

PEPTIDE-BASED LINKERS

FIELD OF THE INVENTION

The invention relates to novel linkers which comprise two or three basic, acidic or hydrophobic natural or non-natural amino acids. The invention also relates to drug conjugates comprising said linkers, to pharmaceutical compositions comprising said drug conjugates and to the use of said drug conjugates in preventing, suppressing or treating cancer.

BACKGROUND OF THE INVENTION

Cancer treating complexes containing a conjugate of a binding agent (i.e. peptides, antibodies etc.) and a cytotoxic agent have been evaluated for a number of years. The concept involves the binding agent being configured to bind to a target, typically an epitope upon a cancer cell and the presence of the cytotoxic agent is intended to act as a payload to destroy the cancer cell. However, synthesis of these drug conjugates will typically involve the incorporation of a linker between the binding agent and the cytotoxic agent and following administration to a subject this linker is often subjected to premature cleavage, i.e. by other proteases recognising the linker sequence. Such cleavage results in the release of the cytotoxic agent prior to binding to the cancer target and increases the risk of undesirable side effects.

This problem has been attempted to be addressed by a number of research groups. For example, WO 98/19705 describes the presence of a branched peptide linker which contains two or more amino acid moieties that provide an enzyme cleavage site. US 2017/360952 describes a linker having an azide containing non-natural amino acid between a cell-binding agent and a cytotoxic agent. US 2016/046721 describe antibody-drug conjugates comprising a Val-Cit linker. US 2015/087810 describes conjugates of antibodies and toxins with a linker containing from 1 to 20 amino acids.

Thus, there is a need to provide alternative linkers which allow for selective cleavage of the cytotoxic agent at, or close to, the site of binding to the target and result in increased stability of the resultant conjugate.

SUMMARY OF THE INVENTION

According to a first aspect of the invention, there is provided a linker comprising a -P1-P2-P3- moiety, wherein:

P1 represents a basic non-natural amino acid or a derivative thereof;

P2 represents a hydrophobic amino acid or a hydrophobic non-natural amino acid;
and

P3 is either absent or represents an acidic amino acid or an acidic non-natural amino acid, such that when P1 represents Cit and P2 represents Val, then P3 must represent an
5 acidic non-natural amino acid.

According to a further aspect of the invention, there is provided a drug conjugate comprising a binding agent which binds to a target and a cytotoxic agent, wherein said binding agent is joined to said cytotoxic agent via a linker as described herein.

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According to a further aspect of the invention, there is provided a pharmaceutical composition comprising the drug conjugate as described herein in combination with one or more pharmaceutically acceptable excipients.

15 According to a further aspect of the invention, there is provided the drug conjugate as described herein for use in preventing, suppressing or treating cancer.

BRIEF DESCRIPTION OF THE FIGURES

- 20 **Figure 1:** Pharmacokinetic analysis of BCY7761 in mouse plasma.
Figure 2: Pharmacokinetic analysis of BCY10980 in mouse plasma.
Figure 3: Pharmacokinetic analysis of BCY10981 in mouse plasma.
Figure 4: Pharmacokinetic analysis of BCY10989 in mouse plasma.
Figure 5: Pharmacokinetic analysis of BCY10984 in mouse plasma.
Figure 6: Pharmacokinetic analysis of BCY10985 in mouse plasma.
25 **Figure 7:** Pharmacokinetic analysis of BCY10984 in rat plasma.
Figure 8: Pharmacokinetic analysis of BCY7761 in rat plasma.
Figure 9: Tumour reduction efficacy of BCY10984.
Figure 10: Tumour reduction efficacy of BCY7761.
Figures 11 to 15: Toxin Level Analysis of BCY10984 and BCY7761.
30 **Figure 16:** Tumor volume traces after administering BCY10984 and BCY12951 to female BALB/c nude mice bearing HT1080 tumor. Error bars represent standard error of the mean (SEM).
Figure 17: Results from Example 6 showing mice from Group 5 and 6 (dosed with 45 μ M BCY10984) demonstrated potent inhibition of tumor growth.

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DETAILED DESCRIPTION OF THE INVENTION

Linkers

According to a first aspect of the invention, there is provided a linker comprising a -P1-P2-P3- moiety, wherein:

P1 represents a basic non-natural amino acid or a derivative thereof;

5 P2 represents a hydrophobic amino acid or a hydrophobic non-natural amino acid;
and

P3 is either absent or represents an acidic amino acid or an acidic non-natural amino acid, such that when P1 represents Cit and P2 represents Val, then P3 must represent an acidic non-natural amino acid.

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Thus, the invention relates to linker molecules containing 2 or 3 amino acids which require the presence of at least one non-natural amino acid and either a -basic-hydrophobic- motif or a -basic-hydrophobic-acidic- motif.

15 The linker molecules of the invention provide the advantage of increased plasma stability as evidenced with the extended half-lives demonstrated in Example 1 when compared with the Cit-Val control linker. In addition, the linker molecules of the invention provide the ability to tailor the CatB cleavage rate to a required level depending upon the requirements (see Example 2). Furthermore, the linker molecules of the invention provide the ability to
20 modulate the plasma protein binding ability of a bicyclic peptide toxin conjugate as evidenced in Example 3. Furthermore, the linker molecules of the invention demonstrated extended half-life and lower relative levels of free toxin in plasma as evidenced by the pharmacokinetic studies in mouse and rat shown in Example 4. Furthermore, one example linker molecule of the invention (BCY10984) demonstrated higher efficacy in tumour volume
25 reduction compared with the Cit-Val reference bicyclic peptide toxin conjugate (BTC) (see Figures 9 and 10 and Example 5). Furthermore, higher levels of toxin are observed in the tumour with one example linker molecule of the invention (BCY10984) compared with the Cit-Val reference bicyclic peptide toxin conjugate (BTC) (see Figures 11 to 15 and Example 5).

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References herein to "basic non-natural amino acid or a derivative thereof" refer to any amino acid other than the standard, natural 20 amino acids which have a basic character. Within the scope of the term "basic" are non-natural amino acids which contain basic side chains at neutral pH. Such basic non-natural amino acids are typically polar and positively
35 charged at pH values below their pKa's, and are very hydrophilic.

In one embodiment, P1 represents a basic non-natural amino acid selected from: 2-amino-4-guanidinobutanoic acid (Agb); 2-amino-4-(3-methylguanidino)butanoic acid (Agb(Me)); 2,4-diaminobutanoic acid (Dab); 2,3-diaminopropanoic acid (Dap); 2-amino-3-guanidinopropanoic acid (Dap(CNNH₂)); and citrulline (Cit). In a further embodiment, P1
5 represents citrulline (Cit).

References herein to the term “hydrophobic amino acid or a hydrophobic non-natural amino acid” include any amino acid, including both the standard, natural 20 amino acids and any non-natural amino acids which have a hydrophobic character. Within the scope of the term
10 “hydrophobic” are both natural amino acids and non-natural amino acids which contain hydrophobic side chains, i.e. those which do not like to reside in an aqueous (i.e. water) environment.

In one embodiment, P2 represents a hydrophobic amino acid selected from Ala, Gly, Ile,
15 Leu, Met, Phe, Pro, Trp and Val or a hydrophobic non-natural amino acid selected from cyclobutyl, diphenylalanine (Dpa), 1-naphthylalanine (1Nal), 2-Naphthylalanine (2Nal) and methyltryptophan (Trp(Me)), such as a hydrophobic amino acid selected from Val or a non-natural amino acid selected from cyclobutyl, Dpa, 1Nal and 2Nal. In a further embodiment, P2 represents 1-naphthylalanine (1Nal).

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References herein to the term “acidic amino acid or an acidic non-natural amino acid” include any amino acid, including both the standard, natural 20 amino acids and any non-natural amino acids which have an acidic character. Within the scope of the term “acidic” are both natural amino acids and non-natural amino acids which contain acidic side chains at
25 neutral pH. Typically, their side chains have carboxylic acid groups whose pKa's are low enough to lose protons, becoming negatively charged in the process.

In one embodiment, P3 is absent. In an alternative embodiment, P3 represents an acidic amino acid selected from Asp and Glu. In a further embodiment, P3 represents Glu.

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In one embodiment, the -P1-P2-P3- moiety represents:

P1	P2	P3	Compound Numbers containing said linkers
Agb	Val	Absent	BCY9423
Agb(Me)	Val	Absent	BCY9477

Dab	Val	Absent	BCY9474, BCY10989
Dap	Val	Absent	BCY10298
Dap(CNNH ₂)	Val	Absent	BCY10300
Agb	Val	Glu	BCY9695, BCY10983
Cit	1Nal	Glu	BCY10984
Dab	cBu	Glu	BCY10988
Dab	Dpa	Glu	BCY10987
Dab	1Nal	Glu	BCY10985
Dab	2Nal	Glu	BCY10986
Dab	Val	Glu	BCY10122, BCY10981
Dap	Val	Glu	BCY10297, BCY10982
Dap (CNNH ₂)	Val	Glu	BCY10299

In a further embodiment, the -P1-P2-P3- moiety represents: Cit-1Nal-Glu (BCY10984).

Drug Conjugates

- 5 According to a further aspect of the invention, there is provided a drug conjugate comprising a binding agent which binds to a target and a cytotoxic agent, wherein said binding agent is joined to said cytotoxic agent via a linker as defined herein.

- 10 In one embodiment, said binding agent is a peptide, such as an antibody or bicyclic peptide, in particular a bicyclic peptide.

Bicyclic Peptides

- It will be apparent to the skilled person that peptides and antibodies are recognised terms in the art, however, references herein to bicyclic peptides (or Bicycles) are intended to refer to a peptide sequence which have two loops via cyclisation at three reactive amino acid groups (i.e. cysteine residues). These bicyclic peptides were identified in 2009 by phage display-based combinatorial approaches to generate and screen large libraries of bicyclic peptides to targets of interest (Heinis *et al.* (2009), Nat Chem Biol 5 (7), 502-7 and WO 2009/098450). Desirably, the bicyclic peptide will be configured to bind to an anti-cancer target. Suitable examples of cancer cell binding bicyclic peptides include those described in WO 2016/067035 (MT1-MMP binding bicyclic peptides), WO 2017/191460 (MT1-MMP binding bicyclic peptides), WO 2019/025811 (CD137 binding bicyclic peptides), PCT/GB2018/053675 (EphA2 binding bicyclic peptides), PCT/GB2018/053676 (EphA2 binding bicyclic peptides), PCT/GB2018/053678 (EphA2 binding bicyclic peptides), PCT/GB2019/050485 (CD137

binding bicyclic peptides), PCT/GB2019/051740 (Nectin-4 binding bicyclic peptides) and PCT/GB2019/051741 (Nectin-4 binding bicyclic peptides) the bicyclic peptides disclosed in said documents being incorporated herein by reference.

- 5 A bicyclic peptide, as referred to herein, refers to a peptide covalently bound to a molecular scaffold. Typically, such peptides comprise two or more reactive groups (i.e. cysteine residues) which are capable of forming covalent bonds to the scaffold, and a sequence subtended between said reactive groups which is referred to as the loop sequence, since it forms a loop when the peptide is bound to the scaffold. In the present case, the peptides
10 comprise at least three cysteine residues and form at least two loops on the scaffold.

Molecular Scaffolds

- In one embodiment, the bicyclic peptide is covalently bound to a non-aromatic molecular scaffold. References herein to the term “non-aromatic molecular scaffold” refer to any
15 molecular scaffold as defined herein which does not contain an aromatic (i.e. unsaturated) carbocyclic or heterocyclic ring system.

- Suitable examples of non-aromatic molecular scaffolds are described in *Heinis et al (2014) Angewandte Chemie, International Edition 53(6) 1602-1606*.
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As noted in the foregoing documents, the molecular scaffold may be a small molecule, such as a small organic molecule.

- In one embodiment the molecular scaffold may be a macromolecule. In one embodiment
25 the molecular scaffold is a macromolecule composed of amino acids, nucleotides or carbohydrates.

- In one embodiment the molecular scaffold comprises reactive groups that are capable of reacting with functional group(s) of the polypeptide to form covalent bonds.
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- The molecular scaffold may comprise chemical groups which form the linkage with a peptide, such as amines, thiols, alcohols, ketones, aldehydes, nitriles, carboxylic acids, esters, alkenes, alkynes, azides, anhydrides, succinimides, maleimides, alkyl halides and acyl halides.
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An example of an $\alpha\beta$ unsaturated carbonyl containing compound is 1,1',1''-(1,3,5-triazinane-1,3,5-triyl)triprop-2-en-1-one (TATA) (Angewandte Chemie, International Edition (2014), 53(6), 1602-1606).

5 In an alternative embodiment, the bicyclic peptide is covalently bound to an aromatic molecular scaffold. References herein to the term "aromatic molecular scaffold" refer to any molecular scaffold as defined herein which contains an aromatic carbocyclic or heterocyclic ring system.

10 It will be appreciated that the aromatic molecular scaffold may comprise an aromatic moiety. Examples of suitable aromatic moieties within the aromatic scaffold include biphenylene, terphenylene, naphthalene or anthracene.

It will also be appreciated that the aromatic molecular scaffold may comprise a
15 heteroaromatic moiety. Examples of suitable heteroaromatic moieties within the aromatic scaffold include pyridine, pyrimidine, pyrrole, furan and thiophene.

It will also be appreciated that the aromatic molecular scaffold may comprise a
halomethylarene moiety, such as a bis(bromomethyl)benzene, a tris(bromomethyl)benzene,
20 a tetra(bromomethyl)benzene or derivatives thereof.

Non-limiting examples of aromatic molecular scaffolds include: bis-, tris-, or
tetra(halomethyl)benzene; bis-, tris-, or tetra(halomethyl)pyridine; bis-, tris-, or
tetra(halomethyl)pyridazine; bis-, tris-, or tetra(halomethyl)pyrimidine; bis-, tris-, or
tetra(halomethyl)pyrazine; bis-, tris-, or tetra(halomethyl)-1,2,3-triazine; bis-, tris-, or tetra-
25 halomethyl)-1,2,4-triazine; bis-, tris-, or tetra(halomethyl)pyrrole, -furan, -thiophene; bis-, tris-,
, or tetra(halomethyl)imidazole, -oxazole, -thiazol; bis-, tris-, or tetra(halomethyl)-3H-
pyrazole, -isooxazole, -isothiazol; bis-, tris-, or tetra(halomethyl)biphenylene; bis-, tris-, or
tetra(halomethyl)terphenylene; 1,8-bis(halomethyl)naphthalene; bis-, tris-, or
tetra(halomethyl)anthracene; and bis-, tris-, or tetra(2-halomethylphenyl)methane.

30 More specific examples of aromatic molecular scaffolds include: 1,2-
bis(halomethyl)benzene; 3,4-bis(halomethyl)pyridine; 3,4-bis(halomethyl)pyridazine; 4,5-
bis(halomethyl)pyrimidine; 4,5-bis(halomethyl)pyrazine; 4,5-bis(halomethyl)-1,2,3-triazine;
5,6-bis(halomethyl)-1,2,4-triazine; 3,4-bis(halomethyl)pyrrole, -furan, -thiophene and other
35 regioisomers; 4,5-bis(halomethyl)imidazole, -oxazole, -thiazol; 4,5-bis(halomethyl)-3H-
pyrazole, -isooxazole, -isothiazol; 2,2'-bis(halomethyl)biphenylene; 2,2''-
bis(halomethyl)terphenylene; 1,8-bis(halomethyl)naphthalene; 1,10-

bis(halomethyl)anthracene; bis(2-halomethylphenyl)methane; 1,2,3-tris(halomethyl)benzene; 2,3,4-tris(halomethyl)pyridine; 2,3,4-tris(halomethyl)pyridazine; 3,4,5-tris(halomethyl)pyrimidine; 4,5,6-tris(halomethyl)-1,2,3-triazine; 2,3,4-tris(halomethyl)pyrrole, -furan, -thiophene; 2,4,5-bis(halomethyl)imidazole, -oxazole, -thiazol; 3,4,5-bis(halomethyl)-
 5 1H-pyrazole, -isooxazole, -isothiazol; 2,4,2'-tris(halomethyl)biphenylene; 2,3',2''-tris(halomethyl)terphenylene; 1,3,8-tris(halomethyl)naphthalene; 1,3,10-tris(halomethyl)anthracene; bis(2-halomethylphenyl)methane; 1,2,4,5-tetra(halomethyl)benzene; 1,2,4,5-tetra(halomethyl)pyridine; 2,4,5,6-tetra(halomethyl)pyrimidine; 2,3,4,5-tetra(halomethyl)pyrrole, -furan, -thiophene; 2,2',6,6'-
 10 tetra(halomethyl)biphenylene; 2,2'',6,6''-tetra(halomethyl) terphenylene; 2,3,5,6-tetra(halomethyl)naphthalene and 2,3,7,8-tetra(halomethyl)anthracene; and bis(2,4-bis(halomethyl)phenyl)methane.

In one embodiment, the molecular scaffold may comprise or may consist of
 15 tris(bromomethyl)benzene, especially 1,3,5-tris(bromomethyl)benzene ('TBMB'), or a derivative thereof.

In one embodiment, the molecular scaffold is 2,4,6-tris(bromomethyl)mesitylene. This molecule is similar to 1,3,5-tris(bromomethyl)benzene but contains three additional methyl
 20 groups attached to the benzene ring. This has the advantage that the additional methyl groups may form further contacts with the polypeptide and hence add additional structural constraint.

The molecular scaffold of the invention contains chemical groups that allow functional
 25 groups of the polypeptide of the encoded library of the invention to form covalent links with the molecular scaffold. Said chemical groups are selected from a wide range of functionalities including amines, thiols, alcohols, ketones, aldehydes, nitriles, carboxylic acids, esters, alkenes, alkynes, anhydrides, succinimides, maleimides, azides, alkyl halides and acyl halides.

30 Scaffold reactive groups that could be used on the molecular scaffold to react with thiol groups of cysteines are alkyl halides (or also named halogenoalkanes or haloalkanes).

Examples include bromomethylbenzene (the scaffold reactive group exemplified by TBMB)
 35 or iodoacetamide. Other scaffold reactive groups that are used to selectively couple compounds to cysteines in proteins are maleimides, $\alpha\beta$ -unsaturated carbonyl containing compounds and α -halomethylcarbonyl containing compounds. Examples of maleimides

which may be used as molecular scaffolds in the invention include: tris-(2-maleimidoethyl)amine, tris-(2-maleimidoethyl)benzene, tris-(maleimido)benzene. An example of an α -halomethylcarbonyl containing compound is N,N',N''-(benzene-1,3,5-triyl)tris(2-bromoacetamide). Selenocysteine is also a natural amino acid which has a similar reactivity to cysteine and can be used for the same reactions. Thus, wherever cysteine is mentioned, it is typically acceptable to substitute selenocysteine unless the context suggests otherwise.

Synthesis

The bicyclic peptides may be manufactured synthetically by standard techniques followed by reaction with a molecular scaffold *in vitro*. When this is performed, standard chemistry may be used. This enables the rapid large scale preparation of soluble material for further downstream experiments or validation. Such methods could be accomplished using conventional chemistry such as that disclosed in Timmerman *et al* (*supra*).

Thus, the invention also relates to manufacture of polypeptides selected as set out herein, wherein the manufacture comprises optional further steps as explained below. In one embodiment, these steps are carried out on the end product polypeptide made by chemical synthesis.

Peptides can also be extended, to incorporate for example another loop and therefore introduce multiple specificities.

To extend the peptide, it may simply be extended chemically at its N-terminus or C-terminus or within the loops using orthogonally protected lysines (and analogues) using standard solid phase or solution phase chemistry. Standard (bio)conjugation techniques may be used to introduce an activated or activatable N- or C-terminus. Alternatively additions may be made by fragment condensation or native chemical ligation e.g. as described in (Dawson *et al.* 1994. Synthesis of Proteins by Native Chemical Ligation. Science 266:776-779), or by enzymes, for example using subtiligase as described in (Chang *et al.* Proc Natl Acad Sci U S A. 1994 Dec 20; 91(26):12544-8 or in Hikari *et al*/ Bioorganic & Medicinal Chemistry Letters Volume 18, Issue 22, 15 November 2008, Pages 6000-6003).

Alternatively, the peptides may be extended or modified by further conjugation through disulphide bonds. This has the additional advantage of allowing the first and second peptide to dissociate from each other once within the reducing environment of the cell. In this case, the molecular scaffold (e.g. TATA) could be added during the chemical synthesis of the first

peptide so as to react with the three cysteine groups; a further cysteine or thiol could then be appended to the N or C-terminus of the first peptide, so that this cysteine or thiol only reacted with a free cysteine or thiol of the second peptide, forming a disulfide –linked bicyclic peptide-peptide conjugate.

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Similar techniques apply equally to the synthesis/coupling of two bicyclic and bispecific macrocycles, potentially creating a tetraspecific molecule.

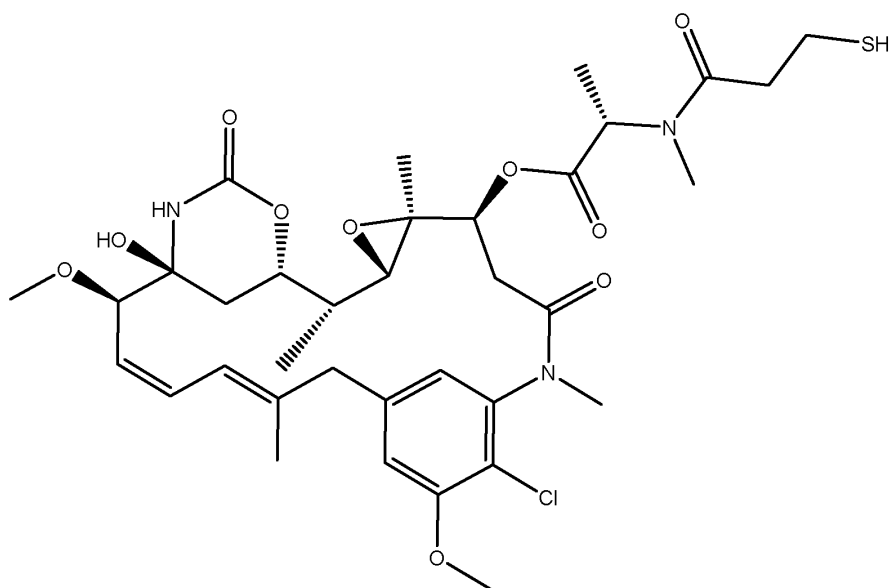
Furthermore, addition of other functional groups or effector groups may be accomplished in the same manner, using appropriate chemistry, coupling at the N- or C-termini or via side chains. In one embodiment, the coupling is conducted in such a manner that it does not block the activity of either entity.

Cytotoxic Agents

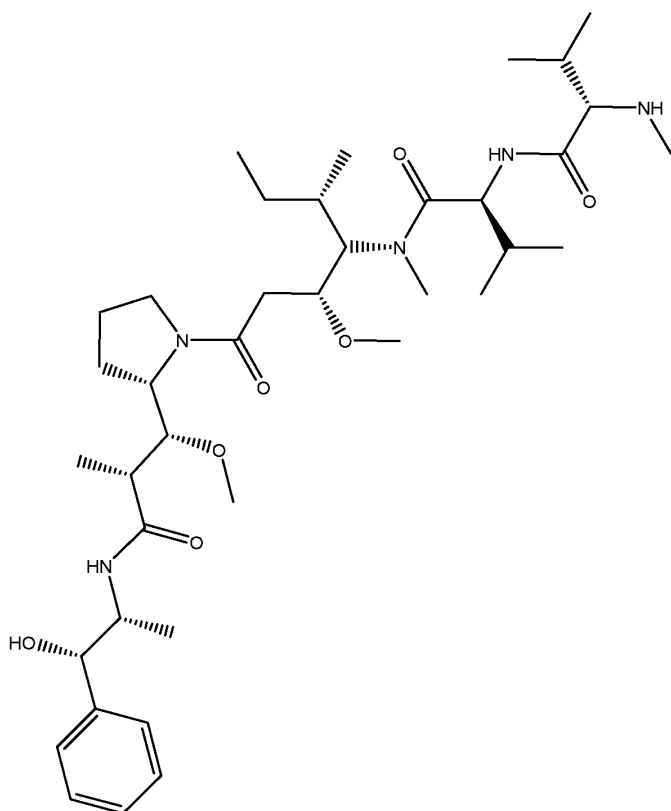
15 Examples of suitable “cytotoxic agents” include: alkylating agents such as cisplatin and carboplatin, as well as oxaliplatin, mechlorethamine, cyclophosphamide, chlorambucil, ifosfamide; Anti-metabolites including purine analogs azathioprine and mercaptopurine or pyrimidine analogs; plant alkaloids and terpenoids including vinca alkaloids such as Vincristine, Vinblastine, Vinorelbine and Vindesine; Podophyllotoxin and its derivatives
20 etoposide and teniposide; Taxanes, including paclitaxel, originally known as Taxol; topoisomerase inhibitors including camptothecins: irinotecan and topotecan, and type II inhibitors including amsacrine, etoposide, etoposide phosphate, and teniposide. Further agents can include antitumour antibiotics which include the immunosuppressant dactinomycin (which is used in kidney transplantations), doxorubicin, epirubicin, bleomycin, calicheamycins,
25 and others.

In one embodiment, said cytotoxic agent is selected from maytansinoids (such as DM1) or monomethyl auristatins (such as MMAE).

30 DM1 is a cytotoxic agent which is a thiol-containing derivative of maytansine and has the following structure:



Monomethyl auristatin E (MMAE) is a synthetic antineoplastic agent and has the following structure:



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In a further embodiment, said cytotoxic agent is MMAE.

Pharmaceutical Compositions

According to a further aspect of the invention, there is provided a pharmaceutical composition comprising the drug conjugate as described herein in combination with one or more pharmaceutically acceptable excipients.

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Generally, the drug conjugates will be utilised in purified form together with pharmacologically appropriate excipients or carriers. Typically, these excipients or carriers include aqueous or alcoholic/aqueous solutions, emulsions or suspensions, including saline and/or buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride and lactated Ringer's. Suitable physiologically-acceptable adjuvants, if necessary to keep a polypeptide complex in suspension, may be chosen from thickeners such as carboxymethylcellulose, polyvinylpyrrolidone, gelatin and alginates.

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Intravenous vehicles include fluid and nutrient replenishers and electrolyte replenishers, such as those based on Ringer's dextrose. Preservatives and other additives, such as antimicrobials, antioxidants, chelating agents and inert gases, may also be present (Mack (1982) Remington's Pharmaceutical Sciences, 16th Edition).

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The drug conjugates of the present invention may be used as separately administered compositions or in conjunction with other agents. These can include antibodies, antibody fragments and various immunotherapeutic drugs, such as cyclosporine, methotrexate, adriamycin or cisplatin and immunotoxins. Pharmaceutical compositions can include "cocktails" of various cytotoxic or other agents in conjunction with the drug conjugates of the present invention, or even combinations of selected drug conjugates according to the present invention having different specificities, such as polypeptides selected using different target ligands, whether or not they are pooled prior to administration.

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The route of administration of pharmaceutical compositions according to the invention may be any of those commonly known to those of ordinary skill in the art. For therapy, the drug conjugates of the invention can be administered to any patient in accordance with standard techniques. The administration can be by any appropriate mode, including parenterally, intravenously, intramuscularly, intraperitoneally, transdermally, via the pulmonary route, or also, appropriately, by direct infusion with a catheter. Preferably, the pharmaceutical compositions according to the invention will be administered by inhalation. The dosage and frequency of administration will depend on the age, sex and condition of the patient,

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concurrent administration of other drugs, counterindications and other parameters to be taken into account by the clinician.

5 The drug conjugates of this invention can be lyophilised for storage and reconstituted in a suitable carrier prior to use. This technique has been shown to be effective and art-known lyophilisation and reconstitution techniques can be employed. It will be appreciated by those skilled in the art that lyophilisation and reconstitution can lead to varying degrees of activity loss and that levels may have to be adjusted upward to compensate.

10 The compositions containing the present drug conjugates or a cocktail thereof can be administered for prophylactic and/or therapeutic treatments. In certain therapeutic applications, an adequate amount to accomplish at least partial inhibition, suppression, modulation, killing, or some other measurable parameter, of a population of selected cells is defined as a "therapeutically-effective dose". Amounts needed to achieve this dosage will
15 depend upon the severity of the disease and the general state of the patient's own immune system, but generally range from 0.005 to 5.0 mg of selected drug conjugate per kilogram of body weight, with doses of 0.05 to 2.0 mg/kg/dose being more commonly used. For prophylactic applications, compositions containing the present drug conjugates or cocktails thereof may also be administered in similar or slightly lower dosages.

20 A composition containing a drug conjugate according to the present invention may be utilised in prophylactic and therapeutic settings to aid in the alteration, inactivation, killing or removal of a select target cell population in a mammal. In addition, the drug conjugates described herein may be used extracorporeally or *in vitro* selectively to kill, deplete or
25 otherwise effectively remove a target cell population from a heterogeneous collection of cells. Blood from a mammal may be combined extracorporeally with the drug conjugates whereby the undesired cells are killed or otherwise removed from the blood for return to the mammal in accordance with standard techniques.

30 ***Therapeutic Uses***

According to a further aspect of the invention, there is provided the drug conjugate as described herein for use in preventing, suppressing or treating cancer.

35 Examples of cancers (and their benign counterparts) which may be treated (or inhibited) include, but are not limited to tumours of epithelial origin (adenomas and carcinomas of various types including adenocarcinomas, squamous carcinomas, transitional cell carcinomas and other carcinomas) such as carcinomas of the bladder and urinary tract,

breast, gastrointestinal tract (including the esophagus, stomach (gastric), small intestine, colon, rectum and anus), liver (hepatocellular carcinoma), gall bladder and biliary system, exocrine pancreas, kidney, lung (for example adenocarcinomas, small cell lung carcinomas, non-small cell lung carcinomas, bronchioalveolar carcinomas and mesotheliomas), head and neck (for example cancers of the tongue, buccal cavity, larynx, pharynx, nasopharynx, tonsil, salivary glands, nasal cavity and paranasal sinuses), ovary, fallopian tubes, peritoneum, vagina, vulva, penis, cervix, myometrium, endometrium, thyroid (for example thyroid follicular carcinoma), adrenal, prostate, skin and adnexae (for example melanoma, basal cell carcinoma, squamous cell carcinoma, keratoacanthoma, dysplastic naevus); haematological malignancies (i.e. leukemias, lymphomas) and premalignant haematological disorders and disorders of borderline malignancy including haematological malignancies and related conditions of lymphoid lineage (for example acute lymphocytic leukemia [ALL], chronic lymphocytic leukemia [CLL], B-cell lymphomas such as diffuse large B-cell lymphoma [DLBCL], follicular lymphoma, Burkitt's lymphoma, mantle cell lymphoma, T-cell lymphomas and leukaemias, natural killer [NK] cell lymphomas, Hodgkin's lymphomas, hairy cell leukaemia, monoclonal gammopathy of uncertain significance, plasmacytoma, multiple myeloma, and post-transplant lymphoproliferative disorders), and haematological malignancies and related conditions of myeloid lineage (for example acute myelogenousleukemia [AML], chronic myelogenousleukemia [CML], chronic myelomonocytic leukemia [CMML], hypereosinophilic syndrome, myeloproliferative disorders such as polycythaemia vera, essential thrombocythaemia and primary myelofibrosis, myeloproliferative syndrome, myelodysplastic syndrome, and promyelocytic leukemia); tumours of mesenchymal origin, for example sarcomas of soft tissue, bone or cartilage such as osteosarcomas, fibrosarcomas, chondrosarcomas, rhabdomyosarcomas, leiomyosarcomas, liposarcomas, angiosarcomas, Kaposi's sarcoma, Ewing's sarcoma, synovial sarcomas, epithelioid sarcomas, gastrointestinal stromal tumours, benign and malignant histiocytomas, and dermatofibrosarcomaprotuberans; tumours of the central or peripheral nervous system (for example astrocytomas, gliomas and glioblastomas, meningiomas, ependymomas, pineal tumours and schwannomas); endocrine tumours (for example pituitary tumours, adrenal tumours, islet cell tumours, parathyroid tumours, carcinoid tumours and medullary carcinoma of the thyroid); ocular and adnexal tumours (for example retinoblastoma); germ cell and trophoblastic tumours (for example teratomas, seminomas, dysgerminomas, hydatidiform moles and choriocarcinomas); and paediatric and embryonal tumours (for example medulloblastoma, neuroblastoma, Wilms tumour, and primitive neuroectodermal tumours); or syndromes, congenital or otherwise, which leave the patient susceptible to malignancy (for example Xeroderma Pigmentosum).

In a further embodiment, the cancer is selected from a hematopoietic malignancy such as selected from: non-Hodgkin's lymphoma (NHL), Burkitt's lymphoma (BL), multiple myeloma (MM), B chronic lymphocytic leukemia (B-CLL), B and T acute lymphocytic leukemia (ALL), T cell lymphoma (TCL), acute myeloid leukemia (AML), hairy cell leukemia (HCL), Hodgkin's
5 Lymphoma (HL), and chronic myeloid leukemia (CML).

References herein to the term "prevention" involves administration of the protective composition prior to the induction of the disease. "Suppression" refers to administration of the composition after an inductive event, but prior to the clinical appearance of the disease.
10 "Treatment" involves administration of the protective composition after disease symptoms become manifest.

Animal model systems which can be used to screen the effectiveness of the drug conjugates in protecting against or treating the disease are available. The use of animal model systems
15 is facilitated by the present invention, which allows the development of drug conjugates which can cross react with human and animal targets, to allow the use of animal models.

The invention is further described below with reference to the following examples.

20 ***Examples***

Materials and Methods

Peptide Synthesis

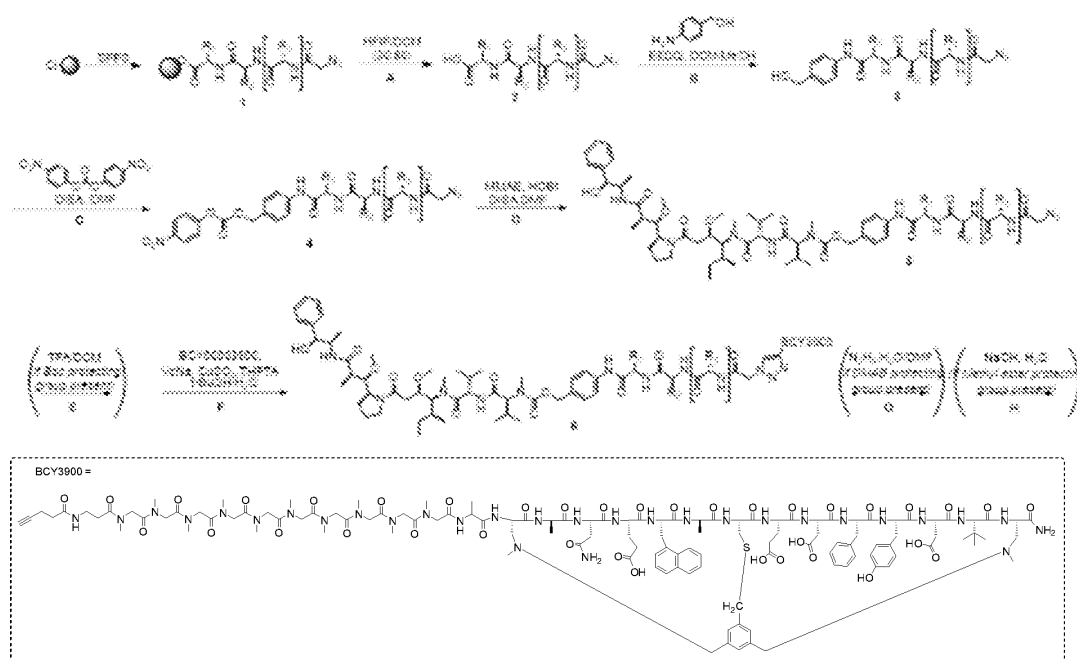
25 Peptide synthesis was based on Fmoc chemistry, using a Symphony peptide synthesiser manufactured by Peptide Instruments and a Syro II synthesiser by MultiSynTech. Standard Fmoc-amino acids were employed (Sigma, Merck), with appropriate side chain protecting groups: where applicable standard coupling conditions were used in each case, followed by deprotection using standard methodology. Peptides were purified using HPLC and following
30 isolation they were modified with 1,3,5-tris(bromomethyl)benzene (TBMB, Sigma). For this, linear peptide was diluted with H₂O up to ~35 mL, ~500 µL of 100 mM TBMB in acetonitrile was added, and the reaction was initiated with 5 mL of 1 M NH₄HCO₃ in H₂O. The reaction was allowed to proceed for ~30 -60 min at RT, and lyophilised once the reaction had completed (judged by MALDI). Following lyophilisation, the modified peptide was purified as
35 above, while replacing the Luna C8 with a Gemini C18 column (Phenomenex), and changing the acid to 0.1% trifluoroacetic acid. Pure fractions containing the correct TMB-modified material were pooled, lyophilised and kept at -20°C for storage.

All amino acids, unless noted otherwise, were used in the L- configurations.

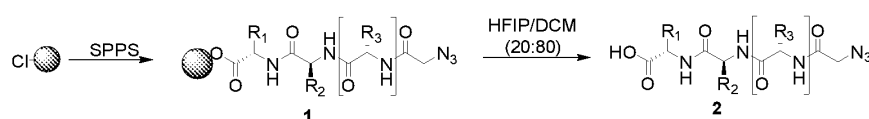
- In some cases peptides are converted to activated disulfides prior to coupling with the free thiol group of a toxin using the following method; a solution of 4-methyl(succinimidyl 4-(2-pyridylthio)pentanoate) (100mM) in dry DMSO (1.25 mol equiv) was added to a solution of peptide (20mM) in dry DMSO (1 mol equiv). The reaction was well mixed and DIPEA (20 mol equiv) was added. The reaction was monitored by LC/MS until complete.

10 Bicycle Conjugate Synthesis

General Method



Step (a): Solid Phase synthesis of peptidic linker compound

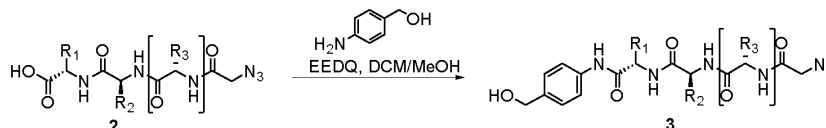


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- Peptides were synthesized on Chlorotrityl resin (2 mmol) using standard Fmoc chemistry. The first amino acid was loaded onto the resin by incubation with a mixture of Fmoc-AA-OH (1 eq) and DIEA (4 eq) in DMF for 2 hours. The resin was drained and washed, then treated with MeOH for 30 min. The rest of the sequence was built up using standard SPPS methods with Fmoc-AA-OH (or acidic capping group e.g. azidoacetic acid) (3 eq), HBTU (2.85 eq) and DIEA (6 eq). Coupling reactions were carried out for 1 hour. Fmoc deprotection was performed using 20% piperidine/DMF for 30 min. The peptides were cleaved from the resin by incubating with

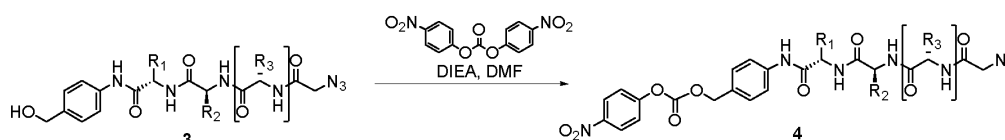
using HFIP/DCM (20:80) for 30 min. The crude peptide was dried and directly used in next step without purification.

Step (b): Addition of (4-aminophenyl)methanol to peptidic linker compound



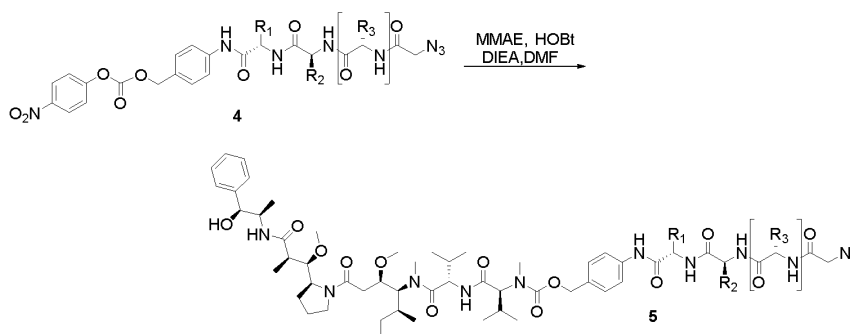
- 5 To a solution of Compound 2 (1.0 eq) in MeOH (100mg/mL) was added a solution of EEDQ (2.0 eq.) and (4-aminophenyl)methanol (2.0 eq) in DCM. The mixture was stirred at 35 °C for 16 hr. Once complete, the reaction mixture was concentrated under reduced pressure and the residue purified by preparative HPLC.

Step (c): Reaction of Bis(2,4-dinitrophenyl)carbonate with peptidic linker-(4-aminophenyl)methanol



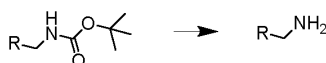
- To a solution of Compound 3 (1.0 eq.) in DMF (50 mg/mL) was added DIEA (5.0 eq.) and bis(4-nitrophenyl) carbonate (4.0 eq.) and the mixture stirred at 25 °C for 1 hr (or until compound 3 was consumed). The reaction mixture was directly purified by preparative HPLC.

Step (d): MMAE conjugation to peptidic linker compounds



- To a solution of Compound 4 (1.5 eq) in DMF (10 mg/mL) was added HOBT (1.5 eq), DIEA (5.0 eq) and MMAE (1.0 eq). The mixture was stirred at 40 °C for 16 hr, until compound 4 was fully consumed. The reaction mixture was directly purified by preparative HPLC.

Step (e): Removal of Boc protecting group

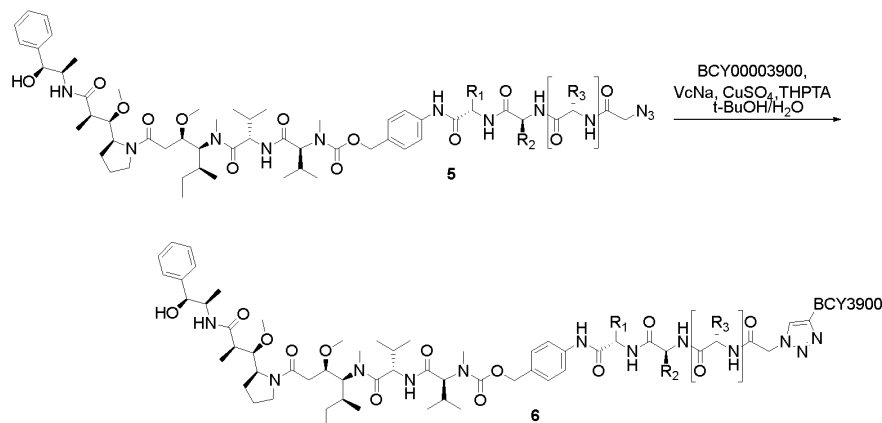


(For linkers synthesised using amino acids with Boc protected side chains).

Boc-protected amine containing linker (1.0 eq) was added to a mixture of 10% TFA/DCM (30 mg/mL). The mixture was stirred at 0 °C for 1 hr, then concentrated under reduced pressure to remove DCM. The crude product was directly used in the next step without purification.

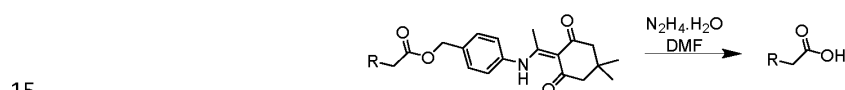
Step (f): Copper catalysed cycloaddition of azide functionalised toxin-linkers to

5 alkyne functionalised Bicycles



To a solution of Compound **5** (1.0 eq) in t-BuOH/H₂O (1:1, 6.5 mg/mL) was added CuSO₄ (0.4 M, 2.0 eq), THPTA (1.0 eq), **BCY3900** (0.9 eq), VcNa (2.0 eq). The mixture was adjusted to pH~7 then stirred at 40 °C for 2 hr (or until consumption of compound **5**). The reaction mixture was concentrated under reduced pressure to remove t-BuOH. If DMAB or Methyl ester protecting group is present in the compound then deprotection was performed on the crude material (general method F or G). Otherwise, the crude residue was purified using preparative HPLC to give the final conjugate.

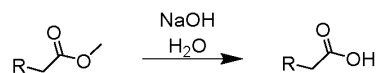
Step (g): Removal of DMAB protecting group



(For linkers synthesised using amino acids with DMAB protected side chains).

To a solution of DMAB protected linker compound (1.0 eq) in DMF (36 mg/mL) was added N₂H₄-H₂O (75 eq). The mixture was stirred at 25 °C for 0.5 hr then the reaction mixture was directly purified by preparative HPLC to give the final conjugate.

20 Step (h): Removal of methyl ester protecting group



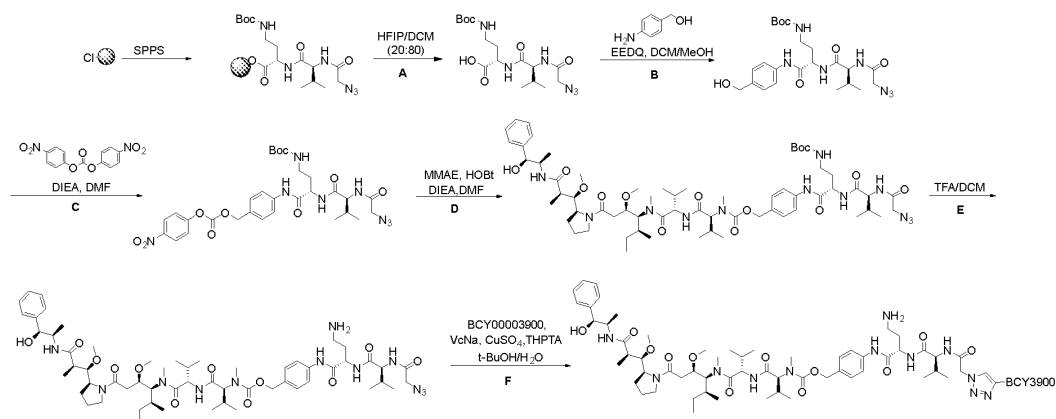
(For linkers synthesised using amino acids with Methyl ester protected side chains).

To a solution of methyl ester protected linker compound (1.0 eq) in H₂O (100 mg/mL) was added NaOH (20.0 eq). The mixture was stirred at 25 °C for 2 hr. The reaction mixture was directly purified by preparative HPLC to give the final conjugate.

The above mentioned General Method was used to prepare the following Bicycle Conjugates:

		Protected amino acids used
BCY10989	MMAE-PAB-(Dab-Val)-BCY3900	Fmoc-Dab(Boc)-OH
BCY10980	<i>MMAE-PAB-(Cit-Val-Glu)-BCY3900</i>	Fmoc-Glu(DMAB)-OH
BCY10982	<i>MMAE-PAB-(Dap-Val-Glu)-BCY3900</i>	Fmoc-Dap(Boc)-OH, Fmoc-Glu(OMe)-OH
BCY10983	<i>MMAE-PAB-(Agp-Val-Glu)-BCY3900</i>	Fmoc-Dap(Boc)-OH Fmoc-Glu(OMe)-OH
BCY10984	MMAE-PAB-(Cit-1Nal-Glu)-BCY3900	Fmoc-Glu(OMe)-OH
BCY10981	<i>MMAE-PAB-(Dab-Val-Glu)-BCY3900</i>	Fmoc-Dab(Boc)-OH Fmoc-Glu(OMe)-OH
BCY10985	MMAE-PAB-(Dab-1Nal-Glu)- BCY3900	Fmoc-Dab(Boc)-OH Fmoc-Glu(OMe)-OH
BCY10986	MMAE-PAB-(Dab-2Nal-Glu)- BCY3900	Fmoc-Dab(Boc)-OH Fmoc-Glu(DMAB)-OH
BCY10987	MMAE-PAB-(Dab-Dpa-Glu)-BCY3900	Fmoc-Dab(Boc)-OH
BCY10988	MMAE-PAB-(Dab-cBu-Glu)-BCY3900	Fmoc-Dab(Boc)-OH Fmoc-Glu(DMAB)-OH

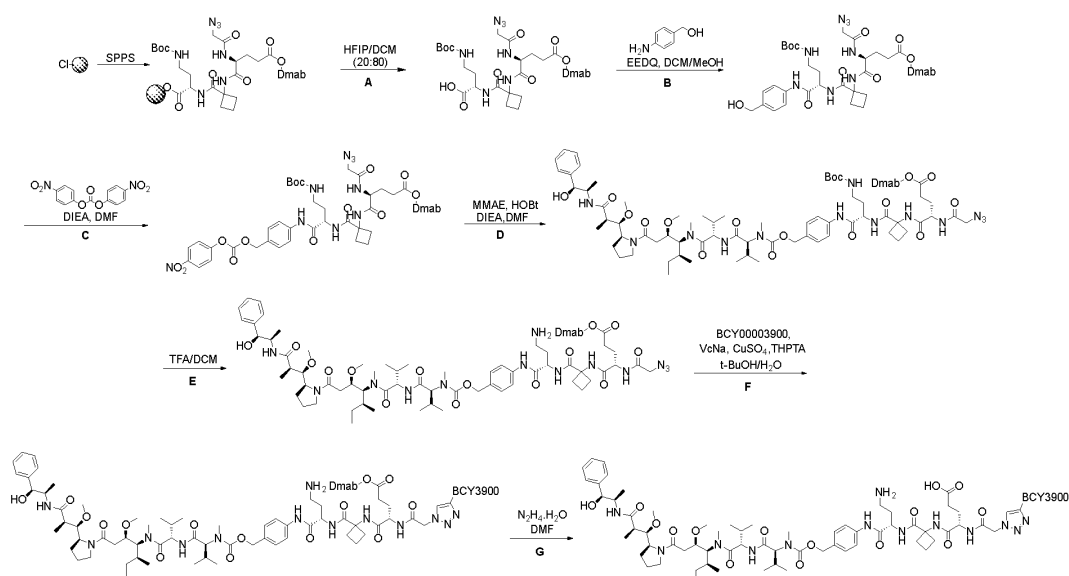
BCY10989 – (Dab-Val)



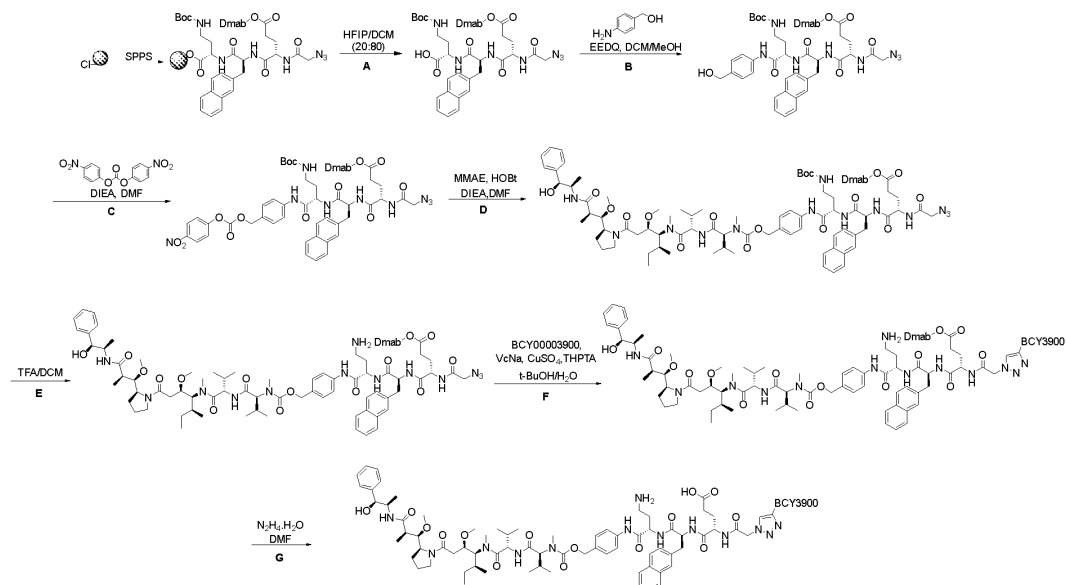
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General methods were followed to yield **BCY10989**. Expected MW = 3882.4, observed m/z: 1294 [M+3H]³⁺, 971 [M+4H]⁴⁺.

BCY10988 – (Dab-cBu-Glu)

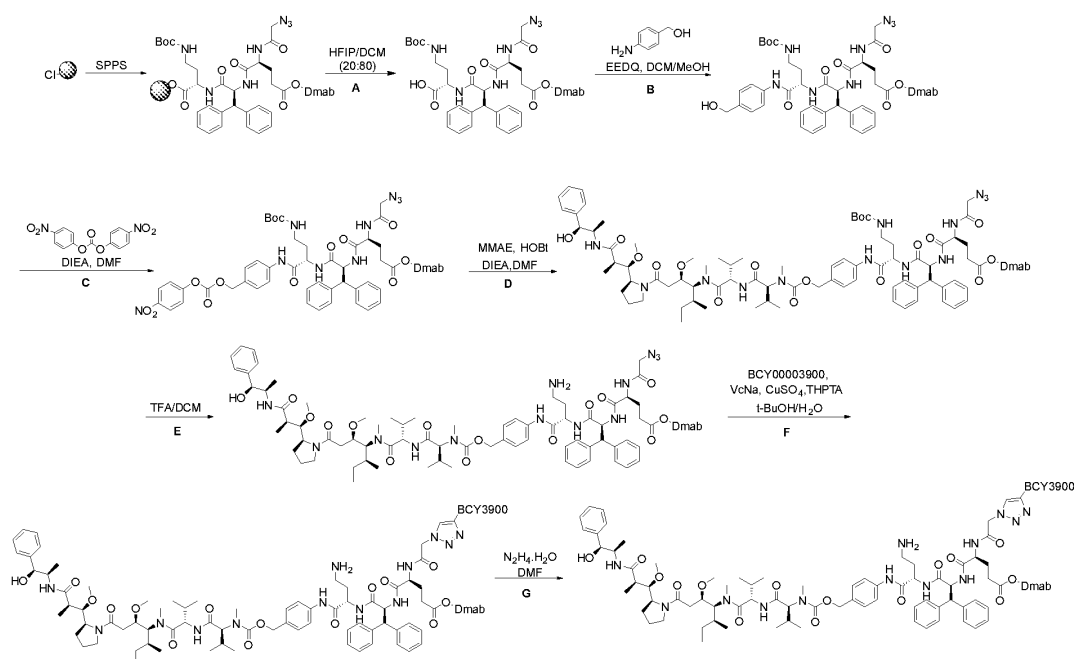


5 BCY10986 (Dab-2Nal-Glu)



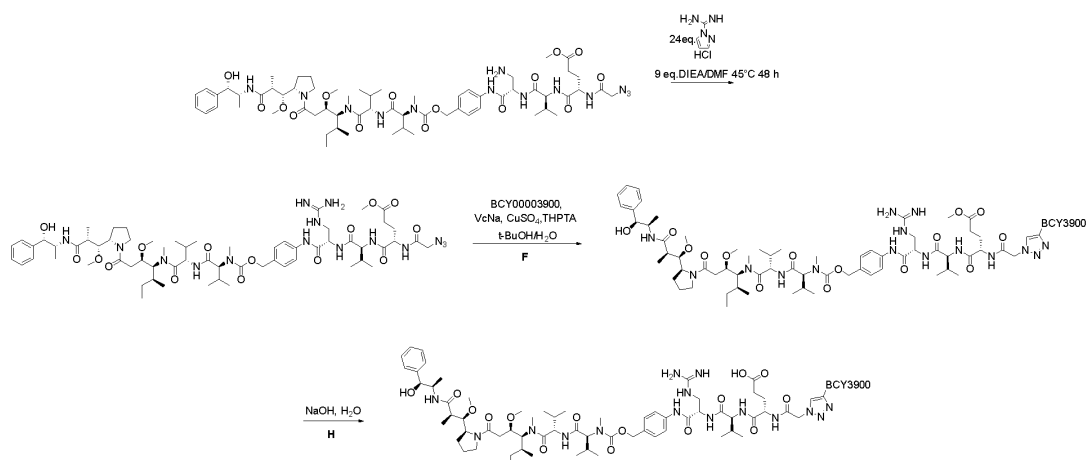
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BCY10987 – (Dab-DPA-Glu)

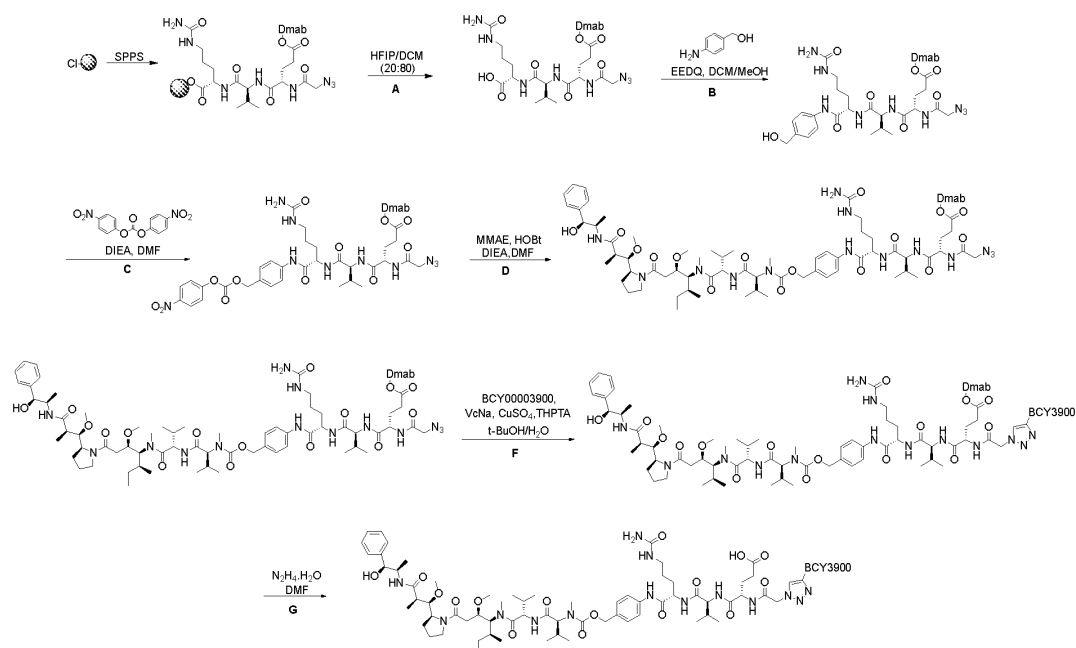


General methods were followed to yield **BCY10987**. Expected MW = 4135.7, observed m/z: 1379 [M+3H]³⁺, 1034 [M+4H]⁴⁺.

5 **BCY10983 – (Agp-Val-Glu)**

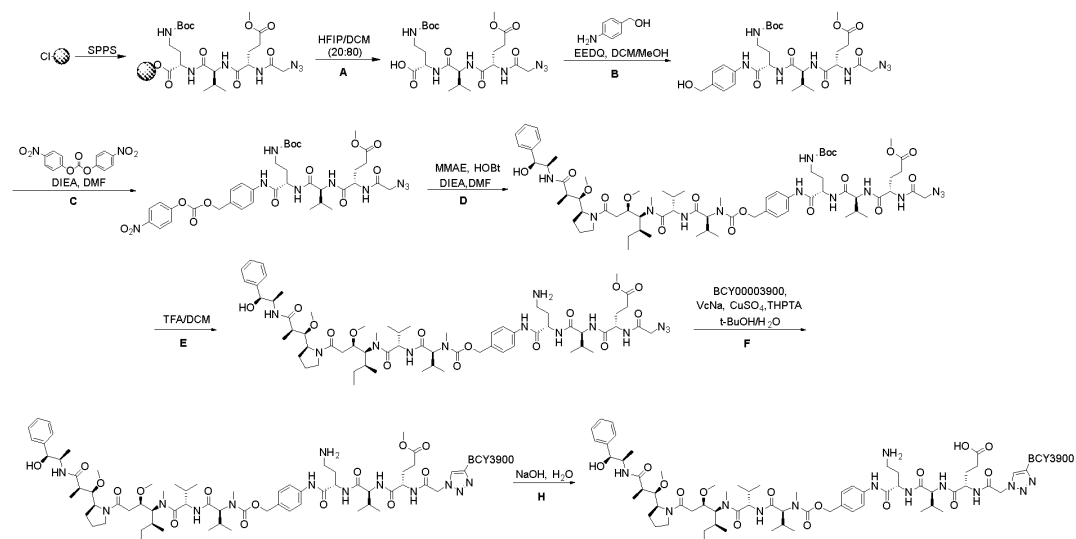


MMAE-PAB-Dap-Val-Glu(OMe)-AcAz intermediate was prepared as described in the synthesis of **BCY10982**. The side chain amine of Dap was then converted to the corresponding guanidine. MMAE-PAB-Dap-Val-Glu(OMe)-AcAz (1eq) was stirred in DMF and to this was added DIEA (9eq) and 1H-Pyrazole-1-carboxamidine hydrochloride (24 eq). The mixture was stirred at 45°C for 24 hours then the mixture diluted and purified by preparative HPLC. The remaining synthetic steps were carried out using general methods to yield **BCY10983**. Expected MW = 4039.5, observed m/z: 1346 [M+3H]³⁺, 1010 [M+4H]⁴⁺.

BCY10980 – (Cit-Val-Glu)

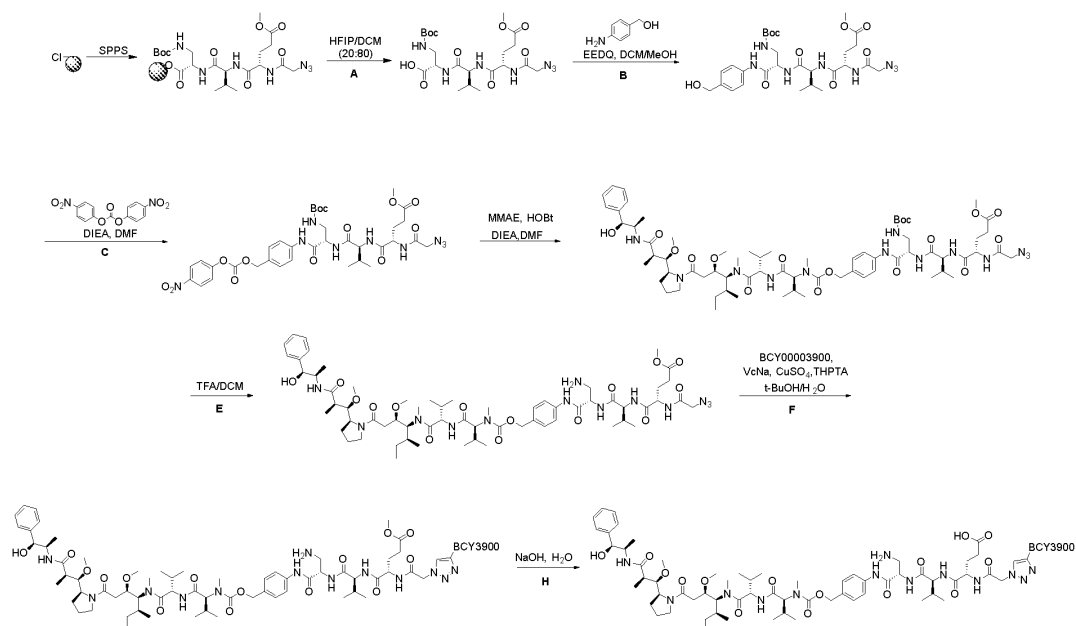
General methods were followed to yield **BCY10980**. Expected MW = 4068.6, observed m/z:

5 1356 [M+3H]³⁺, 1017 [M+4H]⁴⁺.

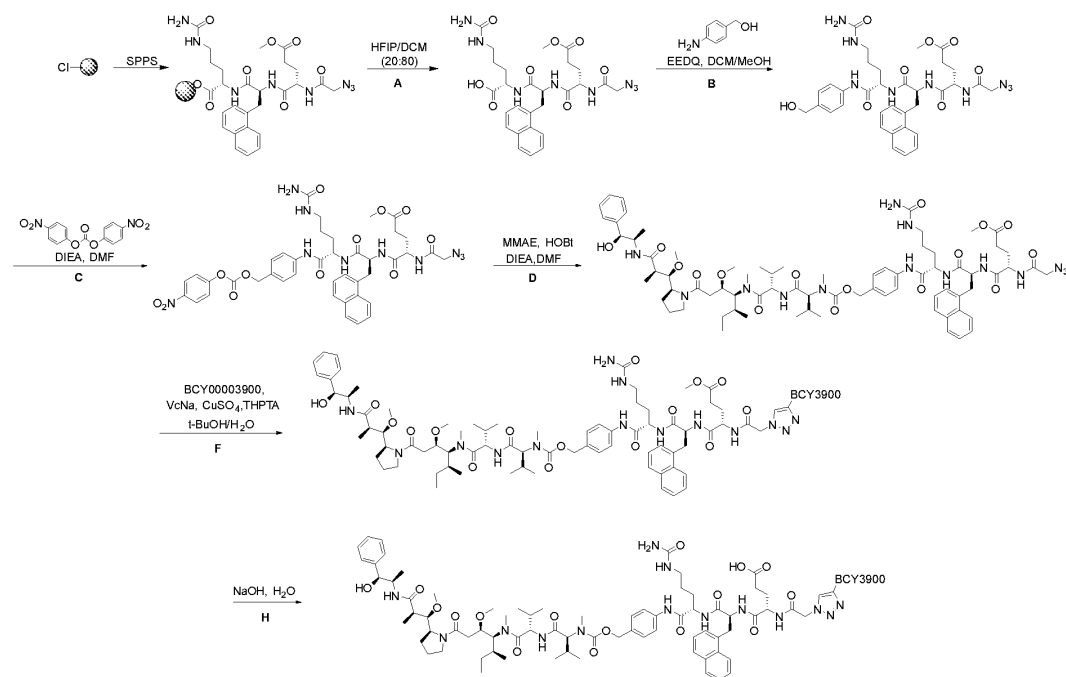
BCY10981 – (Dab-Val-Glu)

10 General methods were followed to yield **BCY10981**. Expected MW = 4011.5, observed m/z:

1338 [M+3H]³⁺, 1003 [M+4H]⁴⁺.

BCY10982 – (Dap-Val-Glu)

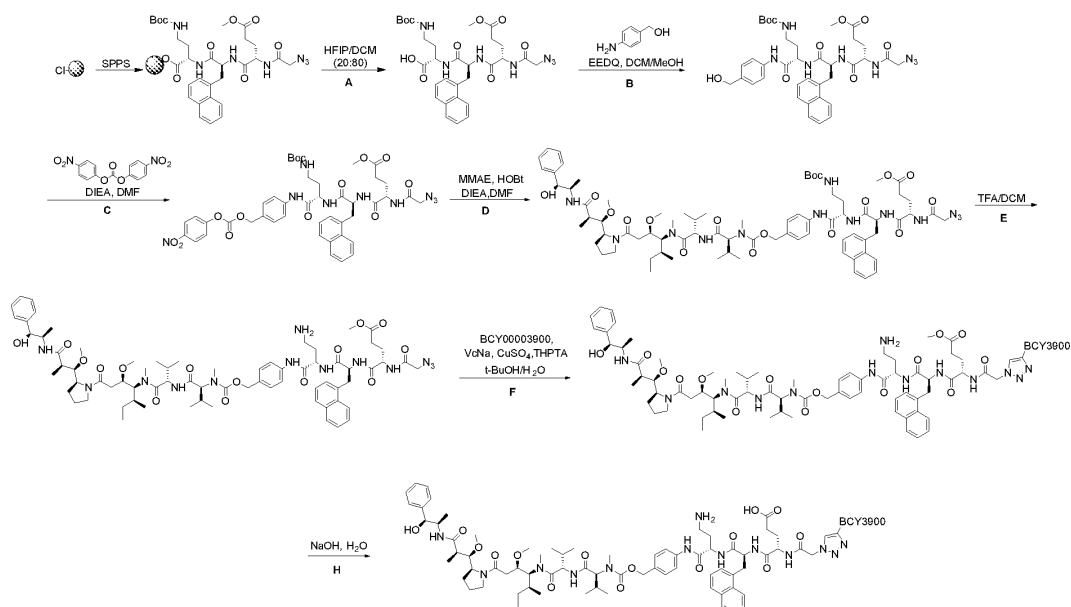
- 5 General methods were followed to yield **BCY10982**. Expected MW = 3997.5, observed m/z: 1333 [M+3H]³⁺, 1000 [M+4H]⁴⁺.

BCY10984 – (Cit-1Nal-Glu)

General methods were followed to yield **BCY10984**. Expected MW = 4166.7, observed m/z: 1388 [M+3H]³⁺, 1042 [M+4H]⁴⁺.

BCY10985 – (Dab-1Nal-Glu)

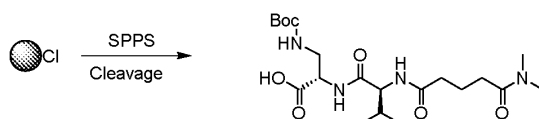
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General methods were followed to yield **BCY10985**. Expected MW = 4109.6, observed m/z: 1370 [M+3H]³⁺, 1028 [M+4H]⁴⁺.

10 **BCY10298 – (Dap-Val)**

Preparation of Compound 1



DCM was added to a vessel containing CTC Resin (5 mmol, 4.50 g, 1.10 mmol/g), then Fmoc-Dap(Boc)-OH (2.13 g, 5 mmol, 1.0 eq) was added with N₂ bubbling. DIEA (4.0 eq) was added
15 dropwise and the resin mixed for 2 hr. MeOH (4.5 mL) was added and the resin again mixed for 30 min. The resin was then drained and washed with DMF 5 times.

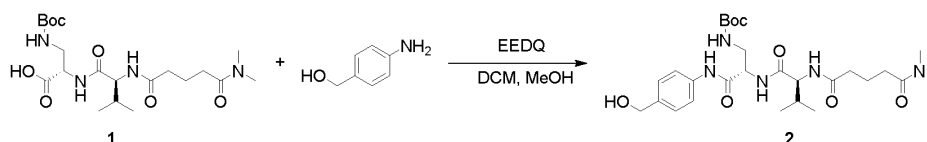
Fmoc groups were removed by adding 20% piperidine/DMF and leaving to react for 30 min, then the resin was drained and washed with DMF 5 times.

20 To couple subsequent amino acids, Fmoc-amino acid solution in DMF was added to the resin and mixed for 30 seconds, then activating agent and base were added. The coupling was left to react for 1 hr with continuous N₂ bubbling. Rounds of coupling and Fmoc deprotection were repeated with the following amino acids

#	Materials	Coupling reagents/base
1	Fmoc-Dap(Boc)-OH (1.0 eq)	DIEA (4.0 eq)
2	Fmoc-Val-OH (3.0 eq)	HBTU (2.85 eq) and DIEA (6.0 eq)
3	Glutaric Anhydride (3.0 eq)	DIEA (6.0 eq)
4	Dimethylamine.HCl(3.0 eq)	HBTU (2.85 eq) and DIEA (6.0 eq)

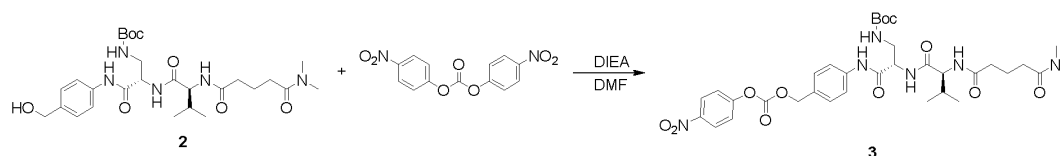
After the final coupling, the resin was washed with MeOH 3 times, and then dried under vacuum. Cleavage from the resin was performed by the addition of 20% HFIP/80% DCM to the flask containing the side chain protected peptide at room temperature. The cleavage was then repeated (1 hr each) with continuous N₂ bubbling. The resin was filtered and the filtrate collected, then concentrated to remove the solvent. The crude peptide was lyophilized to give compound 1 (1.64 g, 94.1% purity, 73.7% yield). Expected MW = 444.53, observed m/z: 445.12 [M+H]⁺.

10 Preparation of Compound 2



To a solution of compound 1 (800 mg, 1.80 mmol, 1.0 eq) in DCM (16.0 mL) and MeOH (8.00 mL) was added (4-aminophenyl)methanol (266 mg, 2.16 mmol, 1.2 eq) and EEDQ (890 mg, 3.60 mmol, 2.0 eq) in the dark. The mixture was stirred at 25 °C for 16 hr. TLC (DCM: MeOH=10: 1, R_f=0.46) indicated compound 1 was consumed completely. LC-MS showed compound 1 was consumed completely and one main peak with desired m/z was detected. The reaction mixture was concentrated under reduced pressure to give a residue. The residue was purified by flash silica gel chromatography (ISCO®; 40 g SepaFlash® Silica Flash Column, Eluent of 0~20% MeOH/DCM@ 60 mL/min) to give compound 2 (550 mg, 1.00 mmol, 55.6% yield) as a pale brown solid. Expected MW = 549.66, observed m/z: 450.04 [(M-Boc)+H]⁺ and 550.08 [M+H]⁺.

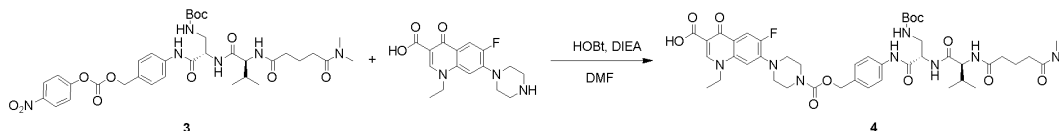
Preparation of Compound 3



To a solution of compound 2 (550 mg, 1.00 mmol, 1.0 eq) in DMF (7.00 mL) was added bis(4-nitrophenyl) carbonate (913 mg, 3.00 mmol, 3.0 eq) and DIEA (517 mg, 4.00 mmol, 697 μL, 4.0 eq) under N₂ atmosphere. The mixture was stirred at 25 °C for 2 hr. LC-MS

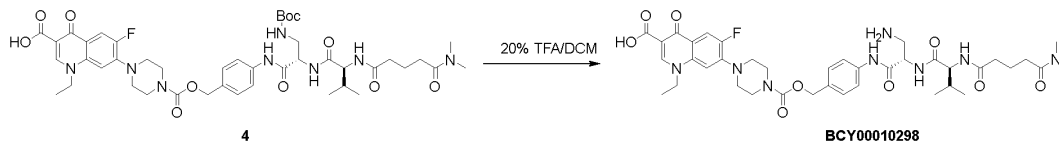
showed compound **2** was consumed completely and one main peak with desired m/z was detected. The reaction mixture was purified by preparative HPLC (neutral condition) to give compound **3** (560 mg, 783 μ mol, 78.30% yield) as a pale yellow solid. Expected MW = 714.76, observed m/z: 614.96 [(M-Boc)+H]⁺ and 715.01 [M+H]⁺.

5 Preparation of Compound 4



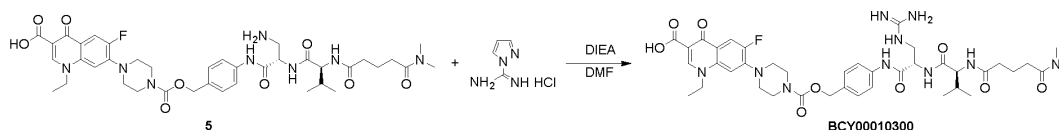
To a solution of compound **3** (550 mg, 769 μ mol, 1.0 eq) in DMF (6.00 mL) was added DIEA (398 mg, 3.08 mmol, 536 μ L, 4.0 eq) and stirred for 10 min under N₂ atmosphere. Then 1-ethyl-6-fluoro-4-oxo-7-piperazin-1-yl-quinoline-3-carboxylic acid (491 mg, 1.54 mmol, 2.0 eq) and HOBt (208 mg, 1.54 mmol, 2.0 eq) were added to the mixture. The mixture was stirred at 25 °C for 2 hr. LC-MS showed compound **3** was consumed completely and one main peak with desired m/z was detected. The reaction mixture was washed by addition H₂O (250 mL) at 25 °C, filtered and concentrated under reduced pressure to give crude product compound **4** (580 mg, crude) as yellow solid, which was used into the next step without further purification. Expected MW = 894.98, observed m/z: 398.04 [(M-Boc)/2+H]⁺, 895.06 [M+H]⁺.

Preparation of BCY10298



To a solution of compound **4** (250 mg, 279 μ mol, 1.0 eq) in DCM (2.40 mL) was added TFA (924 mg, 8.10 mmol, 0.60 mL, 29.0 eq). The mixture was stirred at 25 °C for 2 hr. LC-MS showed compound **4** was consumed completely and one main peak with desired m/z was detected. The reaction mixture was concentrated under reduced pressure to give a residue. The residue was purified by preparative HPLC (A: 0.075% TFA in H₂O, B: ACN) to give **BCY10298** (160 mg, 194 μ mol, 69.6% yield) as a white solid. Expected MW = 794.87, observed m/z: 398.06 [M/2+H]⁺, 795.02 [M+H]⁺.

BCY10300 – (Dap(CNNH₂)-Val)

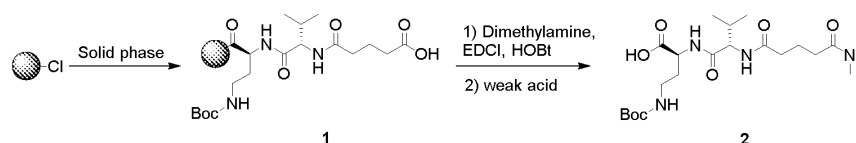


To a solution of **BCY10298** (85.0 mg, 107 μ mol, 1.0 eq) in DMF (1.00 mL) was added chloro(pyrazole-1-carboximidoyl)ammonium (15.6 mg, 107 μ mol, 1.0 eq) and DIEA (41.5

mg, 321 μmol , 60.0 μL , 3.0 eq) under N_2 atmosphere. The mixture was stirred at 25 $^\circ\text{C}$ for 16 hr. LC-MS showed compound **5** was consumed completely and one main peak with desired m/z was detected. The reaction mixture was purified by preparative HPLC (A: 0.075% TFA in H_2O , B: ACN) to give **BCY10300** (39.3 mg, 46.2 μmol , 43.1% yield, 98.3% purity) as a white solid. Expected MW = 836.91, observed m/z: 419.11 $[\text{M}/2+\text{H}]^+$ and 836.95 $[\text{M}+\text{H}]^+$.

BCY9474 – (Dab-Val)

Preparation of Compound 2



DCM was added to a vessel containing CTC Resin (10 mmol, 9.10 g, 1.10 mmol/g), then Fmoc-Dab(Boc)-OH (4.40 g, 10 mmol, 1.0 eq) was added with N_2 bubbling. DIEA (4.0 eq) was added dropwise and the resin mixed for 2 hr. MeOH (9.1 mL) was added and the resin again mixed for 30 min. The resin was then drained and washed with DMF 5 times.

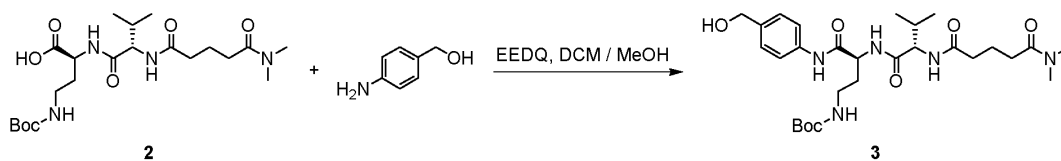
Fmoc groups were removed by adding 20% piperidine/DMF and leaving to react for 30 min, then the resin was drained and washed with DMF 5 times.

To couple subsequent amino acids, Fmoc-amino acid solution in DMF was added to the resin and mixed for 30 seconds, then activating agent and base were added. The coupling was left to react for 1 hr with continuous N_2 bubbling. Rounds of coupling and Fmoc deprotection were carried out with the following amino acids:

#	Materials	Coupling reagents
1	Fmoc-Dab(Boc)-OH (1.0 eq)	DIEA (4.0 eq)
2	Fmoc-Val-OH (3.0 eq)	HBTU (2.85 eq) and DIEA (6.0 eq)
3	tetrahydropyran-2,6-dione (3.0 eq)	HBTU (2.85 eq) and DIEA (6.0 eq)
4	Dimethylamine·HCl (3.0 eq)	HBTU (2.85 eq) and DIEA (6.0 eq)

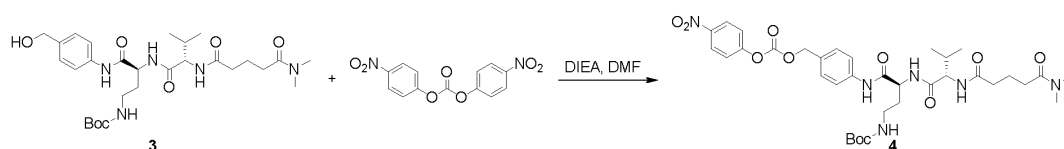
After the final coupling, the resin was washed with MeOH 3 times, and then dried under vacuum. Cleavage from the resin was performed by the addition of 20% HFIP/80% DCM to the flask containing the side chain protected peptide at room temperature. The cleavage was then repeated (1 hr each) with continuous N_2 bubbling. The resin was filtered and the filtrate collected, then concentrated to remove the solvent. The crude peptide was lyophilized to give compound 2 (2.50 g, 96.6 % purity, 54.40% yield). Expected MW = 458.56, observed m/z: 459.4 $[\text{M}+\text{H}]^+$.

Preparation of Compound 3



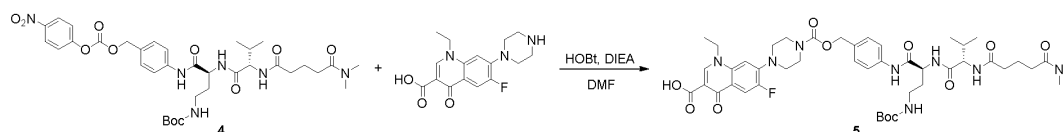
To a solution of compound **2** (2.50 g, 5.45 mmol, 1.0 eq) in DCM (50.0 mL) and MeOH (25.0 mL) was added (4-aminophenyl)methanol (806 mg, 6.54 mmol, 1.2 eq) and EEDQ (2.70 g, 10.9 mmol, 2.0 eq) in the dark. The mixture was stirred at 25 °C for 16 hr in the dark. TLC (DCM: MeOH=10: 1, R_f =0.35) indicated compound **2** was consumed completely. LC-MS showed majority of compound **2** was consumed and one main peak with desired m/z for compound **3** was detected. The reaction mixture was concentrated under reduced pressure to give a residue. The residue was purified by flash silica gel chromatography (ISCO®; 80 g SepaFlash® Silica Flash Column, Eluent of 0~20% MeOH/DCM @ 60 mL/min) to give compound **3** (1.65 g, 2.93 mmol, 53.7% yield) as a pale yellow solid. Expected MW = 563.69, observed m/z : 464.3 [(M-Boc)+H]⁺ and 564.3 [M+H]⁺.

Preparation of Compound 4



To a solution of compound **3** (1.65 g, 2.93 mmol, 1.0 eq) in DMF (10.0 mL) was added bis(4-nitrophenyl)carbonate (2.67 g, 8.78 mmol, 3.0 eq) and DIEA (1.51 g, 11.7 mmol, 2.04 mL, 4.0 eq) under N₂ atmosphere. The mixture was stirred at 25 °C for 2 hr. LC-MS showed one main peak with desired m/z for compound **4** was detected. The reaction mixture was purified by preparative HPLC (neutral condition) to give compound **4** (1.56 g, 1.33 mmol, 45.3% yield, 62.0% purity) as a pale yellow solid. Expected MW = 728.79, observed m/z : 729.3 [M+H]⁺.

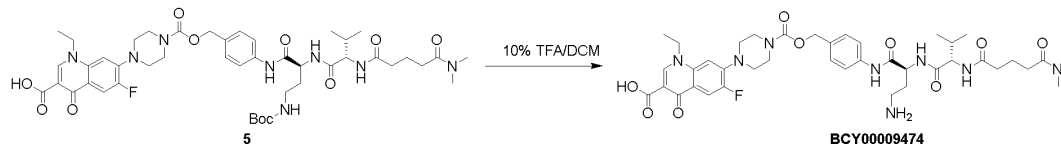
Preparation of Compound 5



To a solution of compound **4** (1.56 g, 2.14 mmol, 1.0 eq) in DMF (10.0 mL) was added DIEA (1.38 g, 10.7 mmol, 1.86 mL, 5.0 eq) and stirred for 10 min. Then 1-ethyl-6-fluoro-4-oxo-7-piperazin-1-yl-quinoline-3-carboxylic acid (1.37 g, 4.28 mmol, 2.0 eq) and HOBt (578 mg, 4.28 mmol, 2.0 eq) were added to the mixture under N₂ atmosphere. The mixture was stirred at 35 °C for 2 hr. LC-MS showed one main peak with desired m/z for compound **5** was detected. The reaction mixture was purified by preparative HPLC (neutral condition) to give

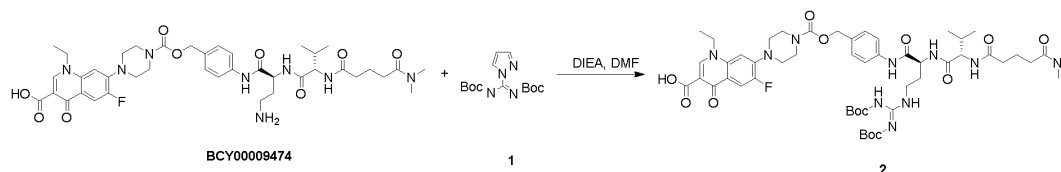
compound **5** (1.45 g, 1.60 mmol, 74.5% yield) as a pale yellow solid. Expected MW = 909.01, observed m/z: 909.3 [M+H]⁺.

Preparation of BCY9474

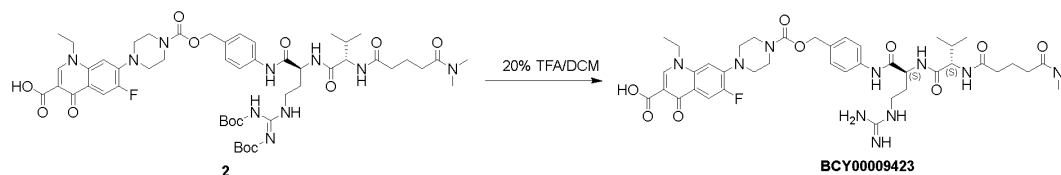


- 5 To a solution of compound **5** (1.45 g, 1.60 mmol, 1.0 eq) in DCM (9.00 mL) was added TFA (1.54 g, 13.5 mmol, 1.00 mL, 8.47 eq). The mixture was stirred at 25 °C for 1 hr. LC-MS showed compound **5** was consumed completely and one main peak with desired m/z was detected. The reaction mixture was concentrated under reduced pressure to give a residue. The residue was purified by preparative HPLC (neutral condition) to give **BCY9474** (850 mg,
- 10 1.05 mmol, 65.9% yield, 98.74% purity) as a pale yellow solid. Expected MW = 808.90, observed m/z: 405.3 [M/2+H]⁺ and 809.3 [M+H]⁺.

BCY9423 – (Agb-Val)



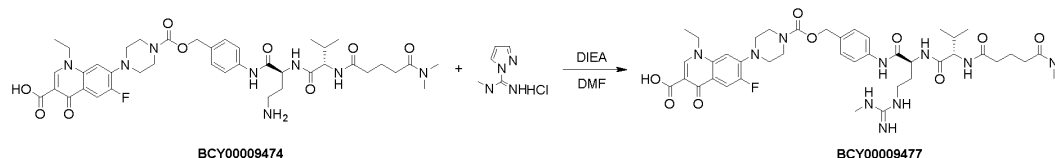
- 15 To a solution of **BCY9474** (150 mg, 185 μmol, 1.0 eq) in DMF (2.00 mL) was added tert-butyl (NZ)-N-[(tert-butoxycarbonylamino)-pyrazol-1-ylmethylene]carbamate (86.3 mg, 278 μmol, 1.5 eq) and DIEA (47.9 mg, 371 μmol, 64.6 μL, 2.0 eq). The mixture was stirred at 25 °C for 16 hr. LC-MS showed majority of **BCY9474** was consumed and one main peak with desired m/z for compound **2** was detected. The reaction mixture was purified by preparative
- 20 HPLC (neutral condition) to give compound **2** (110 mg, 105 μmol, 56.4% yield) as a white solid. Expected MW = 1050.52, observed m/z: 525.70 [M/2+H]⁺ and 1050.82 [M+H]⁺.



- To a solution of compound **2** (110 mg, 105 μmol, 1.0 eq) in DCM (2.00 mL) was added TFA (770 mg, 6.75 mmol, 500 μL, 64.5 eq). The mixture was stirred at 25 °C for 0.5 hr. LC-MS showed compound **2** was consumed completely and one main peak with desired m/z was detected. The reaction mixture was concentrated under reduced pressure to give a residue.
- 25

The residue was purified by preparative HPLC (TFA condition) to give **BCY9423** (20.8 mg, 24.2 μ mol, 23.1% yield, 98.8% purity) as a white solid. Expected MW = 850.94, observed m/z: 425.72 [M/2+H]⁺, 850.67 [M+H]⁺.

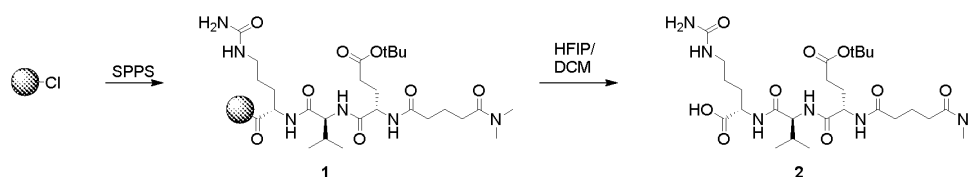
5 BCY9477 – (Aqb(Me)-Val)



To a solution of **BCY9474** (100 mg, 124 μmol , 1.0 eq) in DMF (3 mL) was added N-methylpyrazole-1-carboxamidine (59.6 mg, 371 μmol , 3.0 eq, HCl salt form) and DIEA (95.9 mg, 742 μmol , 129 μL , 6.0 eq) under N_2 atmosphere. The mixture was stirred at 60 $^\circ\text{C}$ for 16 hr. LC-MS showed one main peak with desired m/z was detected. The reaction mixture was purified by preparative HPLC (TFA condition) to give **BCY9477** (40.9 mg, 46.9 μmol , 37.9% yield, 99.2% purity) as a white solid. Expected MW = 864.96, observed m/z : 432.68 $[\text{M}/2+\text{H}]^+$ and 864.62 $[\text{M}+\text{H}]^+$.

15 **BCY9696 – (Cit-Val-Glu)**

Preparation of Compound 2



DCM was added to a vessel containing CTC Resin (5 mmol, 4.50 g, 1.10 mmol/g), then Fmoc-Cit-OH (1.98 g, 5 mmol, 1.0 eq) was added with N₂ bubbling. DIEA (4.0 eq) was added dropwise and the resin mixed for 2 hr. MeOH (4.5 mL) was added and the resin again mixed for 30 min. The resin was then drained and washed with DMF 5 times.

Fmoc groups were removed by adding 20% piperidine/DMF and leaving to react for 30 min, then the resin was drained and washed with DMF 5 times.

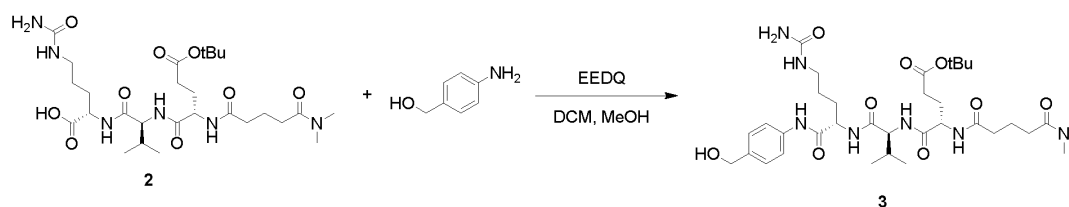
25 To couple subsequent amino acids, Fmoc-amino acid solution in DMF was added to the resin and mixed for 30 seconds, then activating agent and base were added. The coupling was left to react for 1 hr with continuous N₂ bubbling. Rounds of coupling and Fmoc deprotection were carried out with the following amino acids:

#	Materials	Coupling reagents
1	Fmoc-Cit-OH (1.0 eq)	DIEA (4.0 eq)
2	Fmoc-Val-OH (3.0 eq)	HBTU (2.85 eq) and DIEA (6.0 eq)

3	Fmoc-Glu(OtBu)-OH (3.0 eq)	HBTU (2.85 eq) and DIEA (6.0 eq)
4	tetrahydropyran-2,6-dione (3.0 eq)	HBTU (2.85 eq) and DIEA (6.0 eq)
5	Dimethylamine·HCl (3.0 eq)	HBTU (2.85 eq) and DIEA (6.0 eq)

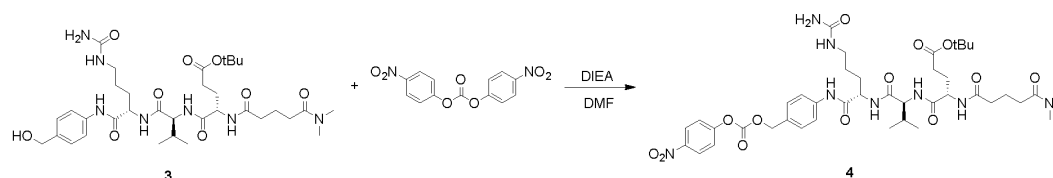
After the final coupling, the resin was washed with MeOH 3 times, and then dried under vacuum. Cleavage from the resin was performed by the addition of 20% HFIP/80% DCM to the flask containing the side chain protected peptide at room temperature. The cleavage was then repeated (1 hr each) with continuous N₂ bubbling. The resin was filtered and the filtrate collected, then concentrated to remove the solvent. The crude peptide was lyophilized to give compound 2 (1.9 g, 100 % purity, 63.3 % yield). Expected MW = 600.71, observed m/z: 601.3 [M+H]⁺.

Preparation of Compound 3



To a solution of compound 2 (500 mg, 832 μmol, 1.0 eq) in DCM (10 mL) and MeOH (5 mL) was added (4-aminophenyl)methanol (123 mg, 999 μmol, 1.2 eq) and EEDQ (412 mg, 1.66 mmol, 2.0 eq) in the dark. The mixture was stirred at 25 °C for 12 hr. TLC (DCM: MeOH=10:1, R_f=0.23) indicated compound 2 was consumed completely and many new spots formed. LC-MS showed compound 2 was consumed completely and one main peak with desired m/z for compound 3 was detected. The reaction mixture was concentrated under reduced pressure to give a residue. The residue was purified by flash silica gel chromatography (ISCO®; 40 g SepaFlash® Silica Flash Column, Eluent of 0~20% MeOH/DCM@ 40 mL/min) to give compound 3 (350 mg, 496 μmol, 59.6% yield) as a pale yellow solid. Expected MW = 705.84, observed m/z: 706.3 [M+H]⁺.

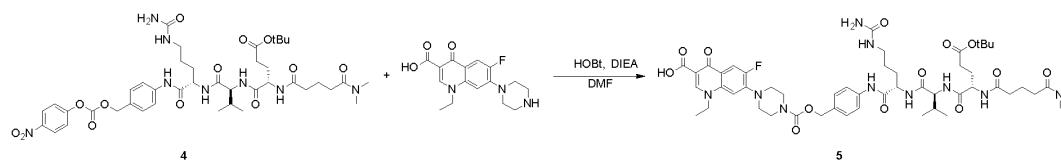
Preparation of Compound 4



To a solution of compound 3 (350 mg, 496 μmol, 1.0 eq) in DMF (4 mL) was added bis(4-nitrophenyl) carbonate (453 mg, 1.49 mmol, 3.0 eq) and DIEA (256 mg, 1.98 mmol, 345 μL, 4.0 eq) under N₂ atmosphere. The mixture was stirred at 25 °C for 2 hr. LC-MS showed compound 3 was consumed completely and one main peak with desired m/z was detected.

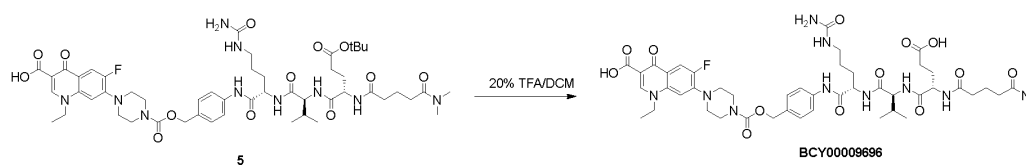
The reaction mixture was purified by preparative HPLC (neutral condition) to give compound **4** (370 mg, 425 μ mol, 85.7% yield) as a white solid. Expected MW = 870.41, observed m/z: 870.66 $[M+H]^+$.

Preparation of Compound 5



- To a solution of compound **4** (360 mg, 413 μ mol, 1.0 eq) in DMF (4 mL) was added DIEA (267 mg, 2.07 mmol, 360 μ L, 5.0 eq) and stirred for 10 min under N_2 atmosphere. Then 1-ethyl-6-fluoro-4-oxo-7-piperazin-1-yl-quinoline-3-carboxylic acid (264 mg, 827 μ mol, 2.0 eq) and HOBt (112 mg, 827 μ mol, 2.0 eq) were added to the mixture. The mixture was stirred at 35 $^{\circ}$ C for 2 hr. LC-MS showed compound **4** was consumed completely and one main peak with desired m/z was detected. The reaction mixture was purified by preparative HPLC (neutral condition) to give compound **5** (390 mg, 371 μ mol, 89.8% yield) as a white solid.

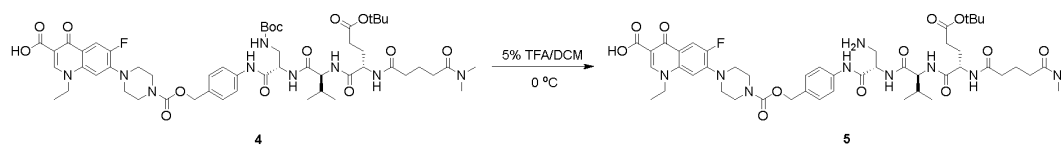
Preparation of BCY9696



- To a solution of compound **5** (150 mg, 143 μ mol, 1.0 eq) in DCM (5 mL) was added TFA (1.93 g, 16.9 mmol, 1.25 mL, 118.0 eq). The mixture was stirred at 25 $^{\circ}$ C for 0.5 hr. LC-MS showed compound **5** was consumed completely and one main peak with desired m/z was detected. The reaction mixture was concentrated under reduced pressure to give a residue. The residue was purified by preparative HPLC (A: 0.075% TFA in H_2O , B: ACN) to give compound **BCY9696** (66.5 mg, 65.8 μ mol, 46.1% yield, 98.4% purity) as a white solid. Expected MW = 994.46, observed m/z: 497.68 $[M/2+H]^+$ and 994.64 $[M+H]^+$.

BCY10299 – (Dap(CNNH₂)-Val-Glu)

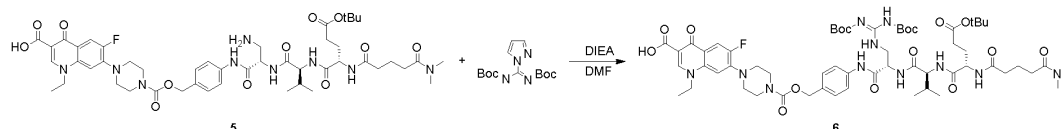
Preparation of Compound 5



- To a solution of compound **4** (which may be prepared as described in BCY10297; 150 mg, 139 μ mol, 1.0 eq) in DCM (1.9 mL) was added TFA (150 mg, 1.32 mmol, 0.10 mL, 9.5 eq) at 0 $^{\circ}$ C and stirred for 1 hr. LC-MS (ES10336-123-P1A3) showed compound **4** was consumed

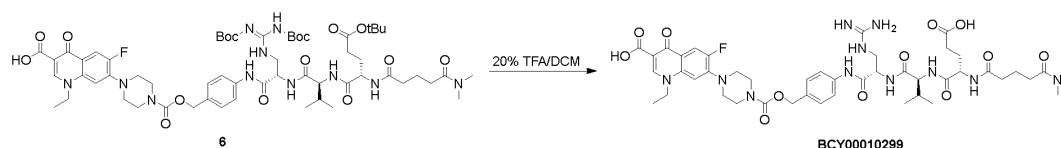
completely and two main peaks formed, where one was **BCY10297** (fully deprotected material) and the other one was desired compound **5**. The reaction mixture was concentrated under reduced pressure to give a residue. The residue was purified by preparative HPLC (neutral condition) to give compound **5** (50.0 mg, 51.0 μ mol, 36.7% yield) as a white solid. Expected MW = 980.09, observed m/z: 490.67 $[M/2+H]^+$ and 980.07 $[M+H]^+$.

Preparation of Compound 6



To a solution of compound **5** (92.0 mg, 93.9 μ mol, 1.0 eq) in DMF (2 mL) was added DIEA (48.5 mg, 375 μ mol, 65.4 μ L, 4.0 eq) and pyrazole-1-carboxamide (13.8 mg, 93.9 μ mol, 1.0 eq) under N_2 atmosphere. The mixture was stirred at 25 $^{\circ}$ C for 16 hr. LC-MS showed most of compound **5** had been consumed and one main peak with desired m/z for compound **6** was detected. The reaction mixture was purified by preparative HPLC (neutral condition) to give compound **6** (72.0 mg, 70.4 μ mol, 75.0% yield) as a white solid. Expected MW = 1222.36, observed m/z: 512.1 $[(M-2*Boc)/2+H]^+$, 1022.7 $[(M-2*Boc)+H]^+$.

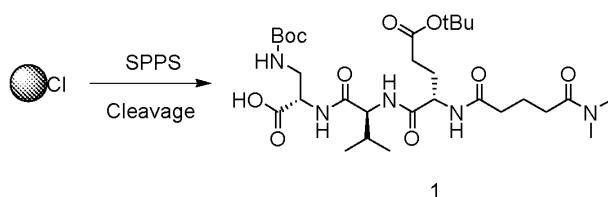
Preparation of BCY10299



To a solution of compound **6** (72.0 mg, 70.4 μ mol, 1.0 eq) in DCM (2.4 mL) was added TFA (900 mg, 8.00 mmol, 0.60 mL, 114.0 eq) and stirred at 25 $^{\circ}$ C for 2 hr. LC-MS showed compound **6** was consumed completely and one main peak with desired m/z was detected. The reaction mixture was concentrated under reduced pressure to remove solvent. The residue was purified by preparative HPLC (A: 0.075% TFA in H_2O , B: ACN) to give **BCY10299** (15.2 mg, 14.0 μ mol, 96.0% purity and 1.1 mg, 1.09 μ mol, 97.1% purity; overall 21.4% yield) as a white solid. Expected MW = 966.02, observed m/z: 483.65 $[M/2+H]^+$ and 966.12 $[M+H]^+$.

BCY10297 – (Dap-Val-Glu)

Preparation of Compound 1



DCM was added to a vessel containing CTC Resin (5 mmol, 4.50 g, 1.10 mmol/g), then Fmoc-Dap(Boc)-OH (2.13 g, 5 mmol, 1.0 eq) was added with N₂ bubbling. DIEA (4.0 eq) was added dropwise and the resin mixed for 2 hr. MeOH (4.5 mL) was added and the resin again mixed for 30 min. The resin was then drained and washed with DMF 5 times.

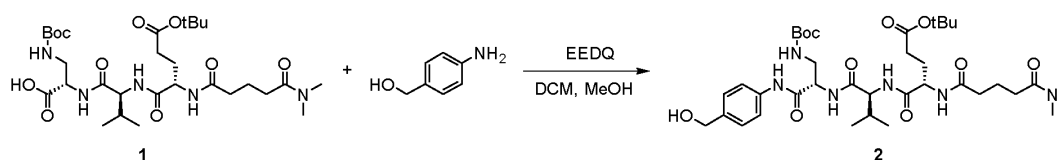
Fmoc groups were removed by adding 20% piperidine/DMF and leaving to react for 30 min, then the resin was drained and washed with DMF 5 times.

To couple subsequent amino acids, Fmoc-amino acid solution in DMF was added to the resin and mixed for 30 seconds, then activating agent and base were added. The coupling was left 10 to react for 1 hr with continuous N₂ bubbling. Rounds of coupling and Fmoc deprotection were carried out with the following amino acids:

#	Materials	Coupling reagents
1	Fmoc-Dap(Boc) -OH (1.0 eq)	DIEA (4.0 eq)
2	Fmoc-Val-OH (3.0 eq)	HBTU(2.85 eq) and DIEA(6.0 eq)
3	Fmoc-Glu(OtBu)-OH (3.0 eq)	HBTU(2.85 eq) and DIEA(6.0 eq)
4	Glutaric Anhydride (3.0 eq)	HBTU(2.85 eq) and DIEA(6.0 eq)
5	Dimethylamine.HCl (3.0 eq)	HBTU(2.85 eq) and DIEA(6.0 eq)

After the final coupling, the resin was washed with MeOH 3 times, and then dried under vacuum. Cleavage from the resin was performed by the addition of 20% HFIP/80% DCM to the flask containing the side chain protected peptide at room temperature. The cleavage was then repeated (1 hr each) with continuous N₂ bubbling. The resin was filtered and the filtrate collected, then concentrated to remove the solvent. The crude peptide was lyophilized to give compound 2 (2.27 g, 93.4 % purity, 69.1% yield). Expected MW = 629.75, observed m/z: 630.10 [(M-Boc)+H]⁺, 630.10 [M+H]⁺.

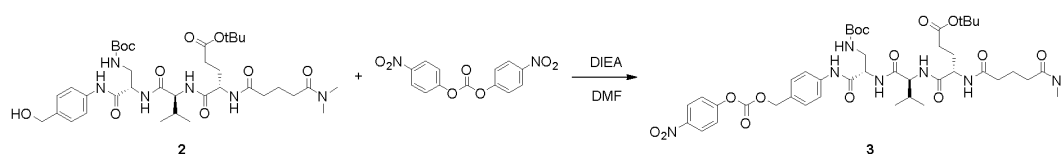
20 Preparation of Compound 2



To a solution of compound **1** (800 mg, 1.27 mmol, 1.0 eq) in DCM (16.0 mL) and MeOH (8.00 mL) was added (4-aminophenyl)methanol (188 mg, 1.52 mmol, 1.2 eq) and EEDQ (628 mg, 2.54 mmol, 2.0 eq) in the dark. The mixture was stirred at 25 °C for 16 hr. TLC

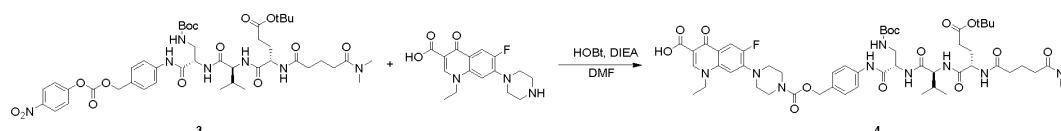
(DCM: MeOH=10: 1, R_f =0.46) indicated compound **1** was consumed completely. LC-MS showed compound **1** was consumed completely and one main peak with desired m/z was detected. The reaction mixture was concentrated under reduced pressure to give a residue. The residue was then purified by flash silica gel chromatography (ISCO®; 40 g SepaFlash®
 5 Silica Flash Column, Eluent of 0~20% MeOH/DCM@ 60 mL/min) to give compound **2** (600 mg, 816 μ mol, 64.3% yield) as a pale brown solid. Expected MW = 734.88, observed m/z : 635.09 [(M-Boc)+H]⁺ and 735.10 [M+H]⁺.

Preparation of Compound 3



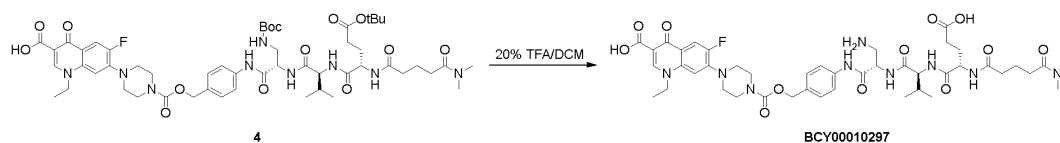
- 10 To a solution of compound **2** (600 mg, 816 μ mol, 1.0 eq) in DMF (8.00 mL) was added bis(4-nitrophenyl) carbonate (745 mg, 2.45 mmol, 3.0 eq) and DIEA (422 mg, 3.27 mmol, 569 μ L, 4.0 eq) under N₂ atmosphere. The mixture was stirred at 25 °C for 2 hr. LC-MS showed compound **2** was consumed completely and one main peak with desired m/z was detected. The reaction mixture was purified by preparative HPLC (neutral condition) to give compound
 15 **3** (630 mg, 700 μ mol, 85.7% yield) as a pale yellow solid. Expected MW = 899.98, observed m/z : 799.99 [(M-Boc)+H]⁺ and 900.02 [M+H]⁺.

Preparation of Compound 4



- To a solution of compound **3** (630 mg, 700 μ mol, 1.0 eq) in DMF (8.00 mL) was added DIEA
 20 (362 mg, 2.80 mmol, 488 μ L, 4.0 eq) and stirred for 10 min under N₂ atmosphere. Then 1-ethyl-6-fluoro-4-oxo-7-piperazin-1-yl-quinoline-3-carboxylic acid (447 mg, 1.40 mmol, 2.0 eq) and HOBT (189 mg, 1.40 mmol, 2.0 eq) were added to the mixture. The mixture was stirred at 25 °C for 2 hr. LC-MS showed compound **3** was consumed completely and one main peak with desired m/z was detected. The reaction mixture was washed by addition 250 mL
 25 of H₂O at 25 °C, filtered and concentrated under reduced pressure to give the crude compound **4** (630 mg, crude) as yellow solid, which was used into the next step without further purification. Expected MW = 1080.20, observed m/z : 490.63 [(M-Boc)/2+H]⁺ and 1080.09 [M+H]⁺.

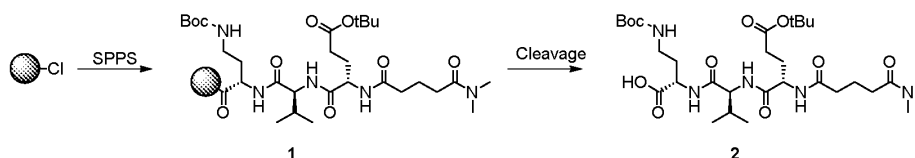
Preparation of BCY10297



To a solution of compound **4** (130 mg, 120 μ mol, 1.0 eq) in DCM (1.60 mL) was added TFA (596 mg, 5.23 mmol, 400 μ L, 43.6 eq). The mixture was stirred at 25 °C for 0.5 hr. LC-MS showed compound **4** was consumed completely and one main peak with desired m/z was detected. The reaction mixture was concentrated under reduced pressure to give a residue. The residue was purified by preparative HPLC (A: 0.075% TFA in H₂O, B: ACN) to give **BCY10297** (45.3 mg, 46.6 μ mol, 38.7% yield, 95.0% purity) as a white solid. Expected MW = 923.68, observed m/z: 462.58 [M/2+H]⁺ and 924.07 [M+H]⁺.

10 **BCY9695 – (Agb-Val-Glu)**

Preparation of Compound 2



DCM was added to a vessel containing CTC Resin (5 mmol, 4.50 g, 1.10 mmol/g), then Fmoc-Dab(Boc)-OH (2.20 g, 5 mmol, 1.0 eq) was added with N₂ bubbling. DIEA (4.0 eq) was added dropwise and the resin mixed for 2 hr. MeOH (4.5 mL) was added and the resin again mixed for 30 min. The resin was then drained and washed with DMF 5 times.

Fmoc groups were removed by adding 20% piperidine/DMF and leaving to react for 30 min, then the resin was drained and washed with DMF 5 times.

To couple subsequent amino acids, Fmoc-amino acid solution in DMF was added to the resin and mixed for 30 seconds, then activating agent and base were added. The coupling was left to react for 1 hr with continuous N₂ bubbling. Rounds of coupling and Fmoc deprotection were carried out with the following amino acids:

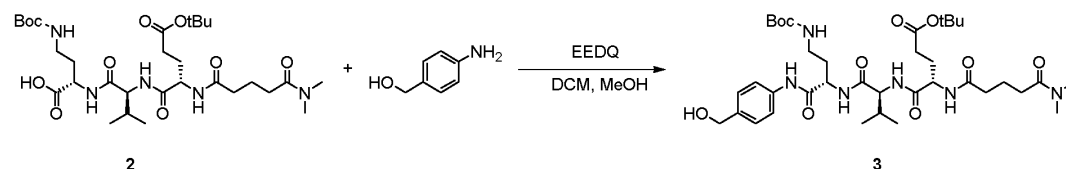
#	Materials	Coupling reagents
1	Fmoc-Dab(Boc)-OH (1.0 eq)	DIEA (4.0 eq)
2	Fmoc-Val-OH (3.0 eq)	HBTU (2.85 eq) and DIEA (6.0 eq)
3	Fmoc-Glu(OtBu)-OH (3.0 eq)	HBTU (2.85 eq) and DIEA (6.0 eq)
4	tetrahydropyran-2,6-dione (3.0 eq)	HBTU (2.85 eq) and DIEA (6.0 eq)
5	Dimethylamine-HCl (3.0 eq)	HBTU (2.85 eq) and DIEA (6.0 eq)

After the final coupling, the resin was washed with MeOH 3 times, and then dried under vacuum. Cleavage from the resin was performed by the addition of 20% HFIP/80% DCM to

the flask containing the side chain protected peptide at room temperature. The cleavage was then repeated (1 hr each) with continuous N₂ bubbling. The resin was filtered and the filtrate collected, then concentrated to remove the solvent. The crude peptide was lyophilized to give compound 2 (2.60 g, 90.0 % purity, 72.6 % yield). Expected MW = 643.78, observed m/z:

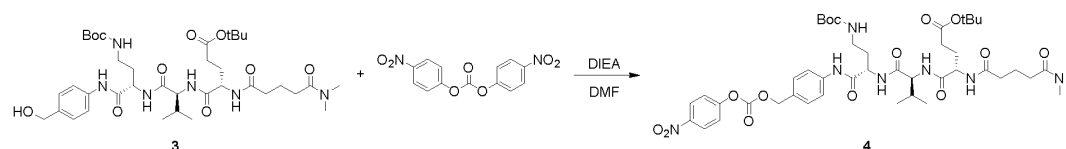
5 644.4 [M+H]⁺.

Preparation of Compound 3



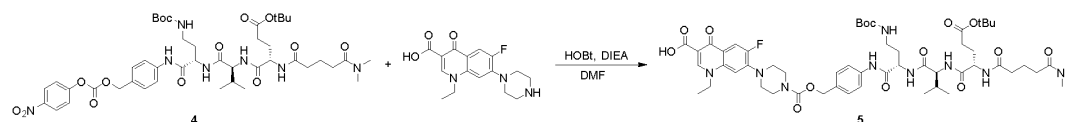
To a solution of compound 2 (500 mg, 777 μmol, 1.0 eq) in DCM (10 mL) and MeOH (5 mL) was added (4-aminophenyl)methanol (115 mg, 932 μmol, 1.2 eq) and EEDQ (384 mg, 1.55 mmol, 2.0 eq) in the dark. The mixture was stirred at 25 °C for 12 hr. TLC (DCM: MeOH=10:1, R_f=0.38) indicated compound 2 was consumed completely and several new spots formed. LC-MS showed compound 2 was consumed completely and one main peak with desired m/z was detected. The reaction mixture was concentrated under reduced pressure to give a residue. The residue was then purified by flash silica gel chromatography (ISCO®; 40 g
15 SepaFlash® Silica Flash Column, Eluent of 0~20% MeOH/DCM@ 40 mL/min) to give compound 3 (390 mg, 521 μmol, 67.1% yield) as a pale yellow solid. Expected MW = 748.44, observed m/z: 749.4 [M+H]⁺.

Preparation of Compound 4



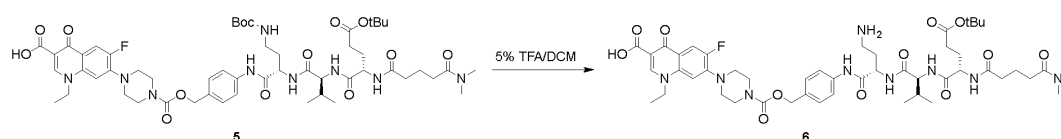
To a solution of compound 3 (390 mg, 521 μmol, 1.0 eq) in DMF (4 mL) was added bis(4-nitrophenyl) carbonate (475 mg, 1.56 mmol, 3.0 eq) and DIEA (269 mg, 2.08 mmol, 363 μL, 4.0 eq) under N₂ atmosphere. The mixture was stirred at 25 °C for 2 hr. LC-MS showed compound 3 was consumed completely and one peak with desired m/z for compound 4 was detected. The residue was purified by preparative HPLC (neutral condition) to give
25 compound 4 (400 mg, 438 μmol, 84.0% yield) as a white solid. Expected MW = 913.44, observed m/z: 913.60 [M+H]⁺.

Preparation of Compound 5



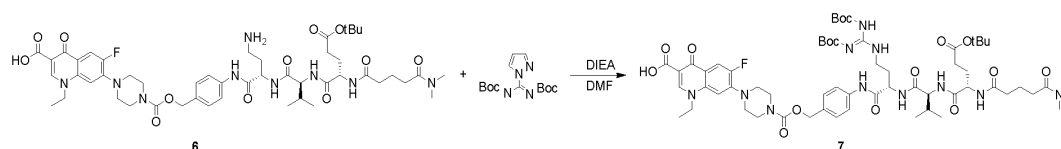
To a solution of compound **4** (400 mg, 438 μ mol, 1.0 eq) in DMF (5 mL) was added DIEA (283 mg, 2.19 mmol, 381 μ L, 5.0 eq) and stirred for 10 min. Then 1-ethyl-6-fluoro-4-oxo-7-piperazin-1-yl-quinoline-3-carboxylic acid (280 mg, 875 μ mol, 2.0 eq) and HOBt (118 mg, 875 μ mol, 2.0 eq) were added to the mixture under N₂ atmosphere. The mixture was stirred at 35 °C for 2 hr. LC-MS showed compound **4** was consumed completely and one main peak with desired m/z for compound **5** was detected. The residue was purified by preparative HPLC (neutral condition) to give compound **5** (310 mg, 283 μ mol, 64.7% yield) as a white solid. Expected MW = 1093.55, observed m/z: 1093.77 [M+H]⁺ and 547.18 [M/2+H]⁺.

10 Preparation of Compound 6



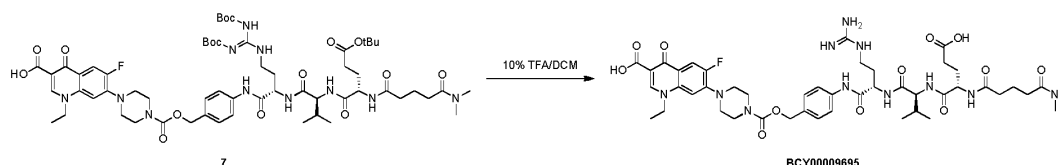
To a solution of compound **5** (305 mg, 279 μ mol, 1.0 eq) in DCM (5.70 mL) was added TFA (462 mg, 4.05 mmol, 0.30 mL, 14.5 eq). The mixture was stirred at 0 °C for 1 hr. LC-MS showed compound **5** was consumed completely and two main peaks formed, where one was desired compound **6** and the other one corresponded to the fully deprotected material. The reaction mixture was concentrated under reduced pressure to give a residue. The residue was purified by preparative HPLC (neutral condition) to give compound **6** (205 mg, 206 μ mol, 74.0% yield) as a white solid. Expected MW = 993.50, observed m/z: 993.72 [M+H]⁺ and 497.26 [M/2+H]⁺.

20 Preparation of Compound 7



To a solution of compound **6** (205 mg, 206 μ mol, 1.0 eq) in DMF (3 mL) was added tert-butyl (NZ)-N-[(tert-butoxycarbonylamino)-pyrazol-1-yl-methylene]carbamate (96.0 mg, 309 μ mol, 1.5 eq) and DIEA (53.3 mg, 412 μ mol, 71.8 μ L, 2.0 eq) under N₂ atmosphere. The mixture was stirred at 25 °C for 16 hr. LC-MS showed compound **6** was consumed completely and one main peak with desired m/z for compound **7** was detected. The residue was purified by preparative HPLC (neutral condition) to give compound **7** (70.0 mg, 56.6 μ mol, 27.5% yield) as a white solid. Expected MW = 1235.62, observed m/z: 1257.66 [M+Na]⁺ and 618.21 [M/2+H]⁺.

30 Preparation of BCY9695



To a solution of compound **7** (70.0 mg, 56.6 μmol , 1.0 eq) in DCM (0.90 mL) was added TFA (154 mg, 1.35 mmol, 100 μL , 23.8 eq). The mixture was stirred at 25 °C for 0.5 hr. LC-MS showed compound **7** was consumed completely and one main peak with desired m/z was detected. The reaction mixture was concentrated under reduced pressure to give a residue. The residue was then purified by preparative HPLC (A: 0.075% TFA in H₂O, B: ACN) to give compound **BCY9695** (21.9 mg, 22.1 μmol , 39.0% yield, 98.9% purity) as a white solid. Expected MW = 979.46, observed m/z: 979.60 [M+H]⁺ and 490.18 [M/2+H]⁺.

10 **BCY10122 – (Dab-Val-Glu)**

Preparation of BCY10122

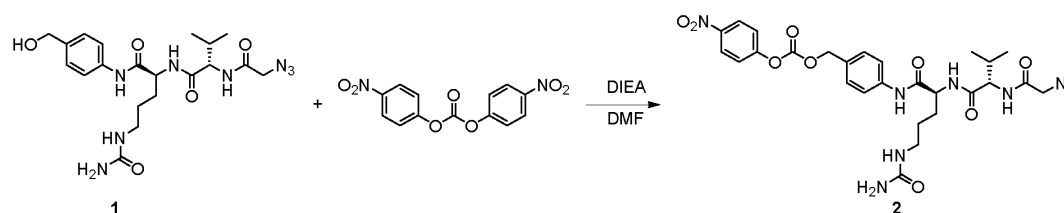


To a solution of compound **5** (305 mg, 279 μmol , 1.0 eq) in DCM (5.70 mL) was added TFA (462 mg, 4.05 mmol, 0.30 mL, 14.5 eq). The mixture was stirred at 0 °C for 1 hr. LC-MS showed compound **5** was consumed completely and one main peak with desired m/z (*calculated* MW:, *observed* m/z:) was detected. The reaction mixture was concentrated under reduced pressure to give a residue. The residue was purified by preparative HPLC (neutral condition) to give **BCY10122** (50.2 mg, 52.0 μmol , 18.6% yield, 97.1% purity) as a white solid. Expected MW = 937.43, observed m/z: 469.24 [M/2+H]⁺, 937.65 [M+H]⁺.

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BCY7761 – (Cit-Val)

Preparation of Compound 2

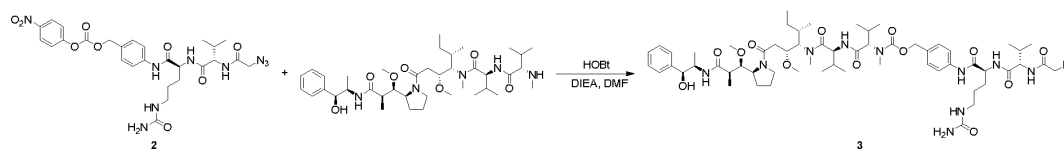


To a solution of compound **1** (1.50 g, 3.24 mmol, 1.0 eq) in DMF (20 mL) was added bis(4-nitrophenyl) carbonate (2.96 g, 9.73 mmol, 3.0 eq) and DIEA (1.68 g, 13.0 mmol, 2.26 mL, 4.0 eq) under N₂ atmosphere. The mixture was stirred at 15 °C for 2 hr. LC-MS showed compound **1** was consumed completely and one peak with desired m/z was detected. The

25

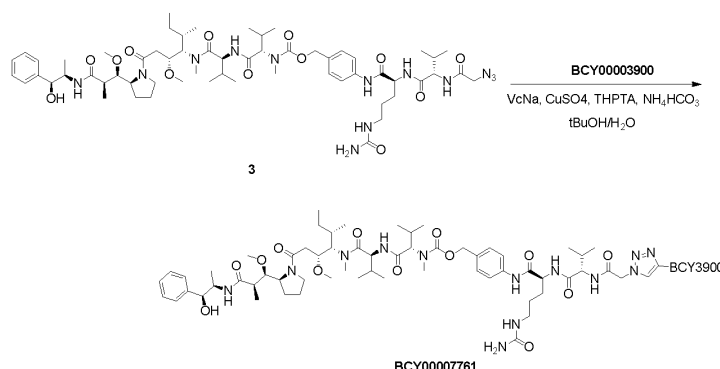
reaction mixture was purified by preparative HPLC (neutral condition) to give compound **2** (1.20 g, 1.91 mmol, 58.9% yield) as a pale yellow solid. Expected MW = 627.61, observed m/z: 627.94 [M+H]⁺.

Preparation of Compound 3



To a solution of compound **2** (905 mg, 1.44 mmol, 1.15 eq) in DMF (10 mL) was added DIEA (486 mg, 3.76 mmol, 655 μ L, 3.0 eq) and stirred for 10 min. Then HOBT (195 mg, 1.44 mmol, 1.15 eq) and **MMAE** (900 mg, 1.25 mmol, 1.0 eq) were added to the mixture. The mixture was stirred at 35 °C for 16 hr. LC-MS showed compound **2** was consumed completely and one main peak with desired m/z was detected. The reaction mixture was purified by preparative HPLC (neutral condition) to give compound **3** (1.08 g, 895 μ mol, 71.6% yield) as a pale yellow solid. Expected MW = 1206.48, observed m/z: 1206.25 [M+H]⁺.

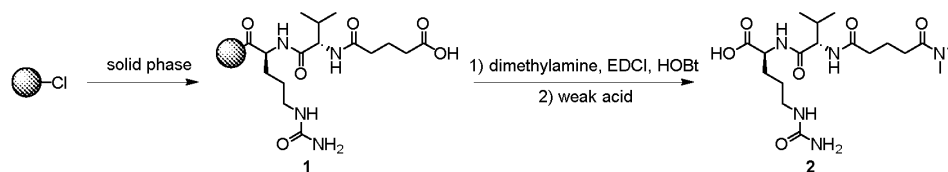
Preparation of BCY7761



To a solution of compound **3** (971 mg, 805 μ mol, 1.1 eq), **BCY3900** (2.00 g, 732 μ mol, 1.0 eq) in t-BuOH (10 mL) and H₂O (10 mL) was added CuSO₄ (0.4 M, 1.83 mL, 1.0 eq) and Tris(3-hydroxypropyl-triazolylmethyl)amine (THPTA, 318 mg, 732 μ mol, 1.0 eq). Then VcNa (0.4 M, 3.66 mL, 2.0 eq) was added to the mixture under N₂ atmosphere. The mixture was stirred at 15 °C for 2 hr. LC-MS showed compound **3** was consumed completely and one main peak with desired m/z was detected. EDTA (0.5 M, 1.5 mL) was added to the reaction mixture to quench reaction. The reaction mixture was then purified by preparative HPLC (A: 0.075% TFA in H₂O, B: ACN) to give **BCY7761** (2.20 g, 539 μ mol, 73.4% yield, 96.5% purity) as a white solid. Expected MW = 3939.45, observed m/z: 985.47 [M/4+H]⁺ and 1313.56 [M/3+H]⁺.

BCY9422 – (Cit-Val)

Preparation of Compound 2



DCM was added to a vessel containing CTC Resin (10 mmol, 9.10 g, 1.10 mmol/g), then Fmoc-Cit-OH (3.98 g, 10 mmol, 1.0 eq) was added with N₂ bubbling. DIEA (4.0 eq) was added dropwise and the resin mixed for 2 hr. MeOH (9.1 mL) was added and the resin again mixed for 30 min. The resin was then drained and washed with DMF 5 times.

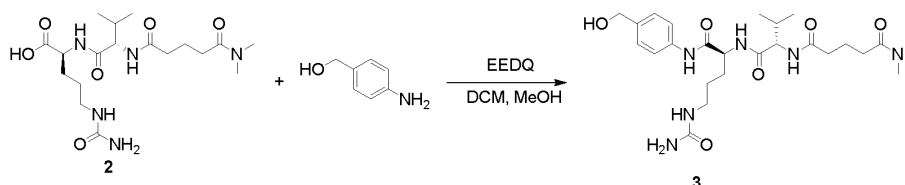
Fmoc groups were removed by adding 20% piperidine/DMF and leaving to react for 30 min, then the resin was drained and washed with DMF 5 times.

To couple subsequent amino acids, Fmoc-amino acid solution in DMF was added to the resin and mixed for 30 seconds, then activating agent and base were added. The coupling was left to react for 1 hr with continuous N₂ bubbling. Rounds of coupling and Fmoc deprotection were carried out with the following amino acids:

#	Materials	Coupling reagents
1	Fmoc-Cit-OH (1.0 eq)	DIEA (4.0 eq)
2	Fmoc-Val-OH (3.0 eq)	HBTU (2.85 eq) and DIEA (6.0 eq)
3	tetrahydropyran-2,6-dione (3.0 eq)	HBTU (2.85 eq) and DIEA (6.0 eq)
4	Dimethylamine·HCl (3.0 eq)	HBTU (2.85 eq) and DIEA (6.0 eq)

After last amino acid coupling, the resin was washed with MeOH 3 times, and then dried under vacuum. Cleavage from the resin was performed by the addition of 20% HFIP/80% DCM to the flask containing the side chain protected peptide at room temperature. The cleavage was then repeated (1 hr each) with continuous N₂ bubbling. The resin was filtered and the filtrate collected, then concentrated to remove the solvent. The crude peptide was lyophilized to give compound 2 (crude, 1.80 g, 91.82 % purity, 39.7 % yield). Expected MW = 415.49, observed m/z: 416.2 [M+H]⁺.

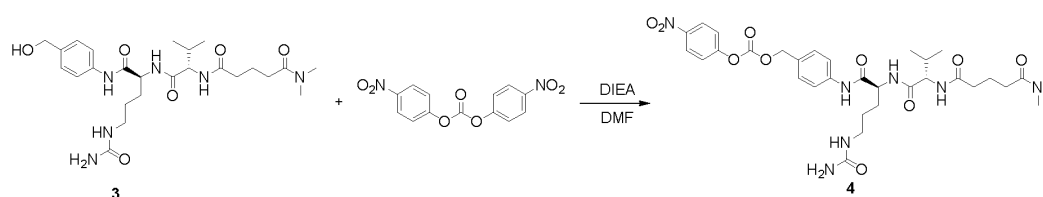
Preparation of Compound 3



To a solution of compound 2 (500 mg, 1.20 mmol, 1.0 eq) in DCM (10 mL) and MeOH (5 mL) was added (4-aminophenyl)methanol (178 mg, 1.44 mmol, 1.2 eq) and EEDQ (595 mg, 2.41 mmol, 2.0 eq) in the dark. The mixture was stirred at 25 °C for 12 hr. TLC (DCM:

MeOH=10: 1, $R_f=0.53$) indicated compound **2** was consumed completely. The reaction mixture was concentrated under reduced pressure to give a residue. The residue was purified by flash silica gel chromatography (ISCO®; 40 g SepaFlash® Silica Flash Column, Eluent of 0~25% MeOH/DCM @ 40 mL/min) to give compound **3** (380 mg, 730 μ mol, 60.7% yield) as a pale yellow solid. ^1H NMR: ES8396-320-P1D1 (400 MHz, DMSO- d_6) δ ppm 9.87 - 9.91 (m, 1 H), 8.09 (br d, $J=7.53$ Hz, 1 H), 7.86 (d, $J=8.53$ Hz, 1 H), 7.49 - 7.57 (m, 2 H), 7.18 - 7.26 (m, 2 H), 5.97 (br t, $J=5.52$ Hz, 1 H), 5.41 (s, 2 H), 5.06 - 5.12 (m, 1 H), 4.34 - 4.44 (m, 3 H), 4.14 - 4.22 (m, 1 H), 3.33 (s, 6 H), 2.91 (s, 3 H), 2.79 (s, 2 H), 2.16 - 2.32 (m, 4 H), 1.97 (dq, $J=13.52, 6.70$ Hz, 1 H), 1.63 - 1.76 (m, 3 H), 0.85 (dd, $J=11.17, 6.90$ Hz, 6 H).

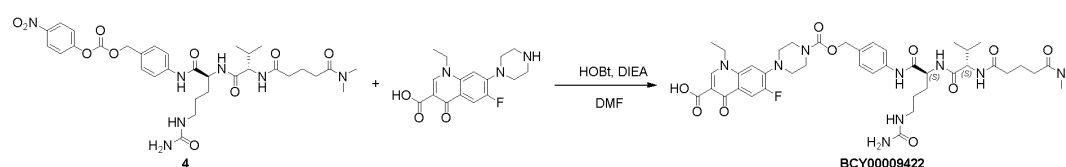
10 Preparation of Compound 4



To a solution of compound **3** (380 mg, 730 μ mol, 1.0 eq) in DMF (8 mL) was added bis(4-nitrophenyl) carbonate (666 mg, 2.19 mmol, 3.0 eq) and DIEA (377 mg, 2.92 mmol, 509 μ L, 4.0 eq). The mixture was stirred at 25 °C for 2 hr. LC-MS showed compound **3** was

15 consumed completely and one main peak with desired m/z for compound **4** was detected. The reaction mixture was purified by preparative HPLC (neutral condition) to give compound **4** (368 mg, 537 μ mol, 73.5% yield) as a white solid. Expected MW = 685.32, observed m/z : 686.1 $[\text{M}+\text{H}]^+$.

Preparation of BCY9422



20 To a solution of compound **4** (150 mg, 219 μ mol, 1.0 eq) in DMF (3 mL) was added DIEA (141 mg, 1.09 mmol, 191 μ L, 5.0 eq) and stirred for 10 min at 25 °C. Then 1-ethyl-6-fluoro-4-oxo-7-piperazin-1-yl-quinoline-3-carboxylic acid (105 mg, 328 μ mol, 1.5 eq) and HOBT (29.6 mg, 219 μ mol, 1.0 eq) were added to the mixture. The mixture was stirred at 35 °C for 16 hr.

25 LC-MS showed one main peak with desired m/z was detected. The reaction mixture was purified by preparative HPLC (A: 0.075% TFA in H_2O , B: ACN) to give **BCY9422** (107.8 mg, 119 μ mol, 54.6% yield, 95.9% purity) as a white solid. Expected MW = 865.95, observed m/z : 433.7 $[\text{M}/2+\text{H}]^+$ and 866.2 $[\text{M}+\text{H}]^+$.

30 Plasma Stability Analysis

Pooled frozen plasma was thawed in a water bath at 37°C. Plasma was centrifuged at 4000 rpm for 5 min and the clots were removed if any. The pH will be adjusted to 7.4 ± 0.1 if required. 1 mM stock solution was prepared with DMSO. Propantheline (positive control) was prepared by making a 100 μ M working solution by diluting 5 μ L of the stock solution (10 mM) with 495 μ L ultra pure water. 100 μ M working solutions of test compounds were made up by diluting 10 μ L of the stock solution (1 mM) with 90 μ L DMSO. 98 μ L of blank plasma was spiked with 2 μ L of dosing solution (100 μ M) to achieve 2 μ M of the final concentration in duplicate and samples were incubated at 37°C in a water bath. At each time point (0, 1, 2, 4, 6 and 24hr), 400 μ L of 200ng/mL tolbutamide and labetalol in 100% MeOH was added and mixed thoroughly to precipitate proteins. Sample plates were centrifuged at 4,000 rpm for 15 min. An aliquot of supernatant (150 μ L) was transferred from each well before submitting for LC-MS/MS analysis.

The % remaining test compound after incubation in plasma was calculated using following equation:

$$\% \text{ Remaining} = 100 \times (\text{PAR at appointed incubation time} / \text{PAR at T0 time})$$

where PAR is the peak area ratio of analyte versus internal standard (IS).

The appointed incubation time points are T0 (0 hr), Tn (n=0,1,2,4,6,24hr). The half-life (T_{1/2}) was calculated from a log linear plot of concentration versus time.

When the %remaining value at the maximal incubation time, which was 24 hr in this study, was higher than 75%, it is considered to be within the acceptable experimental variation. Therefore, a corresponding t_{1/2} of >57.8 hr was reported.

Cathepsin B (CatB) Assay

15 μ L of test compound solution (2 mM in DMSO) was added to the incubation plate in duplicate. 30 μ L of Cathepsin B stock solution (16 μ M) was pre-activated with 1500 μ L activation buffer for 10min at room temperature. The Cathepsin B solution was diluted in 13.17 mL water, then 735 μ L activated enzyme mixture was added to the incubation plate. The mixture was incubated at 37°C in a waterbath. At various time points (e.g. 0h, 1h, 2h, 4h, 6h, 24h) the reaction was terminated by taking 100 μ L aliquots and quenching with 400 μ L cold IS-fortified quenching solution. The samples were mixed and centrifuged at 4000 rpm for 20 min. 50 μ L of supernatant was taken into a new plate containing 150 μ L ultra-pure water and the samples mixed thoroughly before submitting to LC-MS/MS analysis.

Xenograft models

For Cell-Derived Xenograft (CDX) models, mice (balb/c nude, female, 18-23g at study start) were inoculated with HT1080 cells (5.0×10^6 cells/mouse in 0.2ml PBS to the right flank).

- 5 Animals were randomized when the average tumour volume reached the pre-designated start size. Group size is n=4. All studies included a vehicle-treated control.

Dosing was conducted by intravenous bolus. Tumor volume was measured in two dimensions using a caliper, and the volume was expressed in mm^3 using the formula: $V = 0.5 \times a \times b^2$ where a and b are the long and short diameters of the tumor, respectively. All

- 10 Xenograft studies were conducted at Wuxi AppTec Co. Ltd. (Shanghai).

Plasma Pharmacokinetics of Bicycle Conjugates and released payloads in CD-1 Mice

- Male CD-1 mice were dosed with each Bicycle Conjugate formulated in 25 mM histidine HCl, 10% sucrose pH 7 via tail vein injection. Serial bleeding (about 80 μL blood/time point) was performed via submandibular or saphenous vein at each time point. All blood samples were immediately transferred into prechilled microcentrifuge tubes containing 2 μL K2-EDTA (0.5M) as anti-coagulant and placed on wet ice. Blood samples were immediately processed for plasma by centrifugation at approximately 4°C, 3000g. The precipitant including internal standard (350 μL) was immediately added into the 35 μL plasma sample, mixed well and centrifuged at 3220 g, 4°C for 15 minutes. The supernatant was transferred into pre-labeled polypropylene microcentrifuge tubes, and then quick-frozen over dry ice. The samples were stored at 70°C or below as needed until analysis. Supernatant samples were mixed with 50 μL water, vortexed well and centrifuged at 3220 g, 4°C for 15 minutes. A sample of the supernatant was injected for LC-MS/MS analysis using an Acquity UPLC with AB Sciex 6500+ Triple Quad MS in positive ion mode to determine the concentrations of Bicycle Conjugate and released payload. Plasma concentration versus time data were analyzed by non-compartmental approaches using the Phoenix WinNonlin 6.3 software program. C_0 , Cl , Vd_{ss} , $T_{1/2}$, $AUC_{(0-last)}$, $AUC_{(0-inf)}$, $MRT_{(0-last)}$, $MRT_{(0-inf)}$ and graphs of plasma concentration versus time profile were reported.

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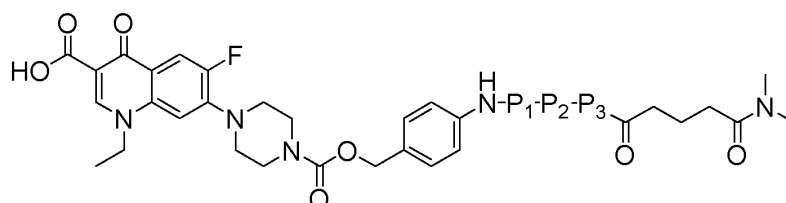
Measurement of MMAE in plasma, muscle and tumour samples

Samples of tumour from *in vivo* xenograft studies were weighed, homogenized (10 x dilution in homogenization buffer including protease inhibitors). Tumour homogenates and plasma were then analysed by LC-MS/MS according to standard procedures.

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Tested Compounds

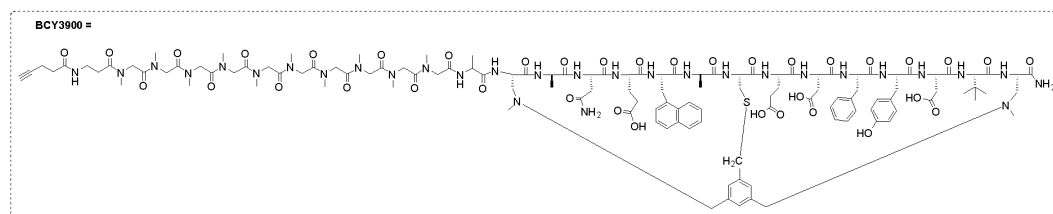
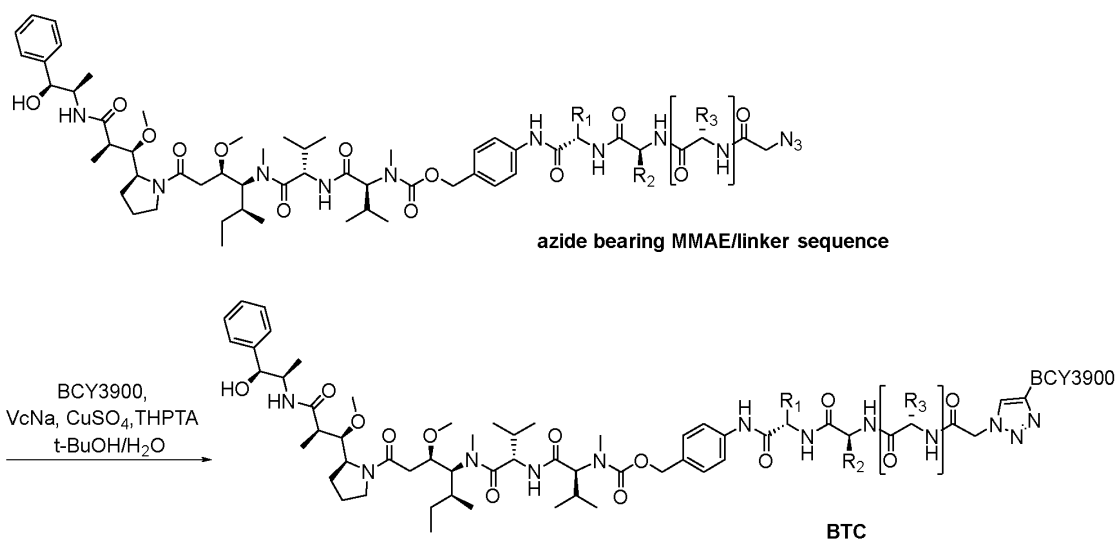
The compounds used for the following studies were constructed as described hereinbefore with Norfloxacin used as a surrogate payload, conjugated to the di/tripeptide linkers of the invention via a PAB self-immolating group. The peptidic linkers were capped at the N-terminus with 5-(dimethylamino)-5-oxopentanoic acid as shown schematically below:



5

Bicycle Toxin Conjugates (BTCs)

BTCs incorporating the di/tripeptide linkers of the invention were synthesised by preparing azide-bearing toxin/linker sequences. Here, MMAE cytotoxin was linked to the peptidic cleavable linkers via PAB self-immolating group, which were conjugated to a bicyclic peptide MT1-MMP binder (BCY3900; as described in WO 2016/067035 as SEQ ID NO: 5) using copper catalysed azide-alkyne cycloaddition.



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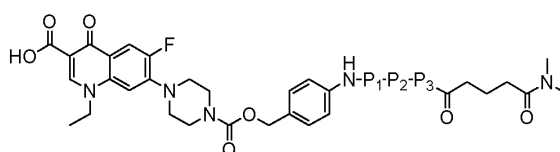
Example 1: Plasma Stability Analysis using Linkers of the Invention

Replacement of the citrulline residue in CatB sensitive dipeptide linker Cit-Val with a basic non-natural amino acid was shown to increase the stability of the linker to non-specific cleavage when incubated with mouse plasma *in vitro*. This is shown by the extended half-lives in Table 1 for the tested compounds when compared with the Cit-Val linker (BCY9422).

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Table 1: Replacement of Cit at P1 with Basic Non-Natural Amino Acids in Dipeptide Linkers

Cit-Val → BAA-Val (BAA = basic non-natural amino acid)



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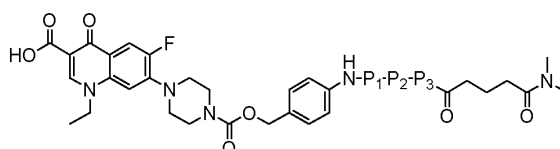
Cpd No	P1	P2	P3	Mouse plasma $t_{1/2}$ (h)	fold increase in half-life vs Cit-Val
BCY9422	Cit	Val	-	0.4	1.0
BCY10300	Dap(CNNH ₂)	Val	-	1.4	3.4
BCY9423	Agb	Val	-	2.4	6.0
BCY9477	Agb(Me)	Val	-	2.5	6.3
BCY10298	Dap	Val	-	2.6	6.5
BCY00009474	Dab	Val	-	4.3	10.8

In some cases, such as with Agb and Dab, when citrulline is replaced within a linker containing a Cit-Val-Glu motif (reported to have a higher mouse plasma stability than Cit-Val), an additive effect is seen where the analogous linkers incorporating a basic residues exhibit further increased mouse plasma stability over Cit-Val-Glu as shown in Table 2.

15

Table 2: Replacement of Cit at P1 with Basic Non-Natural Amino Acids in Tripeptide Linkers

Cit-Val-Glu → BAA-Val-Glu (BAA = basic non-natural amino acid)



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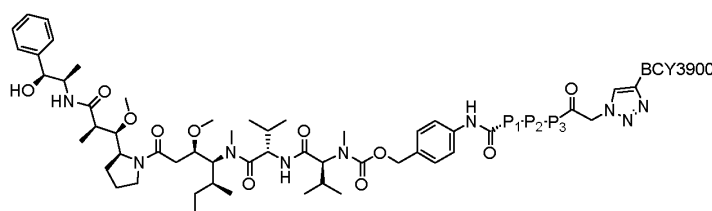
Cpd No	P1	P2	P3	Mouse plasma $t_{1/2}$ (h)	fold increase in half-life vs Cit-Val
BCY9422	Cit	Val	-	0.4	1.0

BCY9696	Cit	Val	Glu	10.8	25.1
BCY10299	Dap(CNNH ₂)	Val	Glu	9.4	11.8
BCY10297	Dap	Val	Glu	15.5	19.3
BCY9695	Agb	Val	Glu	14.3	34.1
BCY10122	Dab	Val	Glu	39.0	107.5

BTCs incorporating linkers which have basic non-natural amino acids in the P1 position show increased stability to mouse plasma, e.g. a BTC with a Dab-Val cleavable linker (see BCY10989 in Table 3) has a half-life of 30.8 hours in mouse plasma (EDTA anticoagulant) compared with 6.8 hours for Cit-Val (see BCY7761 in Table 3).

When Glu is incorporated in the P3 position, linkers with Dab in P1 position show enhanced stability in plasma compared to their Cit counterparts (see Cit-Val-Glu compared with Dab-Val-Glu in human plasma, Cit-1Nal-Glu compared with Dab-1Nal-Glu in rat and mouse plasma EDTA anticoagulant).

Table 3: Plasma Stability of BTCs

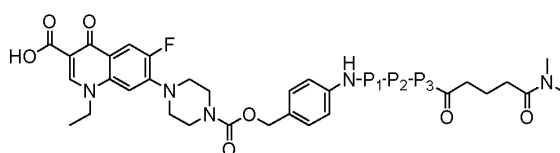


Cpd No	P1	P2	P3	Human plasma t _{1/2} (Heparin)	Rat plasma t _{1/2} (Heparin)	Mouse plasma t _{1/2} (Heparin)	Mouse plasma t _{1/2} (EDTA)	Mouse plasma t _{1/2} expt 2 (EDTA)
BCY7761	Cit	Val	-	>57.8	56.8	9.2	9.1	6.8
BCY10989	Dab	Val	-	NT	NT	NT	NT	30.8
BCY10980	Cit	Val	Glu	40	>57.8	>57.8	>57.8	NT
BCY10981	Dab	Val	Glu	73.8	>57.8	>57.8	>57.8	NT
BCY10984	Cit	1Nal	Glu	>57.8	53.8	47.5	>57.8	NT
BCY10985	Dab	1Nal	Glu	>57.8	>57.8	>57.8	>57.8	NT
BCY10982	Dap	Val	Glu	NT	NT	NT	55.9	NT
BCY10986	Dab	2Nal	Glu	NT	NT	NT	50.1	NT
BCY10987	Dab	Dpa	Glu	NT	NT	NT	>57.8	NT
BCY10988	Dab	cBu	Glu	NT	NT	NT	>57.8	NT

Example 2: CatB Cleavage Rate Analysis using Linkers of the Invention

Replacement of the citrulline residue in CatB sensitive dipeptide linker Cit-Val with a basic non-natural amino acid also modulates the Cathepsin B cleavage rate of the linker. For example, Dab, Agb and Agb(Me) each increase the rate of cleavage by cathepsin B *in vitro* compared with Cit-Val (see Table 4). Dap and Dap(CNNH₂) decreases the cleavage rate (see Table 4).

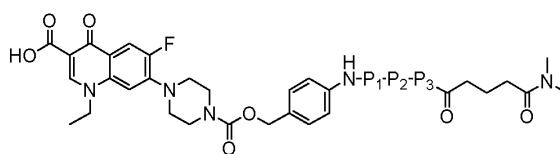
Table 4: Replacement of Cit at P1 with Basic Non-Natural Amino Acids in Dipeptide Linkers



	P1	P2	P3	% Parent remaining after incubation with CatB		
				T = 1 h	T = 4 h	T = 24 h
BCY9422	Cit	Val	-	3	0	0
BCY10300	Dap(CNNH ₂)	Val	-	72	10	0
BCY9423	Agb	Val	-	0.6	0	0
BCY9477	Agb(Me)	Val	-	0	0	0
BCY10298	Dap	Val	-	92	63	6
BCY9474	Dab	Val	-	0.3	0	0

Replacement of the citrulline residue in CatB sensitive tripeptide linker Cit-Val-Glu with a basic non-natural amino acid modulates the Cathepsin B cleavage rate of the linker. For example, Dab and Agb show a similar rate of cleavage as Cit-Val by cathepsin B *in vitro* (see Table 5). Substitution with Dap and Dap(CNNH₂) decreases the cleavage rate (see Table 5).

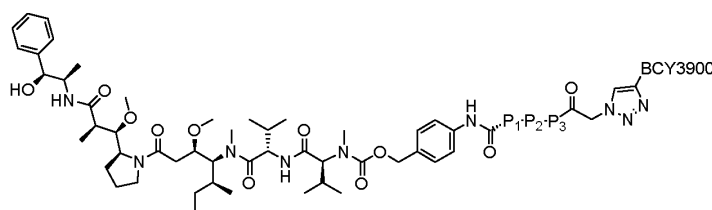
Table 5: Replacement of Cit at P1 with Basic Non-Natural Amino Acids in Tripeptide Linkers



	P1	P2	P3	% Parent remaining after incubation with CatB		
				T = 1 h	T = 4 h	T = 24 h
BCY9696	Cit	Val	Glu	0	0	0
BCY10299	Dap(CNNH2)	Val	Glu	48	1	0
BCY10297	Dap	Val	Glu	75	15	0
BCY9695	Agb	Val	Glu	0	0	0
BCY10122	Dab	Val	Glu	0	0	0

The cathepsin B cleavage rate of linkers can be modulated by introducing different non-natural amino acids at the P1 and P2 position. The results of the CatB cleavage analysis with BTCs can be seen in Table 6 where replacement of Cit with Dab in Cit-Val linker gives a more slowly cleaved linker. When Glu is introduced to these sequences in the P3 position, the cleavage rate between the 2 linkers is comparable. Replacing the P1 position with Dap significantly slows CatB cleavage of the linker. Replacement of Val in the P2 position with 1Nal slows CatB cleavage significantly, whereas its regioisomer 2Nal only slightly reduces cleavage kinetics. Incorporation of Dpa in P2 dramatically reduces CatB cleavage rate and cBu inhibits cleavage altogether.

Table 6: CatB Cleavage of BTCs



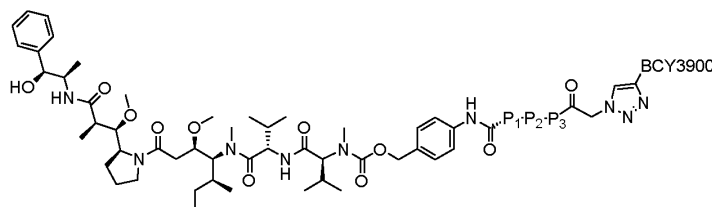
Cpd No	P1	P2	P3	% Parent remaining after incubation with CatB						CatB t _{1/2} (h)
				0 h	1 h	2 h	4 h	6 h	24 h	
BCY7761	Cit	Val	-	100	33.687	15.1	4.93	1.01	0.462	0.9
BCY10989	Dab	Val	-	100	89.4	60.7	40.4	23.2	12.8	2.8
BCY10980	Cit	Val	Glu	100	0	0	0	0	0	<0.2
BCY10981	Dab	Val	Glu	100	0	0	0	0	0	<0.2

BCY10982	Dap	Val	Glu	100	85.435	82.3	73.1	72.3	73.91	>57.8
BCY10984	Cit	1Nal	Glu	100	94.498	83.5	39.2	33.4	32.5	3.3
BCY10985	Dab	1Nal	Glu	100	103.62	94.8	107	92.2	86.82	>57.8
BCY10986	Dab	2Nal	Glu	100	9.7346	0	0	0	0	0.3
BCY10987	Dab	Dpa	Glu	100	104.27	90.6	90.2	94	91.48	>57.8
BCY10988	Dab	cBu	Glu	100	109.88	119	110	105	111	>57.8

Example 3: Plasma Protein Binding Analysis using Linkers of the Invention

Changing the amino acids in P1 and P2 position can modulate the plasma protein binding of a BTC. Table 7 demonstrates that replacement of Cit in P1 position for Dab increases the percentage unbound. Replacement of Val in the P2 position with 1Nal decreases the % unbound

Table 7: Plasma Protein Binding of BTCs



10

	P1	P2	P3	Plasma protein binding (%)			
				Unbound)			
				Mouse	Rat	Cyno	Human
BCY7761	Cit	Val	-	17.3	10.6	18.7	21.1
BCY10980	Cit	Val	Glu	18.7	NT	NT	NT
BCY10981	Dab	Val	Glu	28.8	41.7	45.9	65.3
BCY10984	Cit	1Nal	Glu	0.2	3.5	9.2	3.1
BCY10985	Dab	1Nal	Glu	4.8	NT	NT	23.4
BCY10986	Dab	2Nal	Glu	7.1	11.5	NT	NT

Example 4: Pharmacokinetics Analysis using Linkers of the Invention

Replacement of the dipeptide linker amino acids can alter the pharmacokinetic (PK) profile of a BTC.

15

Mouse

The results shown in Figures 1 to 6 and Table 8 demonstrates that linkers containing 1Nal in the P2 position show extended half-life in mouse PK studies. Linkers with increased mouse plasma stability show lower relative levels of free MMAE in plasma versus Cit-Val (relative to parent compound).

5

Table 8: Summary of Pharmacokinetic Analysis in Mouse

BCY	Sequence	C0 (ng/mL)	CLp (mL/min/kg)	Vdss (L/kg)	t _{1/2} (h)
BCY7761	MMAE-PAB-(Cit-Val)-BCY3900	24753	1.0	0.04	0.48
BCY10984	MMAE-PAB-(Cit-1Nal-Glu)- BCY3900	111807	0.2	0.09	7.55
BCY10980	<i>MMAE-PAB-(Cit-Val-Glu)-BCY3900</i>	40157	4.8	0.20	0.64
BCY10981	<i>MMAE-PAB-(Dab-Val-Glu)- BCY3900</i>	30674	3.9	0.15	0.60
BCY10985	MMAE-PAB-(Dab-1Nal-Glu)- BCY3900	26393	1.0	0.18	2.48

Rat

- 10 The results of the rat PK experiments shown in Figures 7 and 8 and Table 9 show that BCY10984 has an extended half-life compared to the Cit-Val analogue BCY7761. There is also less free MMAE toxin in plasma (relative to intact parent).

Table 9: Summary of Pharmacokinetic Analysis in Rat

15

Compound	Linker	PPB (% Unbound)	t _{1/2} (h)	AUC ratio
BCY7761	Cit-Val	11%	0.49	0.00445
BCY10984	Cit-1Nal-Glu	4%	1.83	0.0000936

Example 5: Tumour Reduction Efficacy and Toxin Levels in Tumour

The results shown in Figures 9 and 10 demonstrate that BCY10984 (Cit-1Nal-Glu linker) shows higher efficacy than BCY7761 (BT1769 – Cit-Val linker) in a mouse CDX model

(HT1080 cells) with full tumour clearance demonstrated at 1mg/kg and 3 mg/kg following one dose. Body weights of the animals are not affected at these doses.

- The results shown in Figures 11 to 15 demonstrate that higher levels of MMAE toxin are observed in the tumour when BCY10984 is administered to mice bearing HT1080 tumours compared to BT1769 at the same dose. Similar levels of MMAE are present in the plasma and muscle tissue.

Example 6: *In vivo* Efficacy Study using Linkers of the Invention in the HT1080

Xenograft Model in BALB/c Nude Mice

(a) Study Objective

- The objective of this study was to evaluate the *in vivo* therapeutic efficacy of BCY10984 and BCY12951 (a drug conjugate containing the same linker as BCY10984 but conjugated to a non-binding bicyclic peptide ligand, i.e. having the composition: MMAE-PAB-(Dab-Val-Glu)-Non-Binding Bicyclic Peptide) in the HT1080 xenograft model in BALB/c nude mice.

(b) Experimental Design

Group	N	Treatment	Dosage	Dosing Volume	Route	Schedule
1	5	Vehicle	--	10 mL/kg	iv	qw×2
2	5	BCY10984	5 µM	10 mL/kg	iv	qw×3
3	5	BCY10984	15 µM	10 mL/kg	iv	qw×3
4	5	BCY10984	45 µM	10 mL/kg	iv	qw×3
5	5	BCY12951	5 µM	10 mL/kg	iv	qw×2
6	5	BCY12951	15 µM	10 mL/kg	iv	qw×2
7	5	BCY12951	45 µM	10 mL/kg	iv	qw×3

Note: Mice were monitored to Day 39 after 2~3 dosing cycles.

Mice from Group 5 and 6 were dosed with 45 µM BCY10984 on Day 14 and Day 28.

(c) Materials

(i) Animals and Housing Condition

Animals

- Species: *Mus Musculus*
 Strain: BALB/c nude
 Age: 6-8 weeks
 Sex: female

Body weight: 18-22 g

Number of animals: 35 mice plus spare

Animal supplier: Shanghai Lingchang Biotechnology Experimental Animal Co., LTD.

Housing condition

- 5 The mice were kept in individual ventilation cages at constant temperature and humidity with 5 animals in each cage.

- Temperature: 20~26°C.
- Humidity 40-70%.

- 10 Cages: Made of polycarbonate. The size is 375 mm x 215 mm x 180 mm. The bedding material is corn cob, which is changed twice per week.

Diet: Animals had free access to irradiation sterilized dry granule food during the entire study period.

Water: Animals had free access to sterile drinking water.

- 15 Cage identification: The identification labels for each cage contained the following information: number of animals, sex, strain, the date received, treatment, study number, group number and the starting date of the treatment.

Animal identification: Animals were marked by ear coding.

(d) Experimental Methods and Procedures

(i) Cell Culture

- 20 The HT1080 cells were maintained in EMEM medium supplemented with 10% heat inactivated fetal bovine serum at 37°C in an atmosphere of 5% CO₂ in air. The tumor cells were routinely sub-cultured twice weekly. The cells growing in an exponential growth phase were harvested and counted for tumor inoculation.

(ii) Tumor Inoculation

- 25 Each mouse was inoculated subcutaneously at the right flank with HT1080 tumor cells (5 x 10⁶) in 0.2 ml of PBS for tumor development. Animals were randomized when the average tumor volume reached 320 mm³ for efficacy study. The test article administration and the animal numbers in each group were shown in the experimental design table.

(iii) Testing Article Formulation Preparation

Treatment	Conc. (mg/mL)	Formulation
Vehicle	--	25 mM Histidine 10% Sucrose pH=7
BCY10984	0.4	Dissolve 2.34 mg BCY10984 in 5.583 mL His-buffer*.

	0.188	Dilute 0.703 mL 0.4 mg/mL BCY10948 stock with 0.797 mL His-buffer.
	0.063	Dilute 0.234 mL 0.4 mg/mL BCY10948 stock with 1.266 mL His-buffer.
	0.021	Dilute 0.078 mL 0.4 mg/mL BCY10948 stock with 1.422 mL His-buffer.
BCY12951	0.4	Dissolve 2.5 mg BCY12951 in 5.645 mL His-buffer.
	0.185	Dilute 0.693 mL 0.4 mg/mL BCY12951 stock with 0.807 mL His-buffer.
	0.062	Dilute 0.231 mL 0.4 mg/mL BCY12951 stock with 1.438 mL His-buffer.
	0.021	Dilute 0.077 mL 0.4 mg/mL BCY12951 stock with 1.479 mL His-buffer.
*His-buffer: 25 mM Histidine 10% Sucrose pH=7		

(iv) Observations

All the procedures related to animal handling, care and the treatment in the study were performed according to the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of WuXi AppTec, following the guidance of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). At the time of routine monitoring, the animals were checked for any effects of tumor growth and treatments on normal behavior such as mobility, food and water consumption (by observation only), body weight gain/loss, eye/hair matting and any other abnormal effect as stated in the protocol. Death and observed clinical signs were recorded on the basis of the numbers of animals within each subset.

(v) Tumor Measurements and the Endpoints

The major endpoint was to see if the tumor growth could be delayed or mice could be cured. Tumor volume was measured three times weekly in two dimensions using a caliper, and the volume was expressed in mm³ using the formula: $V = 0.5 a \times b^2$ where a and b are the long and short diameters of the tumor, respectively. The tumor size was then used for calculations of T/C value. The T/C value (in percent) is an indication of antitumor effectiveness; T and C are the mean volumes of the treated and control groups, respectively, on a given day.

TGI was calculated for each group using the formula: $TGI (\%) = [1 - (T_i - T_0) / (V_i - V_0)] \times 100$; T_i is the average tumor volume of a treatment group on a given day, T_0 is the average tumor volume of the treatment group on the day of treatment start, V_i is the average tumor volume of the

vehicle control group on the same day with T_i , and V_0 is the average tumor volume of the vehicle group on the day of treatment start.

(vi) Statistical Analysis

Summary statistics, including mean and the standard error of the mean (SEM), were provided for the tumor volume of each group at each time point.

Statistical analysis of difference in tumor volume among the groups was conducted on the data obtained at the best therapeutic time point after the final dose.

A one-way ANOVA was performed to compare tumor volume among groups, and when a significant F-statistics (a ratio of treatment variance to the error variance) was obtained, comparisons between groups were carried out with Games-Howell test. A two-tailed T test was performed to compare tumor volume between two groups. All data were analyzed using GraphPad 5.0. $P < 0.05$ was considered to be statistically significant.

(e) Results

(i) Tumor Growth Curve

Tumor growth curves are shown in Figures 16 and 17.

(ii) Tumor Volume Trace

Mean tumor volume over time in female BALB/c nude mice bearing HT1080 tumor is shown in Table 10:

Table 10: Tumor volume trace over time

Group	Treatment	Days after the start of treatment					
		0	2	4	6	9	11
1	Vehicle, iv, qw	321±45	505±42	742±73	920±97	1297±133	1731±194
2	BCY10984, 5 µM, iv, qw	320±48	508±49	681±64	750±50	958±53	1257±95
3	BCY10984, 15 µM, iv, qw	321±43	519±76	428±88	378±128	304±112	288±126
4	BCY10984, 45 µM, iv, qw	320±32	489±68	285±90	139±46	29±10	15±6
5	BCY12951, 5 µM, iv, qw	321±42	529±51	668±68	720±69	818±98	1011±140

6	BCY12951, 15 μ M, iv, qw	321 $\pm$ 37	528 $\pm$ 67	687 $\pm$ 91	830 $\pm$ 113	1026 $\pm$ 88	1354 $\pm$ 145
7	BCY12951, 45 μ M, iv, qw	321 $\pm$ 30	522 $\pm$ 34	545 $\pm$ 20	584 $\pm$ 22	755 $\pm$ 20	901 $\pm$ 59

(iii) Tumor Growth Inhibition Analysis

Tumor growth inhibition rate for BCY10984 and BCY12951 in HT1080 xenograft model was calculated based on tumor volume measurements on day 11 after the start of the treatment.

5

Table 11: Tumor growth inhibition analysis

Group	Treatment	Tumor Volume (mm ³) ^a	T/C ^b (%)	TGI (%)	<i>p</i> value (vs Vehicle)	<i>p</i> value (BCY10984 vs BCY12951)
1	Vehicle, iv, qw	1731 $\pm$ 194	--	--	--	
2	BCY10984, 5 μ M, iv, qw	1257 $\pm$ 95	72.6	33.6	<i>p</i> >0.05	<i>p</i> >0.05 (vs Gr5)
3	BCY10984, 15 μ M, iv, qw	288 $\pm$ 126	16.6	102.3	<i>p</i> <0.001	<i>p</i> <0.001 (vs Gr6)
4	BCY10984, 45 μ M, iv, qw	15 $\pm$ 6	0.9	121.6	<i>p</i> <0.001	<i>p</i> <0.001 (vs Gr7)
5	BCY12951, 5 μ M, iv, qw	1011 $\pm$ 140	58.4	51.1	<i>p</i> <0.01	-
6	BCY12951, 15 μ M, iv, qw	1354 $\pm$ 145	78.2	26.7	<i>p</i> >0.05	-
7	BCY12951, 45 μ M, iv, qw	901 $\pm$ 59	52.0	58.9	<i>p</i> <0.001	-

a. Mean $\pm$ SEM;

b. Tumor Growth Inhibition is calculated by dividing the group average tumor volume for the treated group by the group average tumor volume for the control group (T/C).

(f) Results Summary and Discussion

In this study, the therapeutic efficacy of BCY10984 and BCY12951 in HT1080 xenograft model was evaluated. The measured tumor volume of all treatment groups at various time points are shown in Figures 16 and 17 and Tables 10 and 11.

The mean tumor volume of vehicle treated mice reached 1731 mm³ on day 11 after the start of treatment. BCY10984 at 5 μM qw (TV=1257 mm³, TGI=33.6%, $p>0.05$), 15 μM qw (TV=288 mm³, TGI=102.3%, $p<0.001$) and 45 μM qw (TV=15 mm³, TGI=121.6%, $p<0.001$) showed dose-dependent anti-tumor activity. Among them, the BCY10984 at 45 μM qw eradicated the tumors completely on day 16. BCY12951 at 5 μM qw (TV=1011 mm³, TGI=51.1%, $p<0.01$) and 45 μM qw (TV=901 mm³, TGI=58.9%, $p<0.001$) showed significant anti-tumor activity.

When comparing the anti-tumor efficacy between the two test articles, the BCY10984 at 5 μM showed comparable anti-tumor efficacy with BCY12951 5 μM ($p>0.05$), BCY10984 at 15 μM and 45 μM showed more potent efficacy than BCY12951 at the same molar dosage (BCY10984 15 μM vs BCY12951 15μM, $p<0.001$; BCY10984 45 μM vs BCY12951 45 μM, $p<0.001$).

7 animals in group 5 and 6 were treated with BCY10984 45 μM at an average starting tumor size of 1291 mm³. All mice showed sudden tumor regression after the first dosing, and all tumors were eradicated completely after the second dosing.

CLAIMS

1. A linker comprising a -P1-P2-P3- moiety, wherein:
P1 represents a basic non-natural amino acid or a derivative thereof;
5 P2 represents a hydrophobic amino acid or a hydrophobic non-natural amino acid;
and
P3 is either absent or represents an acidic amino acid or an acidic non-natural amino acid, such that when P1 represents Cit and P2 represents Val, then P3 must represent an acidic non-natural amino acid.
- 10 2. The linker according to claim 1, wherein P1 represents a basic non-natural amino acid selected from: 2-amino-4-guanidinobutanoic acid (Agb); 2-amino-4-(3-methylguanidino)butanoic acid (Agb(Me)); 2,4-diaminobutanoic acid (Dab); 2,3-diaminopropanoic acid (Dap); 2-amino-3-guanidinopropanoic acid (Dap(CNNH₂)); and
15 citrulline (Cit), such as citrulline (Cit).
3. The linker according to claim 1 or claim 2, wherein P2 represents a hydrophobic amino acid selected from Ala, Gly, Ile, Leu, Met, Phe, Pro, Trp and Val or a hydrophobic non-natural amino acid selected from cyclobutyl, diphenylalanine (Dpa), 1-naphthylalanine
20 (1Nal), 2-Naphthylalanine (2Nal) and methyltryptophan (Trp(Me)), such as a hydrophobic amino acid selected from Val or a non-natural amino acid selected from cyclobutyl, Dpa, 1Nal and 2Nal, such as 1-naphthylalanine (1Nal).
4. The linker according to any one of claims 1 to 3, wherein P3 represents an acidic
25 amino acid selected from Asp and Glu, such as an acidic amino acid selected from Glu.
5. The linker according to any one of claims 1 to 4, wherein the -P1-P2-P3- moiety represents:

P1	P2	P3
Agb	Val	Absent
Agb(Me)	Val	Absent
Dab	Val	Absent
Dap	Val	Absent
Dap(CNNH ₂)	Val	Absent
Agb	Val	Glu

Cit	1Nal	Glu
Dab	cBu	Glu
Dab	Dpa	Glu
Dab	1Nal	Glu
Dab	2Nal	Glu
Dab	Val	Glu
Dap	Val	Glu
Dap (CNNH ₂)	Val	Glu

6. A drug conjugate comprising a binding agent which binds to a target and a cytotoxic agent, wherein said binding agent is joined to said cytotoxic agent via a linker according to any one of claims 1 to 5.

5

7. The drug conjugate according to claim 6, wherein said binding agent is a peptide, such as an antibody or bicyclic peptide, in particular a bicyclic peptide.

8. The drug conjugate according to claim 6 or claim 7, wherein said cytotoxic agent is DM1 or MMAE, such as MMAE.

10

9. The drug conjugate according to any one of claims 6 to 8 which is protease resistant when compared with a conjugate in the absence of said linker, such as resistant to Cathepsin B cleavage.

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10. The drug conjugate according to any one of claims 6 to 9 which is plasma stable when compared with a conjugate in the absence of said linker.

11. The drug conjugate according to any one of claims 6 to 10 which is selected from: BCY10989, BCY10980, BCY10982, BCY10983, BCY10984, BCY10981, BCY10985, BCY10986, BCY10987, and BCY10988, such as BCY10984.

20

12. A pharmaceutical composition comprising the drug conjugate according to any one of claims 6 to 11 in combination with one or more pharmaceutically acceptable excipients.

25

13. The drug conjugate according to any one of claims 6 to 11 for use in preventing, suppressing or treating cancer.