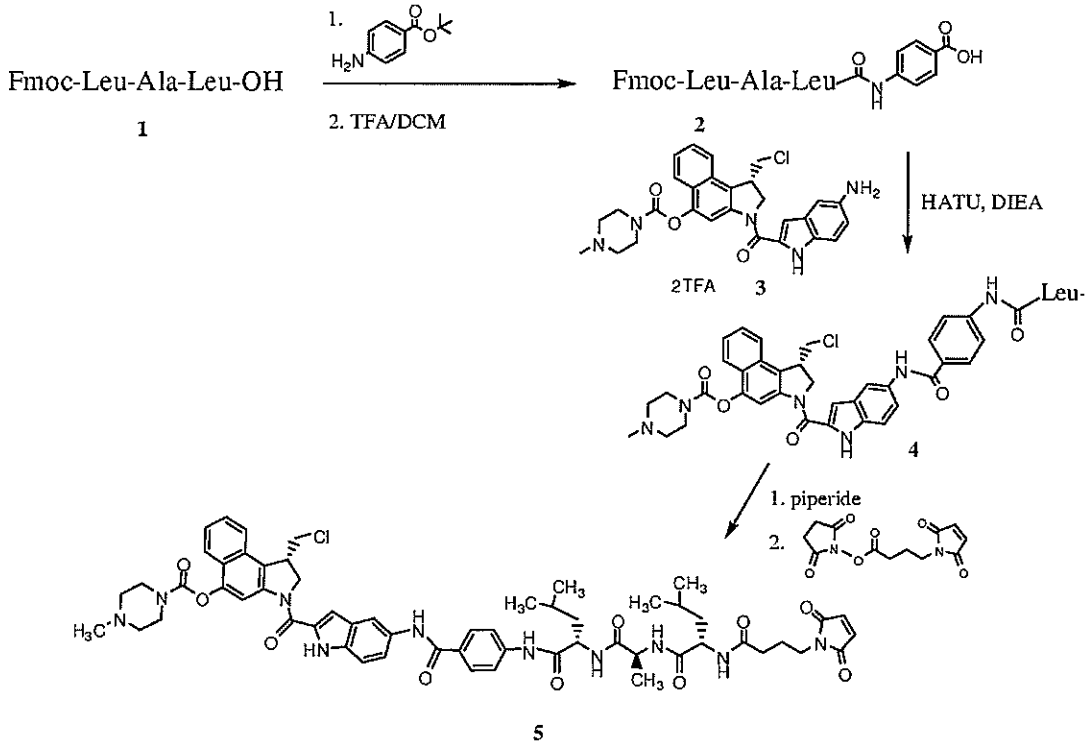
Synthesis of Compound 11: 30 mg of compound **10** (0.02 mMoles) was deprotected by stirring in 2.5 mL of MeOH and 4 μL of 2N NaOH solution for 5 hours. The reaction mixture was neutralized with acetic acid and passed through reverse phase HPLC to give 17 mg (0.014mMoles) of the double TFA salt of compound **11**. M^{+1} = 986.6



12

Synthesis of Compound 12: 17 mg of the double TFA salt compound **11** (0.014 mMoles) was reacted with 5.1 mg (0.018mMoles) of commercially available *N*-succinimidyl 4-maleimidobutyrate in 3 mL DMF and 12.2 μL (0.07 mMoles) of DIPEA for 1 hour. The solvent was evaporated and 14 mg (0.011mMoles) of the TFA salt of the desired compound **12** were obtained after purification by reverse phase Prep HPLC (76% yield). M^{+1} = 1151.8

Example 17:



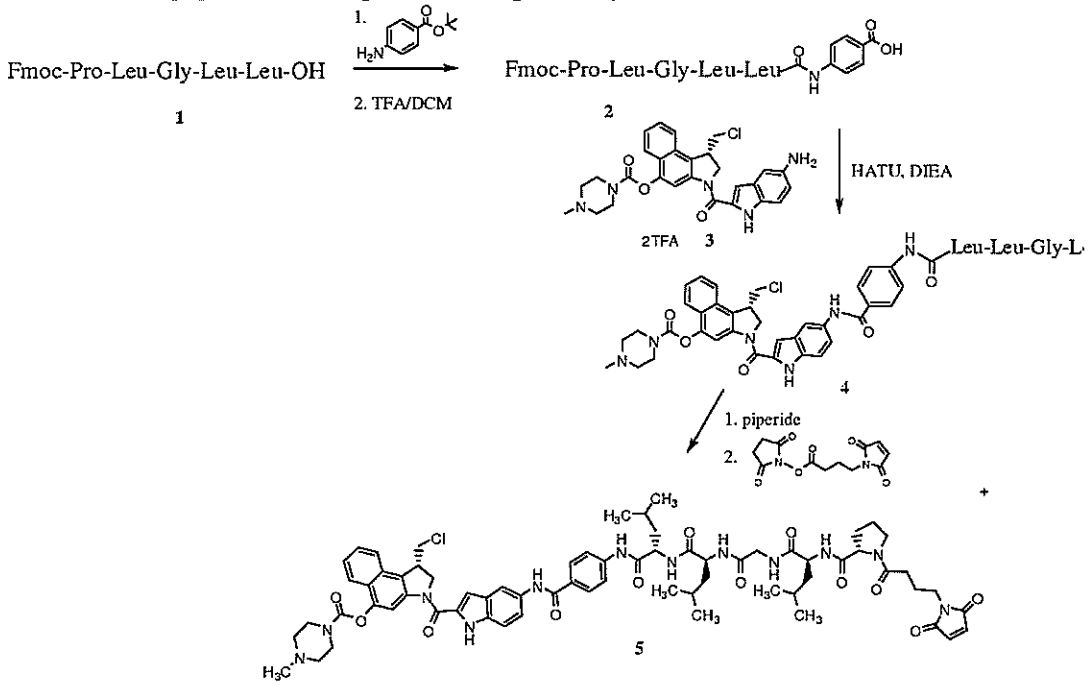
Synthesis of Compound 2: To a solution of Fmoc-Leu-Ala-Leu-OH (200 mg, 0.37 mmole) and ter-butyl-4-amino benzoate (114 mg, 0.59 mmole) in solution of 5% DMF in DCM (5 mL) was added HATU (224 mg, 0.59 mmole) followed by adding diisoproopylethylamine (410 uL, 2.36 mmole) at room temperature. The reaction mixture was stirred for 30 minutes. The solvent evaporated and the residue was purified by flash column chromatography on silica gel eluted with 5% methanol in dichloromethane. The resulting product was dissolved in DMC/TFA (1:1, 10 mL) and the mixture was stirred at room temperature for 90 minutes. Solvent was removed in vacuo. Crude product was precipitated with ether to yield **2** (206 mg, 80 %).

Synthesis of Compound 4: To a solution of compound **2**(20.6 mg) in DMF was added HATU (10.3 mg) and the resulting reaction mixture was stirred for 10 minutes at room temperature followed by **3** (20mg) diisoproopylethylamine (23 uL) at room temperature. The reaction mixture was stirred for 60 minutes. Solvent was removed in vacuo. Crude product was directly submitted for purification by semi-preparative HPLC to gain **4** (28 mg, 76%).

Synthesis of Compound 5: Compound **4** (28 mg) was deprotected using 5% piperidine in DMF (1 mL) at room temperature for 10 minutes. The reaction mixture was

concentrated in vacuo and the crude product was rinsed with ether (5 mL x 2) without further purification. The resulting product was reacted with N-succinimidyl-6-maleimido hexanoate (10 mg) in the presence of diisoproopylethylamine (12 uL) and DMF (1 mL) for 3 hours. The reaction mixture was concentrated and purified by semi-preparative HPLC to gain **5** (17 mg). (MS: 1100), found M+1=1101

Example 18: (peptides in example 18 encompassed by SEQ ID NO:15)



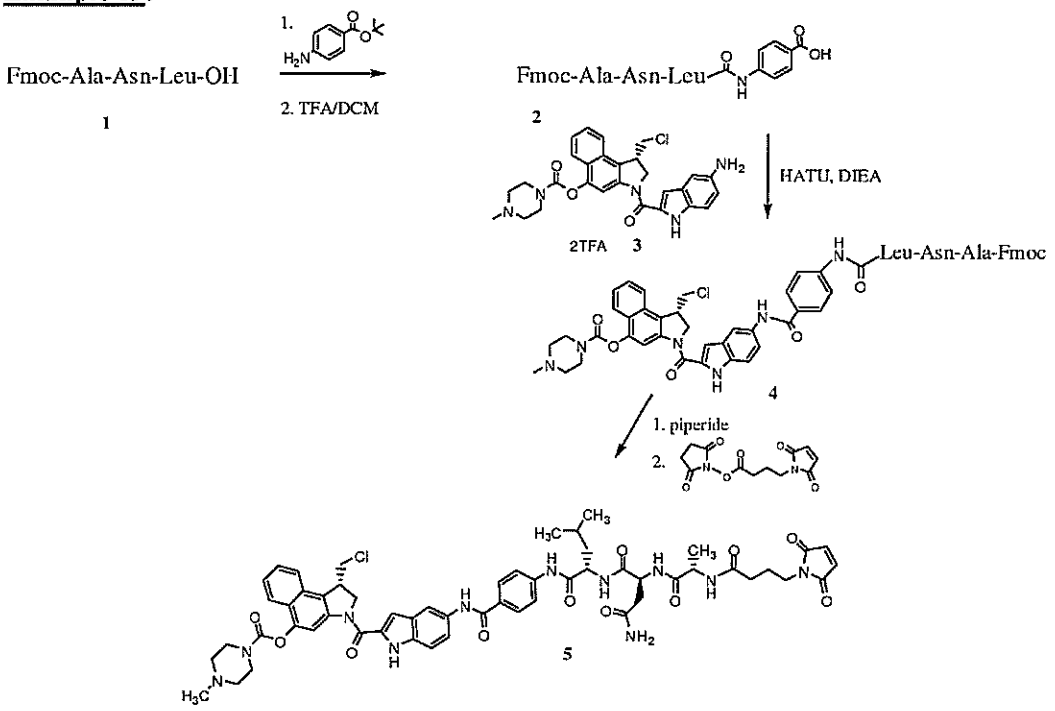
Synthesis of Compound 2: To a solution of Fmoc-Pro-Leu-Gly-Leu-Leu-OH (SEQ ID NO:15) (200 mg, 0.27 mmole) and ter-butyl-4-amino benzoate (114 mg, 0.59 mmole) in solution of 5% DMF in DCM (5 mL) was added HATU (224 mg, 0.59 mmole) followed by adding diisoproopylethylamine (410 uL, 2.36 mmole) at room temperature. The reaction mixture was stirred for 30 minutes. The solvent evaporated and the residue was purified by flash column chromatography on silica gel eluted with 5% methanol in dichloromethane. The resulting product was dissolved in DMC/TFA (1:1, 10 mL) and the mixture was stirred at room temperature for 90 minutes. Solvent was removed under vacuum and the crude product was precipitated with ether to yield **2** (190 mg).

Synthesis of Compound 4: To a solution of compound **2**(42 mg, 0.049 mmol) in DMF was added HATU (20 mg) and the resulting reaction mixture was stirred for 10

minutes at room temperature followed by **3** (40 mg) diisoproopylethylamine (35 uL) at room temperature. The reaction mixture was stirred for 60 minutes. Solvent was removed under vacuum and the crude product was directly submitted for purification by semi-preparative HPLC to gain **4** (33 mg).

Synthesis of Compound 5: Compound **4** (33 mg) was deprotected using 5% piperidine in DMF (1 mL) at room temperature for 10 minutes. The reaction mixture was concentrated under vacuum and the crude product was rinsed with ether (5 mL x 2) without further purification. The resulting product was reacted with N-succinimidyl-6-maleimido hexanoate (10 mg) in the presence of diisoproopylethylamine (12 uL) and DMF (1 mL) for 3 hours. The reaction mixture was concentrated and purified by semi-preparative HPLC to gain **5** (15 mg). (MS: 1296), found M+1=1297

Example 19:



Synthesis of Compound 2: To a solution of Fmoc-Ala-Asn-Leu-OH (200 mg, 0.37 mmole) and ter-butyl-4-amino benzoate (114 mg, 0.59 mmole) in solution of 5% DMF in DCM (5 mL) was added HATU (224 mg, 0.59 mmole) followed by adding diisoproopylethylamine (410 uL, 2.36 mmole) at room temperature. The reaction mixture was stirred for 30 minutes. The solvent evaporated and the residue was purified by flash

column chromatography on silica gel eluted with 5% methanol in dichloromethane. The resulting product was dissolved in DMC/TFA (1:1, 10 mL) and the mixture was stirred at room temperature for 90 minutes. Solvent was removed in vacuo. Crude product was precipitated with ether to yield **2** (190 mg, 72%).

Synthesis of Compound 4: To a solution of compound **2**(20 mg) in DMF was added HATU (13 mg) and the resulting reaction mixture was stirred for 10 minutes at room temperature followed by **3** (25mg) diisoproopylethylamine (22 uL) at room temperature. The reaction mixture was stirred for 60 minutes. Solvent was removed in vacuo. Crude product was directly submitted for purification by semi-preparative HPLC to gain **4** (21 mg, 60%).

Synthesis of Compound 5: Compound **4** (23 mg) was deprotected using 5% piperidine in DMF (1 mL) at room temperature for 10 minutes. The reaction mixture was concentrated in vacuo and the crude product was rinsed with ether (5 mL x 2) without further purification. The resulting product was reacted with N-succinimidyl-6-maleimido hexanoate (8 mg) in the presence of diisoproopylethylamine (12 uL) and DMF (1 mL) for 3 hours. The reaction mixture was concentrated and purified by semi-preparative HPLC to gain **5** (10 mg). (MS: 1101), found M+1=1102.

EXAMPLE 20: Conjugation of Drug-Linker Molecules to Antibodies

For hydrazone linkers: This example describes reaction conditions and methodologies for conjugating a drug-linker molecule of the invention (optionally including other groups, such as spacers, reactive functional groups and the like) to an antibody as a targeting agent, X^4. The conditions and methodologies are intended to be exemplary only and non-limiting. Other approaches for conjugating drug-linker molecules to antibodies are known in the art.

The conjugation method described herein is based on introduction of free thiol groups to the antibody through reaction of lysines of the antibody with 2-iminothiolane, followed by reaction of the drug-linker molecule with an active maleimide group. Initially the antibody to be conjugated was buffer exchanged into 0.1M phosphate buffer pH 8.0 containing 50mM NaCl, 2mM DTPA, pH 8.0 and concentrated to 5-10 mg/ml. Thiolation was achieved through addition of 2-iminothiolane to the antibody. The amount of 2-iminothiolane to be added was determined in preliminary experiments and varies from antibody to antibody. In the preliminary experiments, a titration of increasing

amounts of 2-iminothiolane was added to the antibody, and following incubation with the antibody for one hour at room temperature, the antibody was desalted into 50mM HEPES buffer pH 6.0 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 resulted in liberation of thiopyridine which was monitored at 324nm. Samples at a protein concentration of 0.5-1.0 mg/ml were used. The absorbance at 280nm was used to accurately determine the concentration of protein in the samples, and then an aliquot of each sample (0.9ml) was incubated with 0.1 ml DTDP (5mM stock solution in ethanol) for 10 minutes at room temperature. Blank samples of buffer alone plus DTDP were also incubated alongside. After 10 minutes, absorbance at 324nm was measured and the number of thiols present quantitated using an extinction coefficient for thiopyridine of $19800M^{-1}$.

Typically a thiolation level of three thiol groups per antibody is desired. For example, with one particular antibody this was achieved through adding a 15 fold molar excess of 2-iminothiolane followed by incubation at room temperature for 1 hour. Antibody to be conjugated was therefore incubated with 2-iminothiolane at the desired molar ratio and then desalted into conjugation buffer (50mM HEPES buffer pH 6.0 containing 5mM glycine, 3% Glycerol and 2mM DTPA). The thiolated material was maintained on ice whilst the number of thiols introduced was quantitated as described above.

After verification of the number of thiols introduced, the drug-linker molecule containing an active maleimide group was added at a 3-fold molar excess per thiol. The conjugation reaction was carried out in conjugation buffer also containing a final concentration of 5% ethylene glycol dimethyl ether (or a suitable alternative solvent). Commonly, the drug-linker stock solution was dissolved in 90% ethylene glycol dimethyl ether, 10% dimethyl sulfoxide. For addition to antibody, the stock solution was added directly to the thiolated antibody, which has enough ethylene glycol dimethyl ether added to bring the final concentration to 5%, or pre-diluted in conjugation buffer containing a final concentration of 10% ethylene glycol dimethyl ether, followed by addition to an equal volume of thiolated antibody.

The conjugation reaction was incubated at room temperature for 2 hours with mixing. Following incubation the reaction mix was centrifuged at 14000 RPM for 15 minutes and the pH was adjusted to 7.2 if purification was not immediate. Purification of

conjugate was achieved through chromatography using a number of methods. Conjugate can be purified using size-exclusion chromatography on a Sephacryl S200 column pre-equilibrated with 50mM HEPES buffer pH 7.2 containing 5mM glycine, 50mM NaCl and 3% glycerol. Chromatography was carried out at a linear flow rate of 28 cm/h. Fractions containing conjugate were collected, pooled and concentrated. Alternatively purification can be achieved through ion-exchange chromatography. Conditions vary from antibody to antibody and need to be optimized in each case. For example, antibody-drug conjugate reaction mix was applied to an SP-Sepharose column pre-equilibrated in 50mM HEPES, 5mM glycine, 3%glycerol, pH 6.0. The antibody conjugate was eluted using a gradient of 0-1M NaCl in equilibration buffer. Fractions containing the conjugate were pooled, the pH was adjusted to 7.2 and the sample concentrated as required.

For peptide linkers: This example describes reaction conditions and methodologies for conjugating a drug-linker molecule of the invention (optionally including other groups, such as spacers, reactive functional groups and the like) to an antibody as a targeting agent, X^4. The conditions and methodologies are intended to be exemplary only and non-limiting. Other approaches for conjugating drug-linker molecules to antibodies are known in the art.

The conjugation method described herein is based on introduction of free thiol groups to the antibody through reaction of lysines of the antibody with 2-iminothiolane, followed by reaction of the drug-linker molecule with an active maleimide group. Initially the antibody to be conjugated was buffer exchanged into 0.1M phosphate buffer pH 8.0 containing 50mM NaCl, 2mM DTPA, pH 8.0 and concentrated to 5-10 mg/ml. Thiolation was achieved through addition of 2-iminothiolane to the antibody. The amount of 2-iminothiolane to be added was determined in preliminary experiments and varies from antibody to antibody. In the preliminary experiments, a titration of increasing amounts of 2-iminothiolane was added to the antibody, and following incubation with the antibody for one hour at room temperature, the antibody was desalted into 50mM HEPES buffer pH 6.0 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 resulted in liberation of thiopyridine which was monitored at 324nm. Samples at a protein concentration of 0.5-1.0 mg/ml were used. The absorbance at 280nm was used to accurately determine the concentration of protein in the samples, and then an aliquot of each sample (0.9ml) was incubated with 0.1 ml DTDP (5mM stock solution in

ethanol) for 10 minutes at room temperature. Blank samples of buffer alone plus DTDP were also incubated alongside. After 10 minutes, absorbance at 324nm was measured and the number of thiols present quantitated using an extinction coefficient for thiopyridine of $19800M^{-1}$.

Typically a thiolation level of three thiol groups per antibody is desired. For example, with one particular antibody this was achieved through adding a 15 fold molar excess of 2-iminothiolane followed by incubation at room temperature for 1 hour. Antibody to be conjugated was therefore incubated with 2-iminothiolane at the desired molar ratio and then desalted into conjugation buffer (50mM HEPES buffer pH 6.0 containing 5mM glycine, 0.5% povidone (10k) and 2mM DTPA). The thiolated material was maintained on ice whilst the number of thiols introduced was quantitated as described above.

After verification of the number of thiols introduced, the drug-linker molecule containing an active maleimide group was added at a 3-fold molar excess per thiol. The conjugation reaction was carried out in conjugation buffer also containing a final concentration of 5% DMSO (or a suitable alternative solvent). Commonly, the drug-linker stock solution was dissolved in 100% dimethyl sulfoxide. For addition to antibody, the stock solution was added directly to the thiolated antibody, which has enough DMSO added to bring the final concentration to 10%, or pre-diluted in conjugation buffer containing a final concentration of 10% DMSO, followed by addition to an equal volume of thiolated antibody.

The conjugation reaction was incubated at room temperature for 2 hours with mixing. Following incubation the reaction mix was centrifuged and filtered through 0.2 micrometer filter. Purification of conjugate was achieved through chromatography using a number of methods. Conjugate can be purified using size-exclusion chromatography on a Sephacryl S200 column pre-equilibrated with 50mM HEPES buffer pH 7.2 containing 5mM glycine, 50mM NaCl and 0.5% povidone (10k). Chromatography was carried out at a linear flow rate of 28 cm/h. Fractions containing conjugate were collected, pooled and concentrated. Alternatively purification can be achieved through ion-exchange chromatography. Conditions vary from antibody to antibody and need to be optimized in each case. For example, antibody-drug conjugate reaction mix was applied to an SP-Sepharose column pre-equilibrated in 50mM HEPES, 5mM glycine, 0.5% povidone (10k), pH 5.5. The antibody conjugate was eluted using a gradient of 0-1M NaCl in

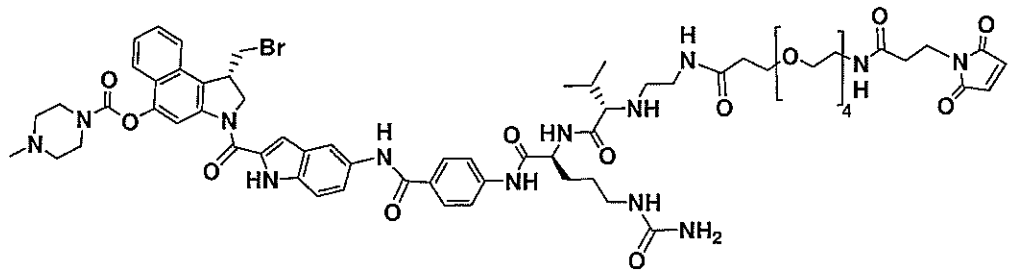
equilibration buffer at pH 5.5. Relevant fractions containing the conjugate were pooled and dialyzed against formulation buffer (with 50mM HEPES buffer pH 7.2 containing 5mM glycine, 100 mM NaCl and 0.5% povidone (10k).

EXAMPLE 21: In Vivo Studies

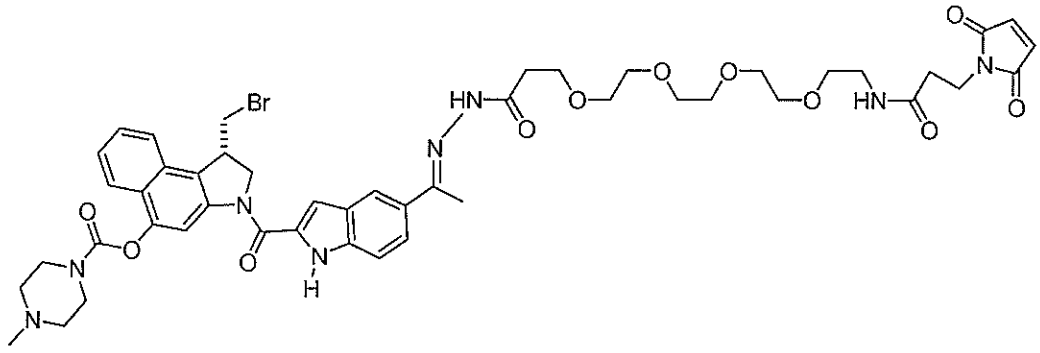
A. Compounds A-G

786-O (ATCC Accession No. CRL-1932) cells were expanded *in vitro* using standard laboratory procedures. Male CB17.SCID mice (Taconic, Hudson, NY) between 6-8 weeks of age were implanted subcutaneously in the right flank with 2.5 million 786-O in 0.2 ml of PBS/Matrigel (1:1) per mouse. Mice were weighed and measured for tumors three dimensionally using an electronic caliper twice weekly after implantation. Tumor volumes were calculated as height x width x length. Mice with tumors averaging 200 mm^3 were randomized into treatment groups. The mice were dosed intraperitoneally with PBS vehicle, cytotoxin-conjugated isotype control antibody or cytotoxin-conjugated anti-CD70 HuMAb 2H5 on Day 0. Each group contained 7 mice.

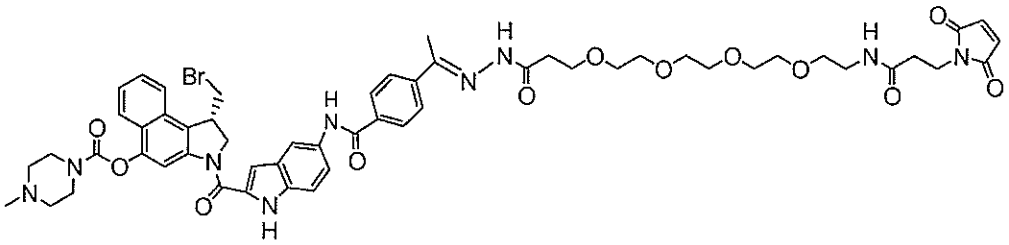
The following cytotoxins were studied:



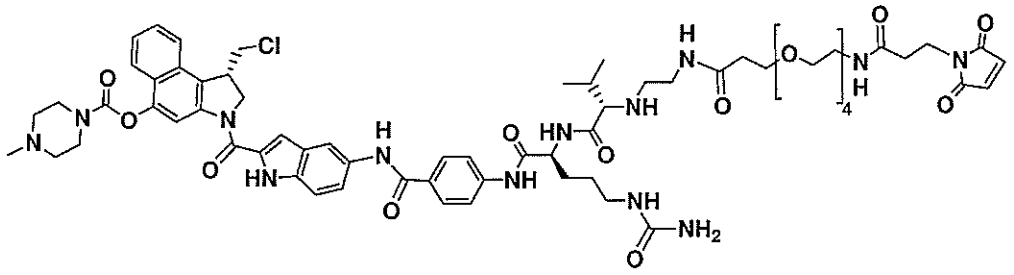
Compound A



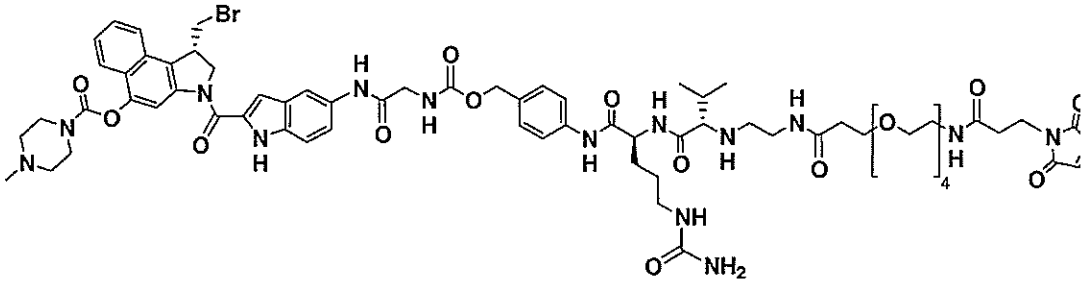
Compound B



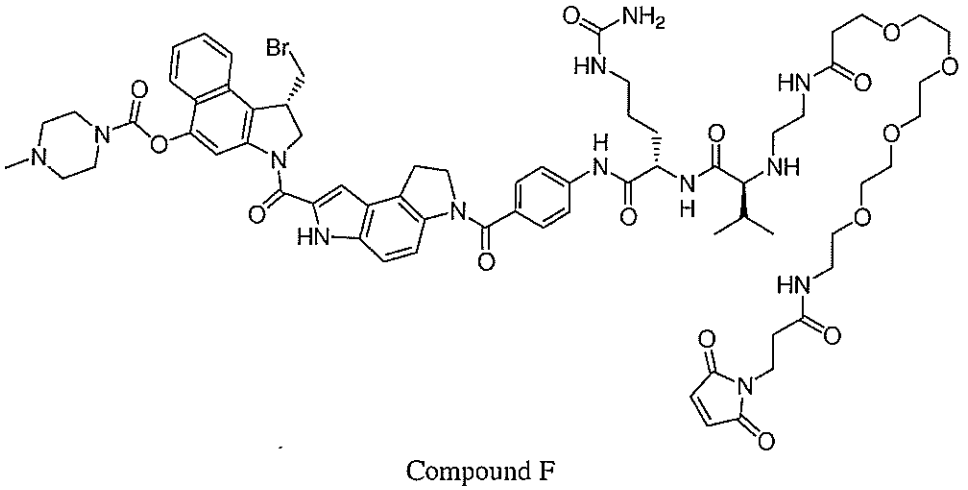
Compound C



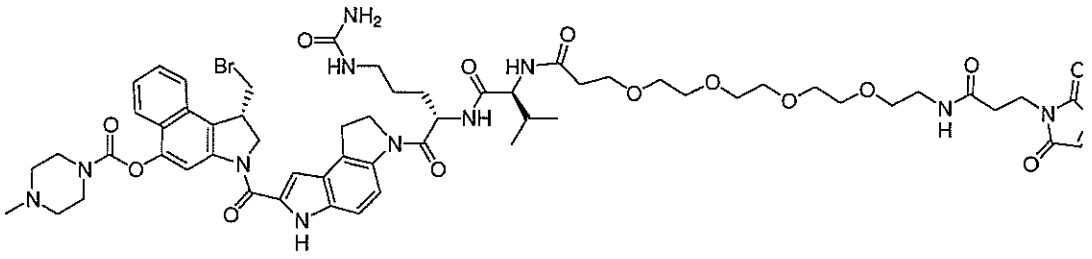
Compound D



Compound E



Compound F



Compound G

Table 1 is a summary of the dosing groups based on mmole of Toxin (Compounds A-G).

Table 1. Study Summary

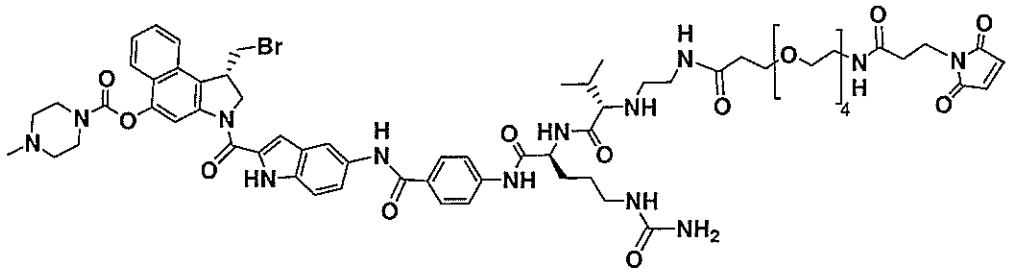
Group		Dose (Toxin μmole)
1	Vehicle IP SD	Matching 0.1 vol
2	CD70.1 0.1 IP SD	0.1
3	CD70.1- Cmpd A IP SD	0.1
4	CD70.1- Cmpd A IP SD	0.01
5	CD70.1- Cmpd B IP SD	0.1
6	CD70.1- Cmpd C IP SD	0.1
7	CD70.1- Cmpd C IP SD	0.01
8	CD70.1- Cmpd D IP SD	0.1
9	CD70.1- Cmpd D IP SD	0.01
10	CD70.1- Cmpd E IP SD	0.1
11	CD70.1- Cmpd F IP SD	0.1
12	CD70.1- Cmpd G IP SD	0.1

Figure 1 shows the mean tumor volume versus days past dosing and Figure 2 shows the median tumor volume versus days past dosing. Efficacy, based on these experiments, appears to be in the following decending order Compound D > Compound A > Compound F > Compound B > Compound C > Compound E > Naked CD70.1/Compound G. Figure 3 shows percent body weight change versus days past dosing.

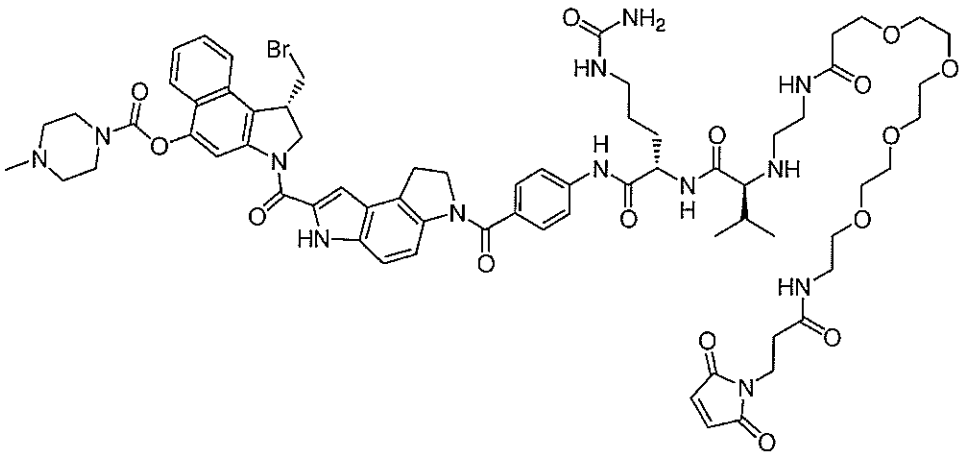
B. Compounds A, F, H-J

786-O (ATCC Accession No. CRL-1932) cells were expanded *in vitro* using standard laboratory procedures. Male CB17.SCID mice (Taconic, Hudson, NY) between 6-8 weeks of age were implanted subcutaneously in the right flank with 2.5 million 786-O in 0.2 ml of PBS/Matrigel (1:1) per mouse. Mice were weighed and measured for tumors three dimensionally using an electronic caliper twice weekly after implantation. Tumor volumes were calculated as height x width x length. Mice with tumors averaging 200 mm^3 were randomized into treatment groups. The mice were dosed intraperitoneally with PBS vehicle, cytotoxin-conjugated isotype control antibody or cytotoxin-conjugated anti-CD70 HuMAb 2H5 on Day 0. Each group contained 8 mice.

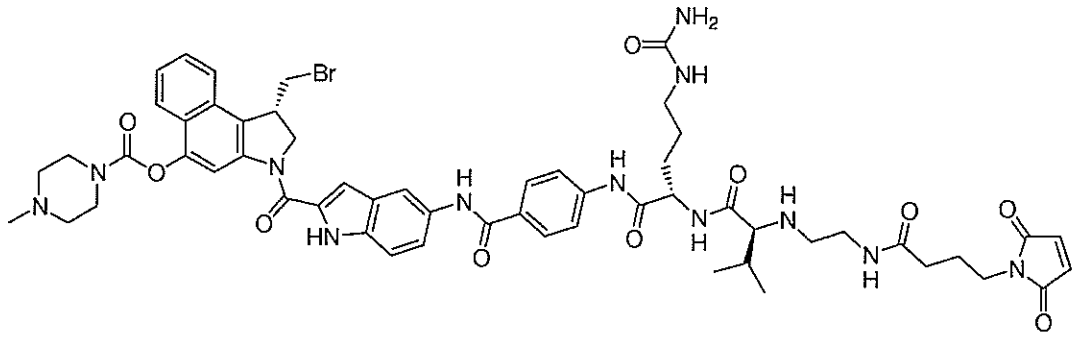
The following cytotoxins were studied:



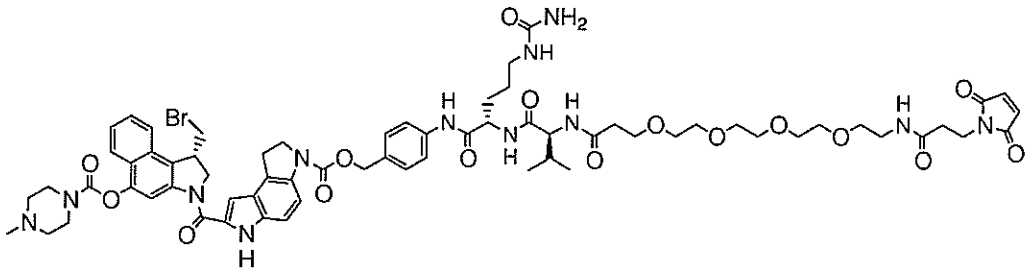
Compound A



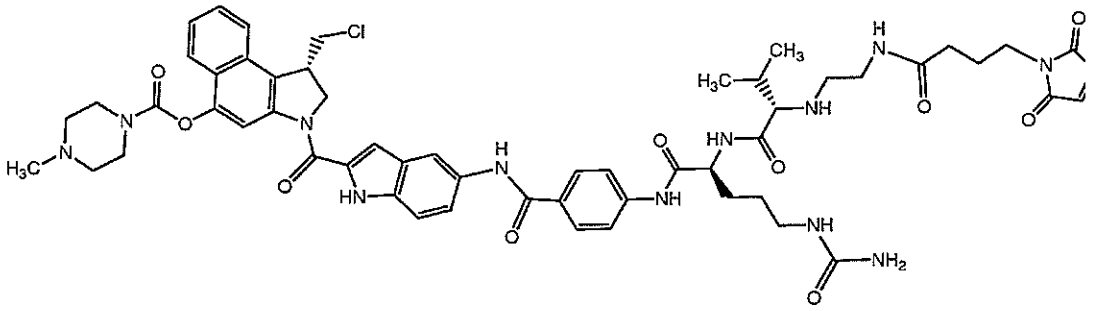
Compound F



Compound H



Compound I



Compound J

Table 2 is a summary of the dosing groups based on mmole of Cytotoxin (Compounds A, F, H-J).

Table 2. Study Summary

Group		Dose (Toxin μmole)
13	Vehicle IP SD	
14	CD70.1 0.1 IP SD	0.03
15	CD70.1- Cmpd A IP SD	0.005
16	CD70.1- Cmpd A IP SD	0.01
17	CD70.1- Cmpd A IP SD	0.03
18	CD70.1- Cmpd H IP SD	0.005
19	CD70.1- Cmpd H IP SD	0.01
20	CD70.1- Cmpd H IP SD	0.03
21	CD70.1- Cmpd I IP SD	0.01
22	CD70.1- Cmpd F IP SD	0.01
23	CD70.1- Cmpd J IP SD	0.005
24	CD70.1- Cmpd J IP SD	0.01
25	CD70.1- Cmpd J IP SD	0.03

Figure 4 shows the mean tumor volume versus days past dosing and Figure 5 shows the median tumor volume versus days past dosing. Efficacy, based on these experiments, appears to be in the following decending order Compound J/Compound C > Compound H > Compound G/Compound I > Naked CD70.1. Figure 6 shows percent body weight change versus days past dosing.

EXAMPLE 22:

Tumor-Activated Activity on LNCaP and 786-O Cells

Adherent cells, LNCaP (prostate carcinoma) and 786-O (renal cell carcinoma), obtained from ATCC, were cultured in RPMI media containing 10% heat inactivated fetal calf serum (FCS) according to ATCC instructions. On the day of the study the cells were detached from the plate with a trypsin solution. The collected cells were washed and resuspended at a concentration of 0.25 or $0.1x10^6$ cells/ml in RPMI containing 10% FCS for LNCaP and 786-0 cells, respectively. 100 µl of cell suspension were added to 96 well plates and the plates were incubated for 3 hours to allow the cells to adhere. Following this incubation, 1:3 serial dilutions of specific antibody-cytotoxin conjugates starting from 300 nM cytotoxin (compound J of Example 21) were added to individual wells. The plates were then incubated for 48 hours, pulsed with 10 µl of a 100 µCi/ml ^{3}H-thymidine and incubated for an additional 72 hours. The plates were harvested using a 96 well Harvester (Packard Instruments) and counted on a Packard Top Count Counter. Four parameter logistic curves were fitted to the ^{3}H-thymidine incorporation as a function of drug molarity using Prism software to determine EC_{50} values. The logistic curves fitted for the various antibody-cytotoxin conjugates and their resulting EC_{50} values, in LNCaP and 786-O cells, respectively, are depicted in Figure 7.

Efficacy Against LNCaP/Prostate Stroma Coculture Tumors in SCID Mice

A LNCaP xenograft study was performed as follows: 120 CB17.SCID mice were each subcutaneously injected with 2 million LNCaP cells and 1 million prostate stroma cells (cat# CC-2508, Cambrex Bio Science Walkersville, Inc, Walkersville, MD) resuspended in 0.2 ml of PBS/Matrigel (1:1) (BD Bioscience) at the flank region. Mice were weighed and measured for tumors three dimensionally using an electronic caliper once weekly after implantation. Tumor volumes were calculated as height x width x length/2. Mice with tumors averaging 50 mm^3 were randomized into 16 treatment groups of seven mice on Day -1 and mice were treated intravenously with vehicle, antibody, or antibody-cytotoxin conjugate according to the dosing regimen described in Table 1 on Day 0. Studies were terminated at Day 62.

Table 3: Dosing of SCID Mice

	Dose (Toxin μmole, Antibody mg/kg)
Vehicle IP SD	
anti-PSMA IP SD	30
anti-RG-1 IP SD	30
anti-EDB IP SD	30
anti-CD70-Toxin IP SD	0.03, 0.1, 0.3
anti-PSMA-Toxin IP SD	0.03, 0.1, 0.3
anti-RG-1-Toxin IP SD	0.03, 0.1, 0.3
anti-EDB-Toxin IP SD	0.03, 0.1, 0.3

Figure 8 depicts the median increase in tumor volume for the seven mice in each of the 16 different groups studied. As indicated in the top left graph, anti-RG-1 and anti-ED-B naked antibodies had no inhibitory effect on tumor growth. Anti-PSMA naked antibody had some anti-tumor growth effect. However, this anti-tumor effect was markedly increased upon conjugation of cytotoxin to the anti-PSMA antibody. Anti-tumor activity similar to that of the conjugated antibody to an internalizing antigen was observed when the previously ineffective antibodies to non-internalizing antigens were conjugated to cytotoxin. These results establish that the anti-tumor activity of cytotoxins can be mediated by antibodies to non-internalizing antigens as well as to internalizing antigens such as PSMA.

Figure 9 depicts the median body weight change for the seven mice in each of the 16 different groups studied. As LNCaP tumors cause cachexia in mice, ineffective treatments (resulting in uncontrolled tumor growth) would be expected to lead to weight loss. This result was observed in mice treated with vehicle or naked antibodies, presumably due to tumor growth. As the mouse models used in this study are immnuno-compromised, they would not be expected to control tumor growth via a robust ADCC response, such as in response to treatment with naked anti-PSMA antibody. In contrast, mice treated with antibody-drug conjugagtes had their lowest body weight right after dosing, indicating that all doses we tested 0.03-0.3 were well tolerated. The fact that the mice in the conjugate groups gained weight points to alleviation of cachexia due to controlled tumor growth.

Efficacy Against LNCaP Tumors in SCID Mice

A LNCaP xenograft study was performed as follows: 120 CB17.SCID mice were each subcutaneously injected with 2.5 million LNCaP cells resuspended in 0.2 ml of PBS/Matrigel (1:1) (BD Bioscience) at the flank region. Mice were weighed and measured for tumors three dimensionally using an electronic caliper once weekly after implantation. Tumor volumes were calculated as height x width x length/2. Mice with tumors averaging 80 mm^3 were randomized into 13 treatment groups of seven mice on Day -1 and mice were treated intravenously with vehicle, antibody, or antibody-cytotoxin conjugate according to the dosing regimen described in Table 2 on Day 0. Studies were terminated at Day 55.

Table 4: Dosing of SCID Mice

	Dose (Toxin μmole, Antibody mg/kg)
Vehicle IP SD	
anti-CD70 IP SD	30
anti-PSMA IP SD	30
anti-RG-1 IP SD	30
anti-CD70-Toxin IP SD	0.03, 0.1, 0.3
anti-PSMA-Toxin IP SD	0.03, 0.1, 0.3
anti-RG-1-Toxin IP SD	0.03, 0.1, 0.3

Figure 10 depicts the mean increase in tumor volume for the eight mice in each of the 13 different groups studied. Similar to the LNCaP/stroma model, anti-RG-1 naked antibody had no inhibitory effect on tumor growth. Anti-PSMA naked antibody had some anti-tumor growth effect. The anti-PSMA antibody's anti-tumor effect was markedly increased upon conjugation of cytotoxin. A similar, but unexpected, anti-tumor activity was observed when non-internalizing anti-RG-1 antibody was conjugated to cytotoxin. These results further confirm that the anti-tumor activity of cytotoxins can be mediated by antibodies to non-internalizing antigens.

Figure 11 depicts the median body weight change for the seven mice in each of the 16 different groups studied. As pointed out above, LNCaP tumors cause cachexia in mice. Again, this cachexia resulted in weight loss in mice treated with vehicle or naked antibodies, presumably due to increased tumor growth and lack of the ability to mount a robust ADCC response to internalized antibodies. In contrast, mice treated with

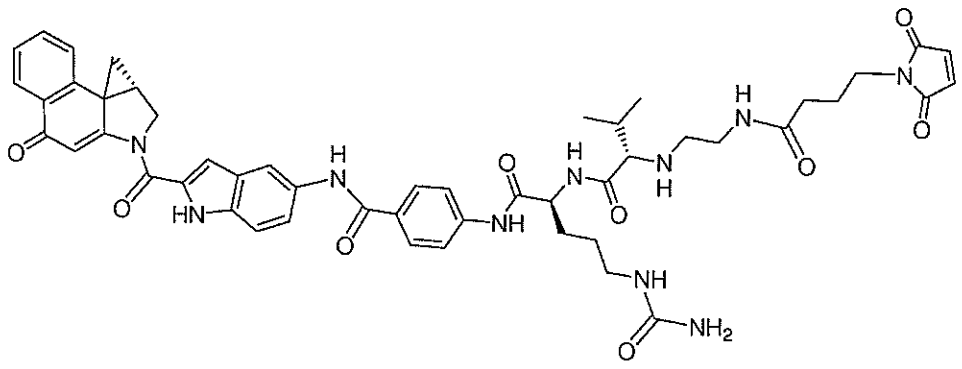
antibody-drug conjugagtes had their lowest body weight right after dosing, indicating that all doses we tested 0.03-0.3 were well tolerated. The fact that the mice in the conjugate groups gained weight points to alleviation of cachexia due to controlled tumor growth.

Efficacy Against Large LNCaP Tumors in SCID Mice

The activity of anti-PSMA-cytotoxin was next demonstrated in SCID mice bearing very large LNCaP cell tumor xenografts. LNCaP cells (2.5 million in 0.1 ml PBS+0.1 ml Matrigel/mouse) were implanted subcutaneously into male SCID mice, and when tumors reached an average size of 310 mm^3, groups of 8 mice were treated by ip injection of a single dose of either anti-PSMA-cytotoxin at either 0.1 or 0.3 μmol/kg body weight. In addition, a control group was injected with vehicle alone. Tumor volumes and weights of mice were recorded throughout the course of the study, which was allowed to proceed for approx. 60 days post dosing. Results, illustrated in Figure 12, demonstrated that a single dose of the anti-PSMA-2460 conjugate was efficacious in mice even when starting with very large tumors.

Example 23. Tumor growth inhibition *in vivo* by anti-CD70-cytotoxin

This example demonstrates the efficacy of anti-CD70-cytotoxin in two xenograft models of kidney cancer. The cytotoxin conjugate is comprised of the CD70 antibody linked to the following cytotoxin compound:



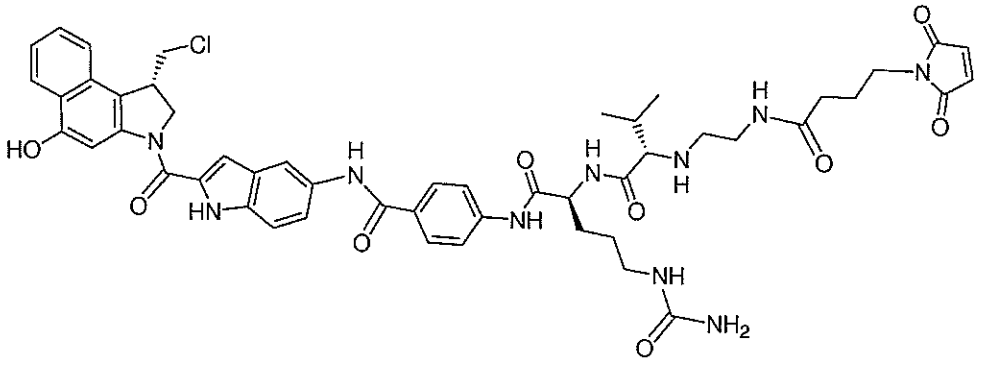
The activity of anti-CD70-cytotoxin was demonstrated in SCID mice bearing A498 tumor xenografts. A498 cells (5 million in 0.1 ml PBS and 0.1 ml Matrigel™/ mouse) were implanted subcutaneously into SCID mice, and when tumors reached an average size of 110 mm3, groups of 8 mice were treated by ip injection of a single dose of either anti-CD70-cytotoxin at 0.1μmol/kg body weight. In addition, a control group was injected with vehicle alone. Tumor volumes (LWH/2) and weights of mice were

recorded throughout the course of the study, which was allowed to proceed for approximately 60 days post dosing. The results are shown in Figure 13. These results indicate that the anti-CD70-cytotoxin conjugate is efficacious against renal cancer.

To demonstrate the activity of anti-CD70-cytotoxin on Caki-1 cell xenografts, 2.5 million Caki-1 cells in 0.1 ml PBS and 0.1 ml Matrigel™ per mouse were implanted subcutaneously into SCID mice, and when tumors reached an average size of 130 mm3, groups of 8 mice were treated by ip injection of a single dose of either anti-CD70-cytotoxin at 0.03, 0.1 or 0.3μmol/kg body weight. In addition, control groups were injected with either vehicle alone, or an isotype control antibody linked to the cytotoxin at doses of 0.1 and 0.3μmol/kg. Tumor volumes (LWH/2) and weights of mice were recorded throughout the course of the study, which was allowed to proceed for 61 days post dosing. The results are shown in Figure 14. These results demonstrate that the anti-CD70 antibody alone and the isotype control conjugates have little effect on the growth of the tumors in this experiment, whereas the anti-CD70-cytotoxin treated mice clearly show dose-dependent anti-tumor efficacy.

Example 24. Tumor growth inhibition *in vivo* by anti-CD70-cytotoxin

This example demonstrates the efficacy of anti-CD70-cytotoxin in two xenograft models of kidney cancer, 786-O cells in SCID mice and Caki-1 cells in nude rats. The cytotoxin conjugate is comprised of the CD70 antibody linked to the following cytotoxin compound:



The activity of anti-CD70-cytotoxin was demonstrated in SCID mice bearing 786-O tumor xenografts. 786-O cells (2.5 million in 0.1 ml PBS and 0.1 ml Matrigel™/ mouse) were implanted subcutaneously into SCID mice, and when tumors reached an average size of 170 mm3, groups of 6 mice were treated by ip injection of a single dose

of anti-CD70-cytotoxin at 0.005μmol/kg body weight. In addition, a control group was injected with vehicle alone. Tumor volumes (LWH/2) and weights of mice were recorded throughout the course of the study. The results are shown in Figure 15. These results demonstrate that the anti-CD70-cytotoxin conjugate is efficacious against renal cancer.

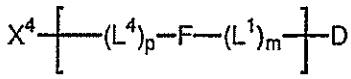
In order to demonstrate that efficacy could be observed in multiple species, a xenograft model in the nude rat was tested. In this model nude rats were implanted subcutaneously with Caki-1 cells (10 million in 0.2 ml RPMI-1640/rat) and when tumors reached an average size of 100 mm3, groups of rats were treated by ip injection of a single dose of either anti-CD70-cytotoxin at 0.3μmol/kg body weight. In addition, control groups were injected with vehicle alone, anti-CD70 antibody alone, or isotype control antibody cytotoxin conjugate at 0.3 μmol/kg body weight as a single dose. Tumor volumes ($LW^2/2$) and weights of rats were recorded throughout the course of the study. The results are shown in Figure 16. These results show that the CD70 antibody alone has little effect on tumor growth, and the isotype control conjugate shows no effect on tumor growth. However, the anti-CD70-cytotoxin conjugate shows a marked anti-tumor effect. Tumor regression was achieved. Therefore, the anti-CD70-cytotoxin conjugate shows an anti-tumor effect in multiple species.

Each of the patent applications, patents, publications, and other published documents mentioned or referred to in this specification is herein incorporated by reference in its entirety, to the same extent as if each individual patent application, patent, publication, and other published document was specifically and individually indicated to be incorporated by reference. The present patent application also incorporates herein by reference U.S. Provisional Patent Application Serial No. 60/882,461.

While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention and the appended claims. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

Claims

1 A compound of the formula

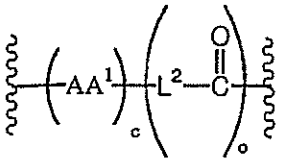


wherein

L^1 is a self-immolative linker;

m is an integer 0, 1, 2, 3, 4, 5, or 6;

F is a linker comprising the structure:



wherein

AA^1 is one or more members independently selected from the group consisting of natural amino acids and unnatural α-amino acids;

c is an integer from 1 to 20;

L^2 is a self-immolative linker;

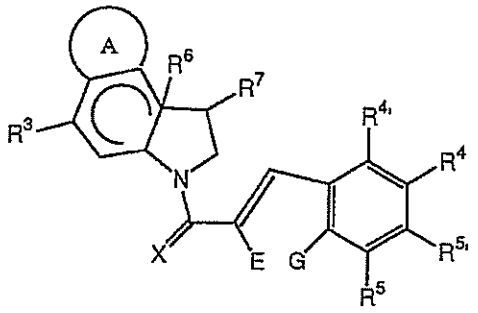
o is 0 or 1;

L^4 is a linker member;

p is 0 or 1;

X^4 is a member selected from the group consisting of protected reactive functional groups, unprotected reactive functional groups, detectable labels, and targeting agents; and

D comprises a structure:



wherein the ring system A is a member selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl groups;

E and G are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, a heteroatom, a single bond, or E and G are joined to form a ring system selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl;

X is a member selected from O, S and NR^{23};

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^3 is a member selected from the group consisting of (=O), SR^{11}, NHR^{11} and OR^{11},

wherein R^{11} is a member selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, monophosphates, diphosphates, triphosphates, sulfonates, acyl, $C(O)R^{12}R^{13}$, $C(O)OR^{12}$, $C(O)NR^{12}R^{13}$, $P(O)(OR^{12})_2$, , $C(O)CHR^{12}R^{13}$, SR^{12} and $SiR^{12}R^{13}R^{14}$,

in which R^{12}, R^{13}, and R^{14} are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl, wherein R^{12} and R^{13} together with the nitrogen or carbon atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

R^4, $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl,

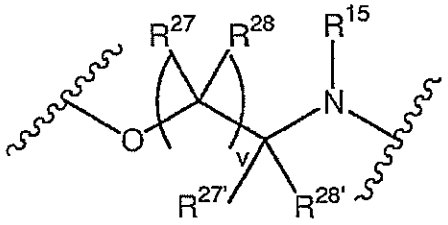
unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, $CR^{15}=NR^{16}$, and $O(CH_2)_nN(CH_3)_2$, or any adjacent pair of R^4, $R^{4'}$, R^5 and $R^{5'}$, together with the carbon atoms to which they are attached, are joined to form a substituted or unsubstituted cycloalkyl or heterocycloalkyl ring system having from 4 to 6 members,

wherein

n is an integer from 1 to 20;

R^{15} and R^{16} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, and substituted or unsubstituted peptidyl, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

wherein at least one of R^4, $R^{4'}$, R^5 and $R^{5'}$ links said drug to L^1, if present, or to F, and comprises



wherein v is an integer from 1 to 6; and

each R^{27}, $R^{27'}$, R^{28}, and $R^{28'}$ is independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, and substituted or unsubstituted heterocycloalkyl;

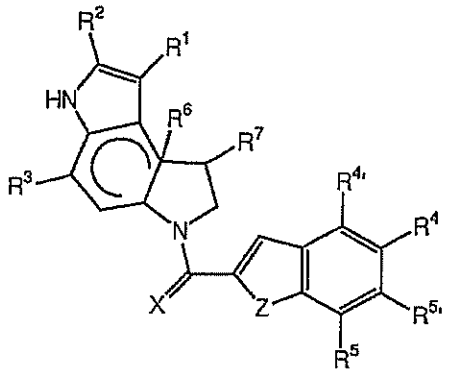
R^6 is a single bond which is either present or absent and when present R^6 and R^7 are joined to form a cyclopropyl ring; and

R^7 is CH_2-X^1 or –CH_2- joined in said cyclopropyl ring with R^6, wherein

X^1 is a leaving group;

or a pharmaceutically acceptable salt thereof.

2. The compound of claim 1, wherein D has the structure:



wherein

Z is a member selected from O, S and NR^{23}

wherein

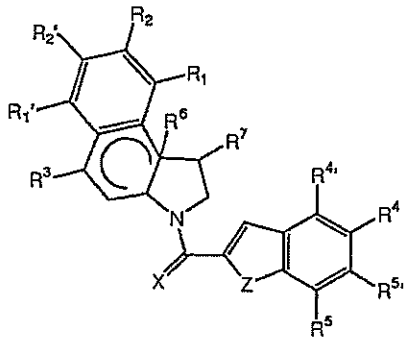
R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^1 is H, substituted or unsubstituted lower alkyl, $C(O)R^8$, or CO_2R^8, wherein R^8 is a member selected from group consisting of substituted alkyl, unsubstituted alkyl, NR^9R^{10}, NR^9NHR^{10}, and OR^9

in which R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl; and

R^2 is H, substituted alkyl or unsubstituted lower alkyl.

3. The compound of claim 1, wherein D has the structure:



wherein

Z is a member selected from O, S and NR^{23}

wherein

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^1 is H, substituted or unsubstituted lower alkyl, $C(O)R^8$, or CO_2R^8, wherein R^8 is a member selected from NR^9R^{10} and OR^9,

in which

R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

$R^{1'}$ is H, substituted or unsubstituted lower alkyl, or $C(O)R^8$, wherein R^8 is a member selected from NR^9R^{10} and OR^9,

in which

R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

R^2 is H, or substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl or cyano or alkoxy; and

$R^{2'}$ is H, or substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl.

4. The compound of claim 1, wherein $(AA^1)_c$ is a peptide sequence cleavable by a protease expressed in tumor tissue.

5. The compound of claim 4, wherein the protease is a lysosomal protease.

6. The compound of claim 1, wherein the amino acid in $(AA^1)_c$ located closest to the drug moiety is selected from the group consisting of: Ala, Asn, Asp, Cit, Cys, Gln, Glu, Gly, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val.

7. The compound of claim 1, . wherein $(AA^1)_c$ is a peptide sequence selected from the group consisting of Val-Cit, Val-Lys, Phe-Lys, Lys-Lys, Ala-Lys, Phe-Cit, Leu-Cit, Ile-Cit, Trp, Cit, Phe-Ala, Phe-N^9-tosyl-Arg, Phe-N^9-nitro-Arg, Phe-Phe-Lys, D-Phe-Phe-Lys, Gly-Phe-Lys, Leu-Ala-Leu, Ile-Ala-Leu, Val-Ala-Val, Ala-Leu-Ala-Leu (SEQ. ID NO: 1), β-Ala-Leu-Ala-Leu (SEQ. ID NO: 2) and Gly-Phe-Leu-Gly (SEQ. ID NO: 3).

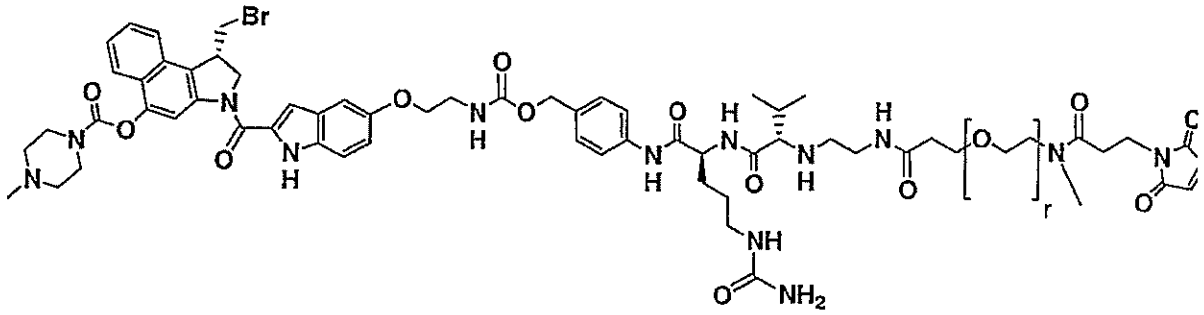
8. The compound of claim 1, wherein $(AA^1)_c$ is Val-Cit or Val-Lys.

9. The compound of claim 1, wherein R^{27}, $R^{27'}$, R^{28}, and $R^{28'}$ are independently H or lower alkyl.

10. The compound of claim 1, wherein v is 1 or 2.

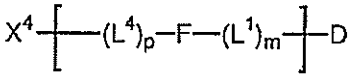
11. The compound of claim 1, wherein R^{15} is H or lower alkyl.

12. The compound of claim 1, wherein the compound is



wherein r is an integer in the range from 0 to 24.

13. A compound of the formula

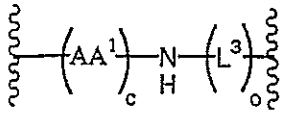


wherein

L^1 is a self-immolative linker;

m is an integer 0, 1, 2, 3, 4, 5, or 6;

F is a linker comprising the structure:



wherein

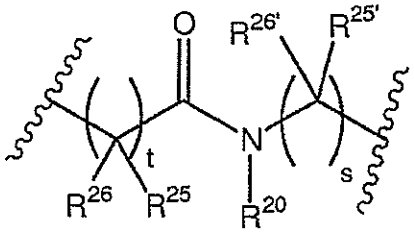
AA^1 is one or more members independently selected from the group consisting of natural amino acids and unnatural α-amino acids;

c is an integer from 1 to 20;

L^3 is a spacer group comprising a primary or secondary amine or a carboxyl functional group; wherein if L^3 is present, m is 0 and either the amine of L^3 forms an amide bond with a pendant carboxyl functional group of D or the carboxyl of L^3 forms an amide bond with a pendant amine functional group of D;

o is 0 or 1;

L^4 is a linker member, wherein L^4 comprises



directly attached to the N-terminus of $(AA^1)_c$, wherein

R^{20} is is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl,

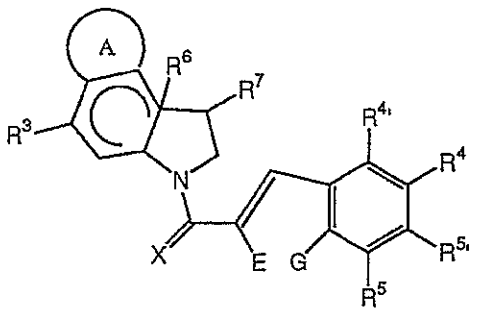
each R^{25}, $R^{25'}$, R^{26}, and $R^{26'}$ is independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, and substituted or unsubstituted heterocycloalkyl;

and s and t are independently integers from 1 to 6;

p is 1;

X^4 is a member selected from the group consisting of protected reactive functional groups, unprotected reactive functional groups, detectable labels, and targeting agents; and

D comprises a structure:



wherein the ring system A is a member selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl groups;

E and G are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, a heteroatom, a single bond, or E and G are joined to form a ring system selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl;

X is a member selected from O, S and NR^{23};

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^3 is a member selected from the group consisting of (=O), SR^{11}, NHR^{11} and OR^{11};

wherein

R^{11} is a member selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, monophosphates, diphosphates, triphosphates, sulfonates, acyl, $C(O)R^{12}R^{13}$, $C(O)OR^{12}$, $C(O)NR^{12}R^{13}$, $P(O)(OR^{12})_2$, , $C(O)CHR^{12}R^{13}$, SR^{12} and $SiR^{12}R^{13}R^{14}$,

in which

R^{12}, R^{13}, and R^{14} are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl, wherein R^{12} and R^{13} together with the nitrogen or carbon atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

R^4 , $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, $CR^{15}{=}NR^{16}$, and $O(CH_2)_nN(CH_3)_2$, or any adjacent pair of R^4, $R^{4'}$, R^5 and $R^{5'}$, together with the

carbon atoms to which they are attached, are joined to form a substituted or unsubstituted cycloalkyl or heterocycloalkyl ring system having from 4 to 6 members,

wherein

n is an integer from 1 to 20;

R^{15} and R^{16} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, and substituted or unsubstituted peptidyl, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

R^6 is a single bond which is either present or absent and when present R^6 and R^7 are joined to form a cyclopropyl ring; and

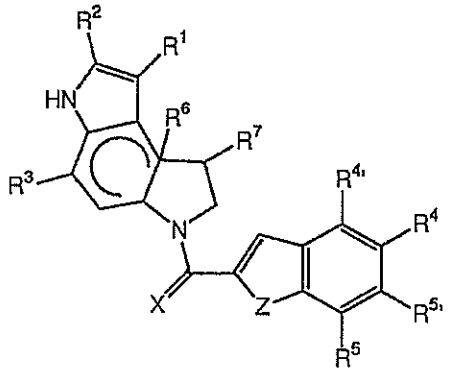
R^7 is CH_2-X^1 or –CH_2- joined in said cyclopropyl ring with R^6, wherein

X^1 is a leaving group,

wherein at least one of R^4, $R^{4'}$, R^5, $R^{5'}$, R^{15} or R^{16} links said drug to L^1, if present, or to F;

or a pharmaceutically acceptable salt thereof.

14. The compound of claim 13, wherein D has the structure:



wherein

Z is a member selected from O, S and NR^{23}

wherein

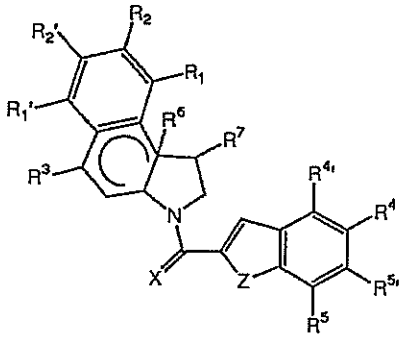
R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^1 is H, substituted or unsubstituted lower alkyl, $C(O)R^8$, or CO_2R^8, wherein R^8 is a member selected from group consisting of substituted alkyl, unsubstituted alkyl, NR^9R^{10}, NR^9NHR^{10}, and OR^9

in which R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl; and

R^2 is H, substituted alkyl or unsubstituted lower alkyl.

15. The compound of claim 13, wherein D has the structure:



wherein

Z is a member selected from O, S and NR^{23}

wherein

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^1 is H, substituted or unsubstituted lower alkyl, $C(O)R^8$, or CO_2R^8, wherein R^8 is a member selected from NR^9R^{10} and OR^9,

in which

R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

$R^{1'}$ is H, substituted or unsubstituted lower alkyl, or $C(O)R^8$, wherein R^8 is a member selected from NR^9R^{10} and OR^9,

in which

R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

R^2 is H, or substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl or cyano or alkoxy; and

$R^{2'}$ is H, or substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl.

16. The compound of claim 13, wherein $(AA^1)_c$ is a peptide sequence cleavable by a protease expressed in tumor tissue.

17. The compound of claim 16, wherein the protease is a lysosomal protease.

18. The compound of claim 13, wherein the amino acid in $(AA^1)_c$ located closest to the drug moiety is selected from the group consisting of: Ala, Asn, Asp, Cit, Cys, Gln, Glu, Gly, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val.

19. The compound of claim 13, wherein $(AA^1)_c$ is a peptide sequence selected from the group consisting of Val-Cit, Val-Lys, Phe-Lys, Lys-Lys, Ala-Lys, Phe-Cit, Leu-Cit, Ile-Cit, Trp, Cit, Phe-Ala, Phe-N^9-tosyl-Arg, Phe-N^9-nitro-Arg, Phe-Phe-Lys, D-Phe-Phe-Lys, Gly-Phe-Lys, Leu-Ala-Leu, Ile-Ala-Leu, Val-Ala-Val, Ala-Leu-Ala-Leu (SEQ. ID NO: 1), β-Ala-Leu-Ala-Leu (SEQ. ID NO: 2) and Gly-Phe-Leu-Gly (SEQ. ID NO: 3).

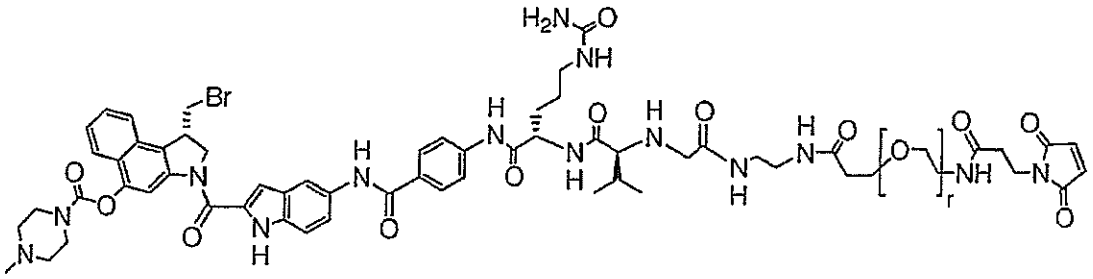
20. The compound of claim 13, wherein $(AA^1)_c$ is Val-Cit or Val-Lys.

21. The compound of claim 13, wherein R^{25}, $R^{25'}$, R^{26}, and $R^{26'}$ are independently H or lower alkyl.

22. The compound of claim 13, wherein s and t are independently 1 or 2.

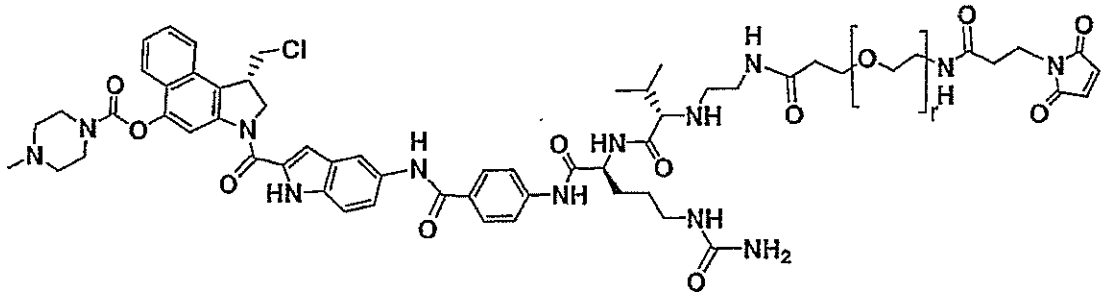
23. The compound of claim 13, wherein R^{20} is H or lower alkyl.

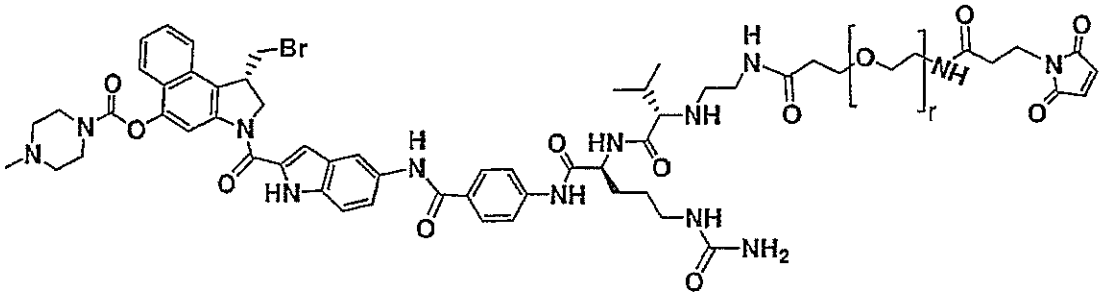
24. The compound of claim 13, wherein the compound is

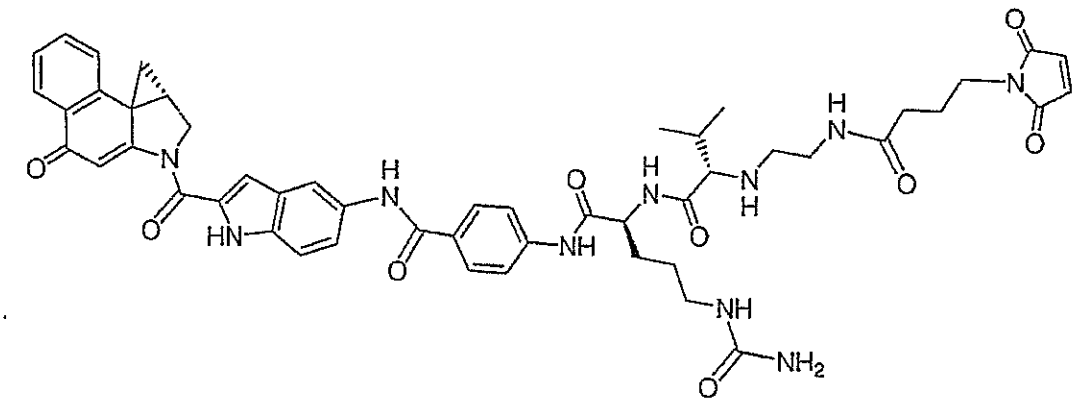


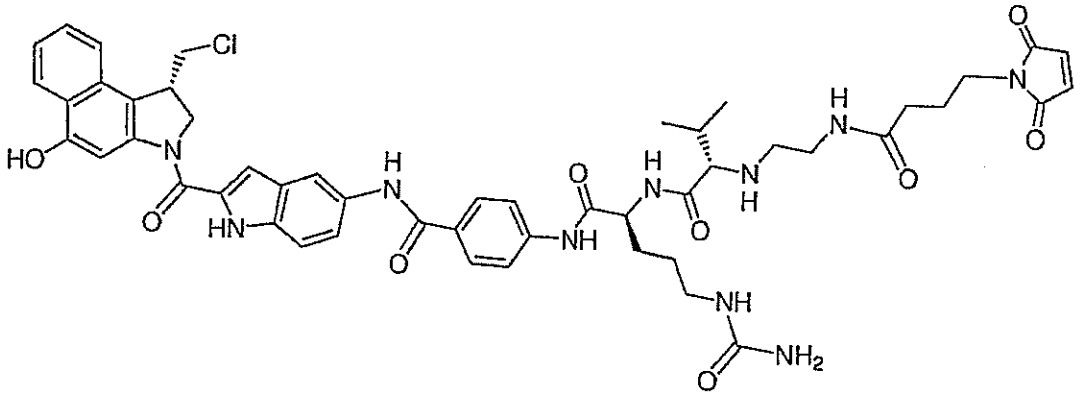
wherein r is an integer in the range from 0 to 24.

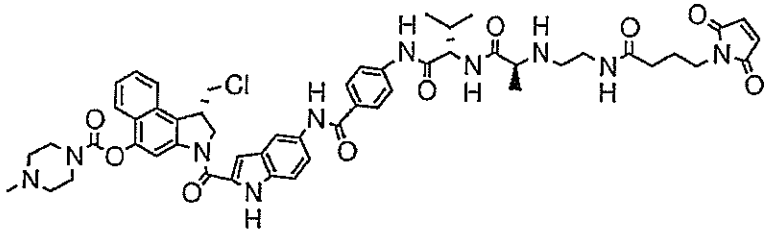
25. The compound of claim 1 or 13 where the compound is selected from:

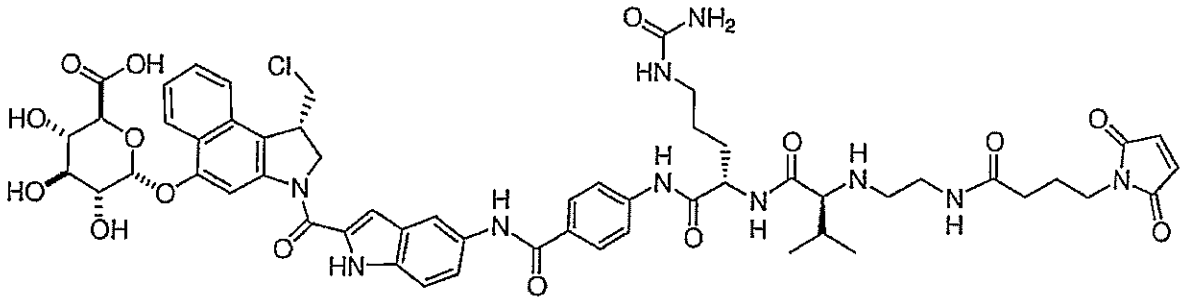


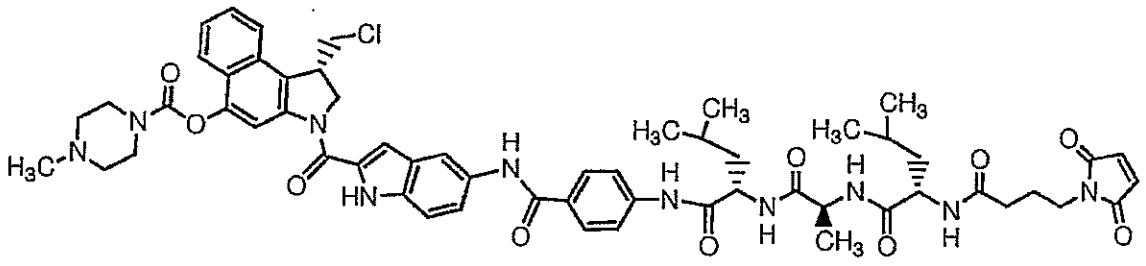


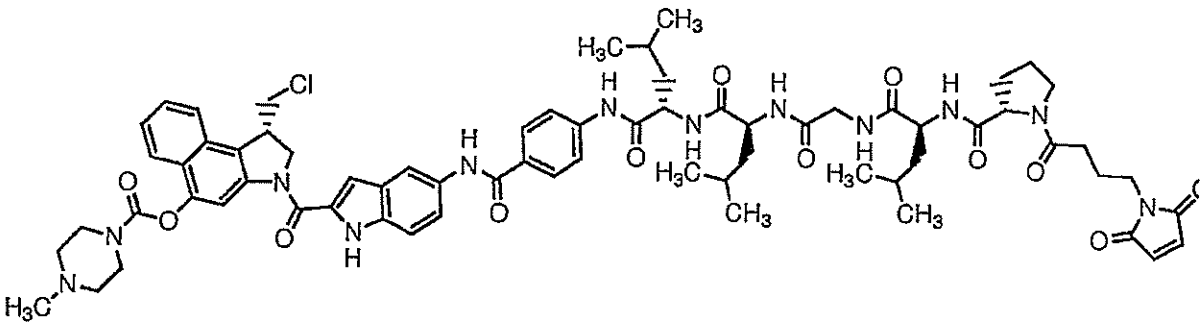


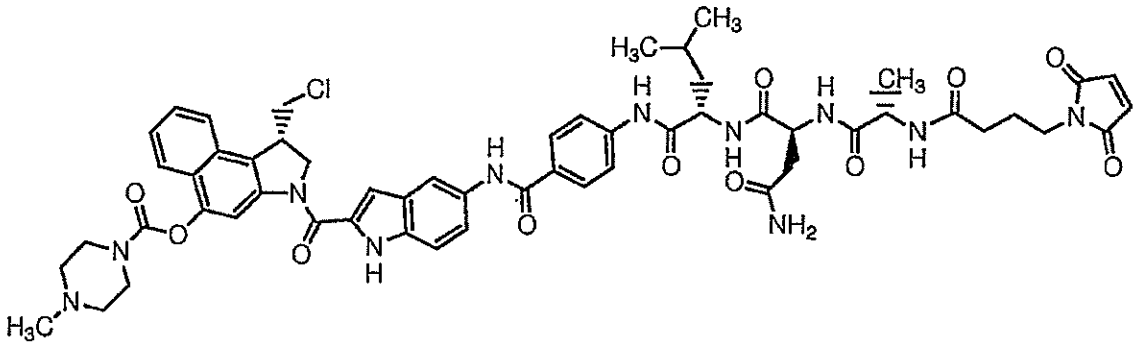


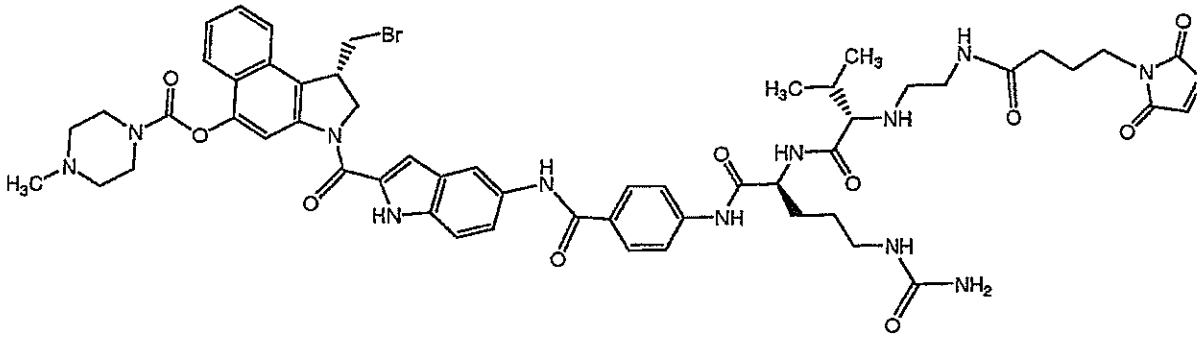




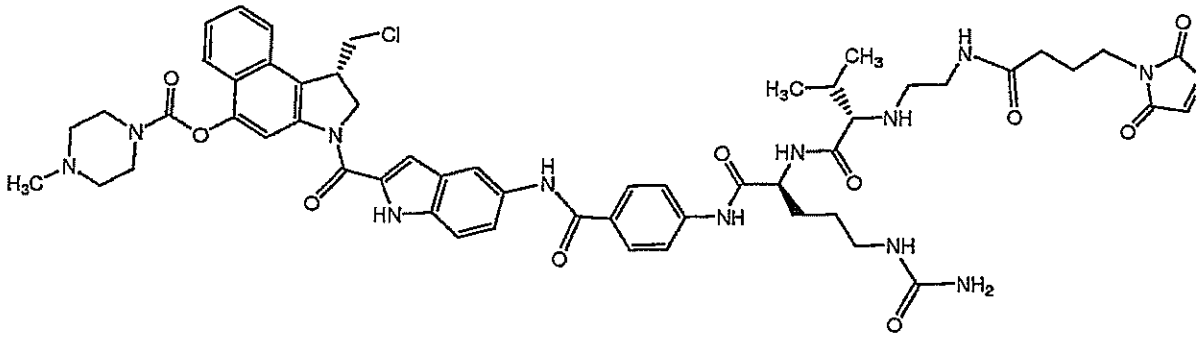


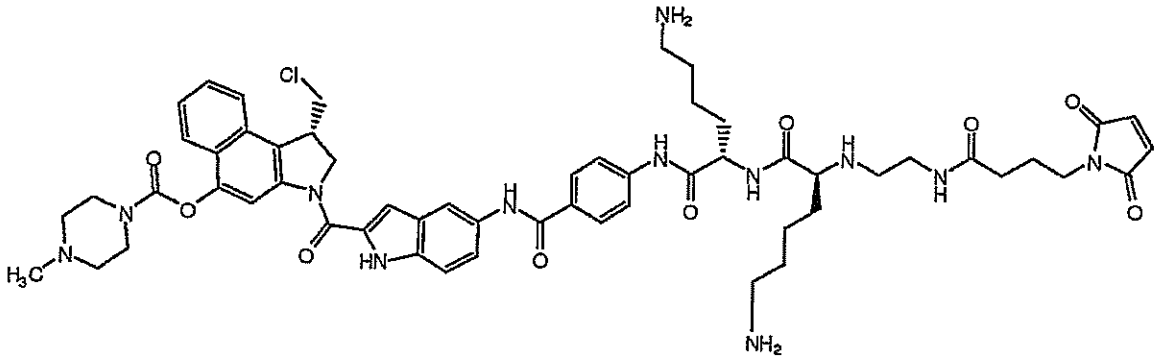


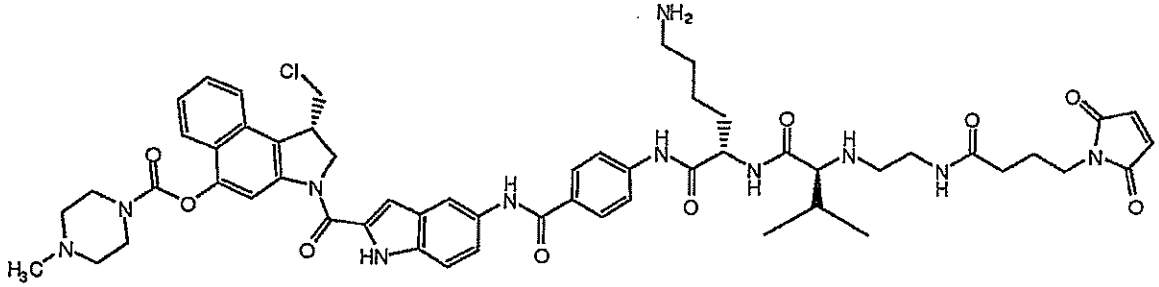


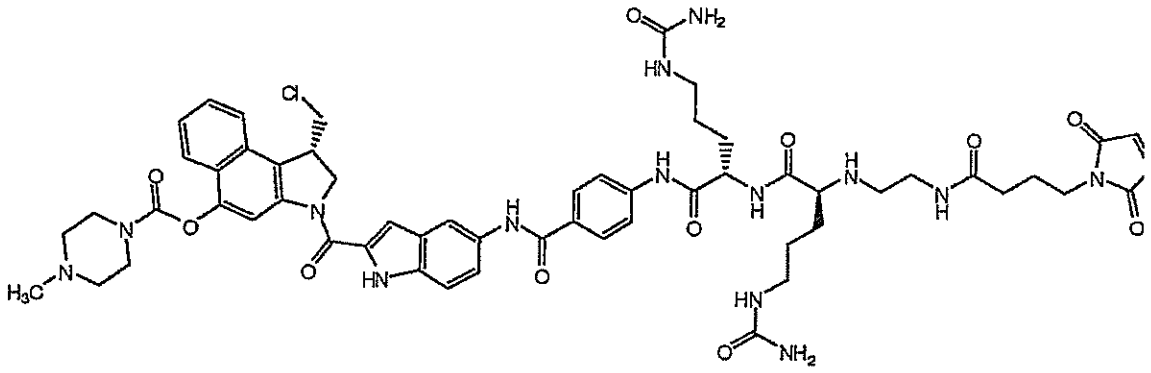


and









wherein r is an integer in the range from 0 to 24.

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