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(71) Demandeur/Applicant:
BICYCLETX LIMITED, GB

(72) Inventeurs/Inventors:
KEEN, NICHOLAS, GB;
MCDONNELL, KEVIN, GB;
PARK, PETER, GB;
UPADHYAYA, PUNIT, GB;
MUDD, GEMMA, GB

(74) Agent: GOWLING WLG (CANADA) LLP

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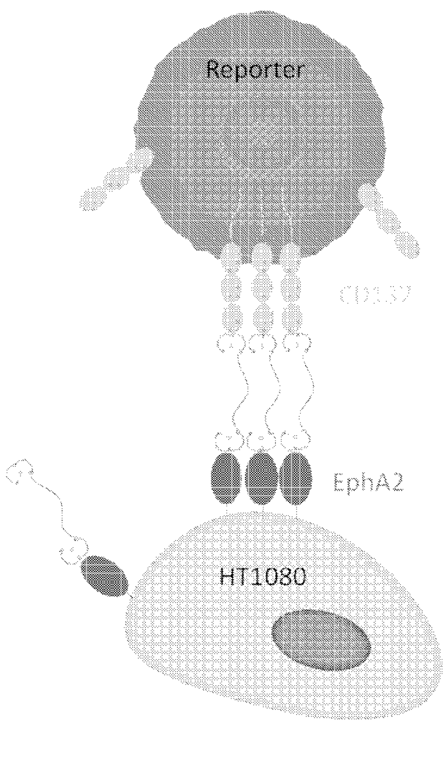

FIGURE 1

(57) Abrégé/Abstract:
The present invention relates to heterotandem bicyclic peptide complexes which comprise a first peptide ligand, which binds to a component present on an immune cell, conjugated via a linker to a second peptide ligand, which binds to a component present on a cancer cell. The invention also relates to the use of said heterotandem bicyclic peptide complexes in preventing, suppressing or treating cancer.

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(71) Applicant: **BICYCLETX LIMITED** [—/GB]; B900, Babraham Research Campus, Cambridge CB22 3AT (GB).

(72) Inventors: **KEEN, Nicholas**; c/o BicycleTx Limited, Building B900, Babraham Research Campus, Cambridge CB22 3AT (GB). **MCDONNELL, Kevin**; c/o BicycleTx Limited, Building B900, Babraham Research Campus, Cambridge CB22 3AT (GB). **PARK, Peter**; c/o BicycleTx Limited, Building B900, Babraham Research Campus, Cambridge CB22 3AT (GB). **UPADHYAYA, Punit**; c/o BicycleTx Limited, B900, Babraham Research Campus, Cambridge CB22 3AT (GB).

(74) Agent: **GIBSON, Mark** et al.; Sagittarius IP, Marlow International, Parkway, Marlow Buckinghamshire SL7 1YL (GB).

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(54) Title: HETEROTANDEM BICYCLIC PEPTIDE COMPLEXES

(57) Abstract: The present invention relates to heterotandem bicyclic peptide complexes which comprise a first peptide ligand, which binds to a component present on an immune cell, conjugated via a linker to a second peptide ligand, which binds to a component present on a cancer cell. The invention also relates to the use of said heterotandem bicyclic peptide complexes in preventing, suppressing or treating cancer.

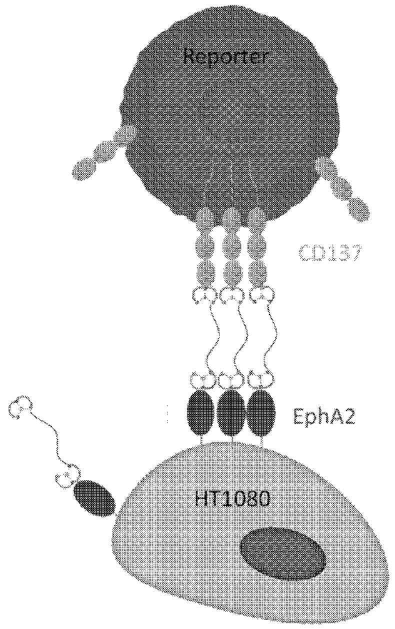

FIGURE 1

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HETEROTANDEM BICYCLIC PEPTIDE COMPLEXES

FIELD OF THE INVENTION

The present invention relates to heterotandem bicyclic peptide complexes which comprise a first peptide ligand, which binds to a component present on an immune cell, conjugated via a linker to a second peptide ligand, which binds to a component present on a cancer cell. The invention also relates to the use of said heterotandem bicyclic peptide complexes in preventing, suppressing or treating cancer.

BACKGROUND OF THE INVENTION

Cyclic peptides are able to bind with high affinity and target specificity to protein targets and hence are an attractive molecule class for the development of therapeutics. In fact, several cyclic peptides are already successfully used in the clinic, as for example the antibacterial peptide vancomycin, the immunosuppressant drug cyclosporine or the anti-cancer drug octreotide (Driggers *et al*. (2008), Nat Rev Drug Discov *7* (7), 608-24). Good binding properties result from a relatively large interaction surface formed between the peptide and the target as well as the reduced conformational flexibility of the cyclic structures. Typically, macrocycles bind to surfaces of several hundred square angstrom, as for example the cyclic peptide CXCR4 antagonist CVX15 (400 Å^2; Wu *et al*. (2007), Science 330, 1066-71), a cyclic peptide with the Arg-Gly-Asp motif binding to integrin αVb3 (355 Å^2) (Xiong *et al*. (2002), Science *296* (5565), 151-5) or the cyclic peptide inhibitor upain-1 binding to urokinase-type plasminogen activator (603 Å^2; Zhao *et al*. (2007), J Struct Biol 160 (1), 1-10).

Due to their cyclic configuration, peptide macrocycles are less flexible than linear peptides, leading to a smaller loss of entropy upon binding to targets and resulting in a higher binding affinity. The reduced flexibility also leads to locking target-specific conformations, increasing binding specificity compared to linear peptides. This effect has been exemplified by a potent and selective inhibitor of matrix metalloproteinase 8, MMP-8) which lost its selectivity over other MMPs when its ring was opened (Cherney *et al*. (1998), J Med Chem 41 (11), 1749-51). The favorable binding properties achieved through macrocyclization are even more pronounced in multicyclic peptides having more than one peptide ring as for example in vancomycin, nisin and actinomycin.

Different research teams have previously tethered polypeptides with cysteine residues to a synthetic molecular structure (Kemp and McNamara (1985), J. Org. Chem; Timmerman *et al*. (2005), ChemBioChem). Meloen and co-workers had used tris(bromomethyl)benzene and related molecules for rapid and quantitative cyclisation of multiple peptide loops onto synthetic scaffolds for structural mimicry of protein surfaces (Timmerman *et al.* (2005), ChemBioChem). Methods for the generation of candidate drug compounds wherein said compounds are generated by linking cysteine containing polypeptides to a molecular scaffold as for example tris(bromomethyl)benzene are disclosed in WO 2004/077062 and WO 2006/078161.

Phage display-based combinatorial approaches have been developed 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). Briefly, combinatorial libraries of linear peptides containing three cysteine residues and two regions of six random amino acids (Cys-$(Xaa)_6$-Cys-$(Xaa)_6$-Cys) were displayed on phage and cyclised by covalently linking the cysteine side chains to a small molecule (tris-(bromomethyl)benzene).

SUMMARY OF THE INVENTION

According to a first aspect of the invention, there is provided a heterotandem bicyclic peptide complex comprising:

(a) a first peptide ligand which binds to a component present on an immune cell; conjugated via a linker to

(b) a second peptide ligand which binds to a component present on a cancer cell; wherein each of said peptide ligands comprise a polypeptide comprising at least three cysteine residues, separated by at least two loop sequences, and a molecular scaffold which forms covalent bonds with the cysteine residues of the polypeptide such that at least two polypeptide loops are formed on the molecular scaffold.

According to a further aspect of the invention, there is provided a pharmaceutical composition comprising a heterotandem bicyclic peptide complex as defined herein in combination with one or more pharmaceutically acceptable excipients.

According to a further aspect of the invention, there is provided a heterotandem bicyclic peptide complex as defined herein for use in preventing, suppressing or treating cancer.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Schematic representation of a heterotandem bicyclic peptide complex comprising an EphA2 and CD137 peptide ligand binding to both an immune cell and a cancer cell.

Figure 2: Structure and composition of the EphA2-CD137 heterotandem bicyclic peptide complex BCY7985.

Figure 3: Analysis of the EphA2-CD137 heterotandem bicyclic peptide complex BCY7985 in the Promega CD137 luciferase reporter assay (CS196008) in the presence of EphA2-expressing HT1080 cells.

Figure 4: EphA2/CD137 heterotandems are active in CD137 reporter assay and the fold induction of activation is dependent on tumour target expression level on the cell line used in co-culture.

Figure 5: EphA2/CD137 heterotandems induce tumour cell killing in primary human T-cell and cancer cell co-culture assay. Tumour cell killing is evaluated by counting viable Nuclight red positive tumour cells over time. A Caspase 3/7 dye is used to identify apoptotic tumour cells.

Figure 6: Nectin-4/CD137 heterotandems are active in CD137 reporter assay and the fold induction of activation is dependent on tumour target expression level on the cell line (HT1376:Nectin-4 high and NCI-H292: Nectin-4 Medium) used in co-culture.

Figure 7: Nectin-4/CD137 heterotandems induce IL-2 and IFN-γ cytokine secretion in a PBMC-4T1 co-culture assay. BCY9350 and BCY9351 are non-binding controls for Nectin-4 and CD137 respectively.

Figure 8: Nectin-4/CD137 heterotandems induce target dependent cytokine release in ex-vivo cultures of primary patient-derived lung tumours. (A) *Ex vivo* patient derived tumour cells form 3D spheroids within 4h in culture, 10X image under light microscope. (B) Flow analysis of Nectin-4 expression in patient derived tumour samples from 3 donors. (C) Table indicates %$CD137^+$ T cells and Nectin-4^+ cells in 3 donor samples. (D) Heatmap indicating % change in immune markers (normalized to vehicle) in response to treatment with control/test compounds. (E) %$CD8^+ki67^+$ T cells in response to treatment with control/test compounds (vehicle indicated with dotted line).

Figure 9: PD-L1/CD137 heterotandems are active in CD137 reporter assay in presence of PD-L1 expressing cell line RKO.

Figure 10: Pharmacokinetics of heterotandems in SD Rats: BCY10572 and BCY10000 were dosed IV at 2 mg/kg (n =3).

DETAILED DESCRIPTION OF THE INVENTION

According to a first aspect of the invention, there is provided a heterotandem bicyclic peptide complex comprising:

(a) a first peptide ligand which binds to a component present on an immune cell; conjugated via a linker to

(b) a second peptide ligand which binds to a component present on a cancer cell; wherein each of said peptide ligands comprise a polypeptide comprising at least three cysteine residues, separated by at least two loop sequences, and a molecular scaffold which forms covalent bonds with the cysteine residues of the polypeptide such that at least two polypeptide loops are formed on the molecular scaffold.

First Peptide Ligands

References herein to the term “immune cell” includes any cell within the immune system. Suitable examples include white blood cells, such as lymphocytes (e.g. T lymphocytes or T cells, B cells or natural killer cells). In one embodiment, the T cell is CD8 or CD4. In a further embodiment, the T cell is CD8. Other examples of immune cells include dendritic cells, follicular dendritic cells and granulocytes.

In one embodiment, the component present on an immune cell is CD137.

CD137 is a member of the tumour necrosis factor (TNF) receptor family. Its alternative names are tumour necrosis factor receptor superfamily member 9 (TNFRSF9), 4- IBB and induced by lymphocyte activation (ILA). CD137 can be expressed by activated T cells, but to a larger extent on CD8+ than on CD4+ T cells. In addition, CD137 expression is found on dendritic cells, follicular dendritic cells, natural killer cells, granulocytes and cells of blood vessel walls at sites of inflammation. One characterized activity of CD137 is its costimulatory activity for activated T cells. Crosslinking of CD137 enhances T cell proliferation, IL-2 secretion, survival and cytolytic activity. Further, it can enhance immune activity to eliminate tumours in mice.

CD137 is a T-cell costimulatory receptor induced on TCR activation (Nam et al., Curr. Cancer Drug Targets, 5:357-363 (2005); Waits et al., Annu. Rev, Immunol., 23:23-68 (2005)). In addition to its expression on activated CD4+ and CD8+ T cells, CD137 is also expressed on CD4+CD25+ regulatory T cells, natural killer (NK) and NK-T cells, monocytes, neutrophils, and dendritic cells. Its natural ligand, CD137L, has been described on antigen- presenting cells including B cells, monocyte/macrophages, and dendritic cells (Watts et al. Annu. Rev. Immunol, 23:23-68 (2005)). On interaction with its ligand, CD137 leads to increased TCR-induced T-cell proliferation, cytokine production, functional maturation, and prolonged CD8+ T-cell survival (Nam et al, Curr. Cancer Drug Targets, 5:357-363 (2005), Watts et d - l., Annu. Rev. Immunol, 23:23-68 (2005)).

Signalling through CD137 by either CD137L or agonistic monoclonal antibodies (mAbs) against CD137 leads to increased TCR-induced T cell proliferation, cytokine production and functional maturation, and prolonged CD8+ T cell survival. These effects result from: (1) the activation of the NF-κB, c-Jun NH2-terminal kinase/stress-activated protein kinase

(JNK/SAPK), and p38 mitogen-activated protein kinase (MAPK) signalling pathways, and (2) the control of anti-apoptotic and cell cycle -related gene expression.

Experiments performed in both CD137 and CD137L-deficient mice have additionally demonstrated the importance of CD137 costimulation in the generation of a fully competent T cell response.

IL-2 and IL-15 activated NK cells express CD137, and ligation of CD137 by agonistic mAbs stimulates NK cell proliferation and IFN-γ secretion, but not their cytolytic activity.

Furthermore, CD137-stimulated NK cells promote the expansion of activated T cells *in vitro*.

In accordance with their costimulatory function, agonist mAbs against CD137 have been shown to promote rejection of cardiac and skin allografts, eradicate established tumours, broaden primary antiviral CD8+ T cell responses, and increase T cell cytolytic potential. These studies support the view that CD137 signalling promotes T cell function which may enhance immunity against tumours and infection.

In one embodiment, the first peptide ligand comprises a CD137 binding bicyclic peptide ligand.

Suitable examples of CD137 binding bicyclic peptide ligands are disclosed in GB Patent Application Nos. 1712589.9 and 1802934.8, the peptides of which are incorporated herein by reference.

In one embodiment, the CD137 binding bicyclic peptide ligand comprises an amino acid sequence:

C_iIEEGQYC_{ii}FADPY[Nle]C_{iii} (SEQ ID NO: 1);

C_i[tBuAla]PE[D-Ala]PYC_{ii}FADPY[Nle]C_{iii} (SEQ ID NO: 3);

C_iIEEGQYC_{ii}F[D-Ala]DPY[Nle]C_{iii} (SEQ ID NO: 4);

C_i[tBuAla]PK[D-Ala]PYC_{ii}FADPY[Nle]C_{iii} (SEQ ID NO: 5);

C_i[tBuAla]PE[D-Lys]PYC_{ii}FADPY[Nle]C_{iii} (SEQ ID NO: 6);

C_i[tBuAla]P[K(PYA)][D-Ala]PYC_{ii}FADPY[Nle]C_{iii} (SEQ ID NO: 7);

C_i[tBuAla]PE[D-Lys(PYA)]PYC_{ii}FADPY[Nle]C_{iii} (SEQ ID NO: 8);

C_iIEE[D-Lys(PYA)]QYC_{ii}FADPY(Nle)C_{iii} (SEQ ID NO: 9); and

[dC_i][dI][dE][dE][K(PYA)][dQ][dY][dC_{ii}][dF][dA][dD][dP][dY][dNle][dC_{iii}] (SEQ ID NO: 10);

wherein C_i, C_{ii} and C_{iii} represent first, second and third cysteine residues, respectively, Nle represents norleucine, tBuAla represents t-butyl-alanine, PYA represents 4-pentynoic acid, or a pharmaceutically acceptable salt thereof.

In one particular embodiment which may be mentioned, the CD137 binding bicyclic peptide ligand comprises an amino acid sequence:

C_iIEEGQYC_{ii}FADPY[Nle]C_{iii} (SEQ ID NO: 1);

wherein C_i, C_{ii} and C_{iii} represent first, second and third cysteine residues, respectively, Nle represents norleucine, or a pharmaceutically acceptable salt thereof.

In a further embodiment, the CD137 binding bicyclic peptide ligand comprises N- and C-terminal modifications and comprises:

Ac-A-(SEQ ID NO: 1)-Dap (hereinafter referred to as BCY7732);
Ac-A-(SEQ ID NO: 1)-Dap(PYA) (hereinafter referred to as BCY7741);
Ac-(SEQ ID NO: 3)-Dap (hereinafter referred to as BCY9172);
Ac-(SEQ ID NO: 3)-Dap(PYA) (hereinafter referred to as BCY11014);
Ac-A-(SEQ ID NO: 4)-Dap (hereinafter referred to as BCY8045);
Ac-(SEQ ID NO: 5)-A (hereinafter referred to as BCY8919);
Ac-(SEQ ID NO: 6)-A (hereinafter referred to as BCY8920);
Ac-(SEQ ID NO: 7)-A (hereinafter referred to as BCY8927);
Ac-(SEQ ID NO: 8)-A (hereinafter referred to as BCY8928);
Ac-A-(SEQ ID NO: 9)-A (hereinafter referred to as BCY7744); and
Ac-[dA]-(SEQ ID NO: 10)-[dA]-NH_2 (hereinafter referred to as BCY11506);

wherein Ac represents an acetyl group, Dap represents diaminopropionic acid and PYA represents 4-pentynoic acid, or a pharmaceutically acceptable salt thereof.

In a further embodiment which may be mentioned, the CD137 binding bicyclic peptide ligand comprises N- and C-terminal modifications and comprises:

Ac-A-(SEQ ID NO: 1)-Dap (hereinafter referred to as BCY7732);

wherein Ac represents an acetyl group and Dap represents diaminopropionic acid, or a pharmaceutically acceptable salt thereof.

Second Peptide Ligands

References herein to the term "cancer cell" includes any cell which is known to be involved in cancer. Cancer cells are created when the genes responsible for regulating cell division are damaged. Carcinogenesis is caused by mutation and epimutation of the genetic material of normal cells, which upsets the normal balance between proliferation and cell death. This results in uncontrolled cell division and the evolution of those cells by natural selection in the body. The uncontrolled and often rapid proliferation of cells can lead to benign or malignant tumours (cancer). Benign tumors do not spread to other parts of the body or invade other tissues. Malignant tumors can invade other organs, spread to distant locations (metastasis) and become life-threatening.

In one embodiment, the cancer cell is selected from an HT1080, SC-OV-3, PC3, H1376, NCI-H292, LnCap, MC38, 4T1-D02 and RKO tumor cell.

In one embodiment, the component present on a cancer cell is EphA2.

Eph receptor tyrosine kinases (Ephs) belong to a large group of receptor tyrosine kinases (RTKs), kinases that phosphorylate proteins on tyrosine residues. Ephs and their membrane bound ephrin ligands (ephrins) control cell positioning and tissue organization (Poliakov *et al.* (2004) Dev Cell 7, 465-80). Functional and biochemical Eph responses occur at higher ligand oligomerization states (Stein *et al.* (1998) Genes Dev 12, 667-678).

Among other patterning functions, various Ephs and ephrins have been shown to play a role in vascular development. Knockout of EphB4 and ephrin-B2 results in a lack of the ability to remodel capillary beds into blood vessels (Poliakov *et al.*, *supra*) and embryonic lethality. Persistent expression of some Eph receptors and ephrins has also been observed in newly-formed, adult micro-vessels (Brantley-Sieders *et al.* (2004) Curr Pharm Des 10, 3431-42; Adams (2003) J Anat 202, 105-12).

The de-regulated re-emergence of some ephrins and their receptors in adults also has been observed to contribute to tumor invasion, metastasis and neo-angiogenesis (Nakamoto *et al.* (2002) Microsc Res Tech 59, 58-67; Brantley-Sieders *et al.*, *supra*). Furthermore, some Eph family members have been found to be over-expressed on tumor cells from a variety of human tumors (Brantley-Sieders *et al.*, *supra*); Marme (2002) Ann Hematol 81 Suppl 2, S66; Booth *et al.* (2002) Nat Med 8, 1360-1).

EPH receptor A2 (ephrin type-A receptor 2) is a protein that in humans is encoded by the *EPHA2* gene.

EphA2 is upregulated in multiple cancers in man, often correlating with disease progression, metastasis and poor prognosis e.g.: breast (Zelinski *et al* (2001) Cancer Res. 61, 2301–2306; Zhuang *et al* (2010) Cancer Res. 70, 299–308; Brantley-Sieders *et al* (2011) PLoS

One 6, e24426), lung (Brannan *et al* (2009) Cancer Prev Res (Phila) 2, 1039–1049; Kinch *et al* (2003) Clin Cancer Res. 9, 613-618; Guo *et al* (2013) J Thorac Oncol. 8, 301-308), gastric (Nakamura *et al* (2005) Cancer Sci. 96, 42-47; Yuan *et al* (2009) Dig Dis Sci 54, 2410-2417), pancreatic (Mudali *et al* (2006) Clin Exp Metastasis 23, 357-365), prostate (Walker-Daniels *et al* (1999) Prostate 41, 275–280), liver (Yang *et al* (2009) Hepatol Res. 39, 1169–1177) and glioblastoma (Wykosky *et al* (2005) Mol Cancer Res. 3, 541–551; Li *et al* (2010) Tumour Biol. 31, 477–488).

The full role of EphA2 in cancer progression is still not defined although there is evidence for interaction at numerous stages of cancer progression including tumour cell growth, survival, invasion and angiogenesis. Downregulation of EphA2 expression suppresses tumour cancer cell propagation (Binda *et al* (2012) Cancer Cell 22, 765-780), whilst EphA2 blockade inhibits VEGF induced cell migration (Hess *et al* (2001) Cancer Res. 61, 3250–3255), sprouting and angiogenesis (Cheng *et al* (2002) Mol Cancer Res. 1, 2–11; Lin *et al* (2007) Cancer 109, 332-40) and metastatic progression (Brantley-Sieders *et al* (2005) FASEB J. 19, 1884–1886).

An antibody drug conjugate to EphA2 has been shown to significantly diminish tumour growth in rat and mouse xenograft models (Jackson *et al* (2008) Cancer Research 68, 9367-9374) and a similar approach has been tried in man although treatment had to be discontinued for treatment related adverse events (Annunziata *et al* (2013) Invest New drugs 31, 77-84).

In one embodiment, the second peptide ligand comprises an EphA2 binding bicyclic peptide ligand.

Suitable examples of EphA2 binding bicyclic peptide ligands are disclosed in GB Patent Application Nos. 1721259.8 and 1804102.0, the peptides of which are incorporated herein by reference.

In one embodiment, the EphA2 binding bicyclic peptide ligand comprises an amino acid sequence:

C_i[HyP]LVNPLC_{ii}LHP[dD]W[HArg]C_{iii} (SEQ ID NO: 2); and

C_iLWDPTPC_{ii}ANLHL[HArg]C_{iii} (SEQ ID NO: 11);

wherein C_i, C_{ii} and C_{iii} represent first, second and third cysteine residues, respectively, HyP represents hydroxyproline, dD represents aspartic acid in D-configuration and HArg represents homoarginine, or a pharmaceutically acceptable salt thereof.

In one embodiment which may be mentioned, the EphA2 binding bicyclic peptide ligand comprises an amino acid sequence:

C_i[HyP]LVNPLC_{ii}LHP[dD]W[HArg]C_{iii} (SEQ ID NO: 2);

wherein C_i, C_{ii} and C_{iii} represent first, second and third cysteine residues, respectively, HyP represents hydroxyproline, dD represents aspartic acid in D-configuration and HArg represents homoarginine, or a pharmaceutically acceptable salt thereof.

In a further embodiment, the EphA2 binding bicyclic peptide ligand comprises N-terminal modifications and comprises:

A-HArg-D-(SEQ ID NO: 2) (hereinafter referred to as BCY9594);

[B-Ala]-[Sar_{10}]-A-[HArg]-D-(SEQ ID NO: 2) (hereinafter referred to as BCY6099);

[PYA]-[B-Ala]-[Sar_{10}]-A-[HArg]-D-(SEQ ID NO: 2) (hereinafter referred to as BCY6169); and

[PYA]-[B-Ala]-[Sar_{10}]-VGP-(SEQ ID NO: 11) (hereinafter referred to as BCY8941);

wherein HArg represents homoarginine, PYA represents 4-pentynoic acid, Sar_{10} represents 10 sarcosine units, B-Ala represents beta-alanine, or a pharmaceutically acceptable salt thereof.

In a further embodiment which may be mentioned, the EphA2 binding bicyclic peptide ligand comprises N-terminal modifications and comprises:

A-HArg-D-(SEQ ID NO: 2) (hereinafter referred to as BCY9594).

wherein HArg represents homoarginine, or a pharmaceutically acceptable salt thereof.

In an alternative embodiment, the component present on a cancer cell is PD-L1.

Programmed cell death 1 ligand 1 (PD-L1) is a 290 amino acid type I transmembrane protein encoded by the CD274 gene on mouse chromosome 19 and human chromosome 9. PD-L1 expression is involved in evasion of immune responses involved in chronic infection, e.g., chronic viral infection (including, for example, HIV, HBV, HCV and HTLV, among others), chronic bacterial infection (including, for example, Helicobacter pylori, among others), and chronic parasitic infection (including, for example, Schistosoma mansoni). PD-L1 expression has been detected in a number of tissues and cell types including T-cells, B-cells, macrophages, dendritic cells, and nonhaematopoietic cells including endothelial cells, hepatocytes, muscle cells, and placenta.

PD-L1 expression is also involved in suppression of anti-tumour immune activity. Tumours express antigens that can be recognised by host T-cells, but immunologic clearance of tumours is rare. Part of this failure is due to immune suppression by the tumour microenvironment. PD-L1 expression on many tumours is a component of this suppressive milieu and acts in concert with other immunosuppressive signals. PD-L1 expression has been shown *in situ* on a wide variety of solid tumours including breast, lung, colon, ovarian, melanoma, bladder, liver, salivary, stomach, gliomas, thyroid, thymic epithelial, head, and neck (Brown JA *et al*. 2003 Immunol. 170:1257-66; Dong H *et al*. 2002 Nat. Med. 8:793-800; Hamanishi J, *et al*. 2007 Proc. Natl. Acad. Sci. USA 104:3360-65; Strome SE *et al*. 2003 Cancer Res. 63:6501-5; Inman BA *et al*. 2007 Cancer 109:1499-505; Konishi J *et al*. 2004 Clin. Cancer Res. 10:5094-100; Nakanishi J *et al*. 2007 Cancer Immunol. Immunother. 56:1173-82; Nomi T *et al*. 2007 Clin. Cancer Res. 13:2151-57; Thompson RH *et al*. 2004 Proc. Natl. Acad. Sci. USA 101: 17174-79; Wu C *et al*. 2006 Acta Histochem. 108:19-24). In addition, the expression of the receptor for PD-L1, Programmed cell death protein 1 (also known as PD-1 and CD279) is upregulated on tumour infiltrating lymphocytes, and this also contributes to tumour immunosuppression (Blank C *et al*. 2003 Immunol. 171:4574-81). Most importantly, studies relating PD-L1 expression on tumours to disease outcome show that PD-L1 expression strongly correlates with unfavourable prognosis in kidney, ovarian, bladder, breast, gastric, and pancreatic cancer (Hamanishi J *et al*. 2007 Proc. Natl. Acad. Sci. USA 104:3360-65; Inman BA *et al*. 2007 Cancer 109:1499-505; Konishi J *et al*. 2004 Clin. Cancer Res. 10:5094-100; Nakanishi J *et al*. 2007 Cancer Immunol. Immunother. 56:1173-82; Nomi T *et al*. 2007 Clin. Cancer Res. 13:2151-57; Thompson RH *et al*. 2004 Proc. Natl. Acad. Sci. USA 101:17174-79; Wu C *et al*. 2006 Acta Histochem. 108:19-24). In addition, these studies suggest that higher levels of PD-L1 expression on tumours may facilitate advancement of tumour stage and invasion into deeper tissue structures.

The PD-1 pathway can also play a role in haematologic malignancies. PD-L1 is expressed on multiple myeloma cells but not on normal plasma cells (Liu J *et al*. 2007 Blood 110:296-304). PD-L1 is expressed on some primary T-cell lymphomas, particularly anaplastic large cell T lymphomas (Brown JA *et al*, 2003 Immunol. 170:1257-66). PD-1 is highly expressed on the T-cells of angioimmunoblastic lymphomas, and PD-L1 is expressed on the associated follicular dendritic cell network (Dorfman DM *et al*. 2006 Am. J. Surg. Pathol. 30:802-10). In nodular lymphocyte-predominant Hodgkin lymphoma, the T-cells associated with lymphocytic or histiocytic (L&H) cells express PD-1. Microarray analysis using a readout of genes induced by PD-1 ligation suggests that tumour-associated T-cells are responding to PD-1 signals *in situ* in Hodgkin lymphoma (Chemnitz JM *et al*. 2007 Blood 110:3226-33). PD-1 and PD-L1 are expressed on CD4 T-cells in HTLV-1 -mediated adult T-cell leukaemia and lymphoma

(Shimauchi T *et al*. 2007 Int. J. Cancer 121: 2585-90). These tumour cells are hyporesponsive to TCR signals.

Studies in animal models demonstrate that PD-L1 on tumours inhibits T-cell activation and lysis of tumour cells and in some cases leads to increased tumour-specific T-cell death (Dong H *et al*. 2002 Nat. Med. 8:793-800; Hirano F *et al*. 2005 Cancer Res. 65:1089-96). Tumour-associated APCs can also utilise the PD-1:PD-L1 pathway to control antitumour T-cell responses. PD-L1 expression on a population of tumour-associated myeloid DCs is upregulated by tumour environmental factors (Curiel TJ *et al*. 2003 Nat. Med. 9:562-67). Plasmacytoid dendritic cells (DCs) in the tumour-draining lymph node of B16 melanoma express IDO, which strongly activates the suppressive activity of regulatory T-cells. The suppressive activity of IDO-treated regulatory T-cells required cell contact with IDO-expressing DCs (Sharma MD *et al*. 2007 Clin. Invest. 117:2570-82).

In one embodiment, the second peptide ligand comprises a PD-L1 binding bicyclic peptide ligand.

Suitable examples of PD-L1 binding bicyclic peptide ligands are disclosed in GB Patent Application Nos. 1820956.9 and 1820969.2, the peptides of which are incorporated herein by reference.

In one embodiment, the PD-L1 binding bicyclic peptide ligand comprises an amino acid sequence selected from:

C_i[HArg]DWC_{ii}HWTFSHGHPC_{iii} (SEQ ID NO: 12);

C_iSAGWLTMC_{ii}QKLHLC_{iii} (SEQ ID NO: 13); and

C_iSAGWLTMC_{ii}Q[K(PYA)]LHLC_{iii} (SEQ ID NO: 14);

wherein C_i, C_{ii} and C_{iii} represent first, second and third cysteine residues, respectively, HArg represents homoarginine and PYA represents 4-pentynoic acid, or a pharmaceutically acceptable salt thereof.

In a further embodiment, the PD-L1 binding bicyclic peptide ligand comprises N-terminal and/or C-terminal modifications and comprises:

[PYA]-[B-Ala]-[Sar$_{10}$]-(SEQ ID NO: 12) (hereinafter referred to as BCY8938);

[PYA]-[B-Ala]-[Sar$_{10}$]-SDK-(SEQ ID NO: 13) (hereinafter referred to as BCY10043);

NH_2-SDK-(SEQ ID NO: 13)-[Sar$_{10}$]-[K(PYA)] (hereinafter referred to as BCY10044);

NH_2-SDK-(SEQ ID NO: 14) (hereinafter referred to as BCY10045); and

Ac-SDK-(SEQ ID NO: 14)-PSH (hereinafter referred to as BCY10861);

wherein PYA represents 4-pentynoic acid, B-Ala represents beta-alanine, Sar_{10} represents 10 sarcosine units, or a pharmaceutically acceptable salt thereof.

In an alternative embodiment, the component present on a cancer cell is Nectin-4.

Nectin-4 is a surface molecule that belongs to the nectin family of proteins, which comprises 4 members. Nectins are cell adhesion molecules that play a key role in various biological processes such as polarity, proliferation, differentiation and migration, for epithelial, endothelial, immune and neuronal cells, during development and adult life. They are involved in several pathological processes in humans. They are the main receptors for poliovirus, herpes simplex virus and measles virus. Mutations in the genes encoding Nectin-1 (PVRL1) or Nectin-4 (PVRL4) cause ectodermal dysplasia syndromes associated with other abnormalities. Nectin-4 is expressed during foetal development. In adult tissues its expression is more restricted than that of other members of the family. Nectin-4 is a tumour-associated antigen in 50%, 49% and 86% of breast, ovarian and lung carcinomas, respectively, mostly on tumours of bad prognosis. Its expression is not detected in the corresponding normal tissues. In breast tumours, Nectin-4 is expressed mainly in triple-negative and ERBB2+ carcinomas. In the serum of patients with these cancers, the detection of soluble forms of Nectin-4 is associated with a poor prognosis. Levels of serum Nectin-4 increase during metastatic progression and decrease after treatment. These results suggest that Nectin-4 could be a reliable target for the treatment of cancer. Accordingly, several anti-Nectin-4 antibodies have been described in the prior art. In particular, Enfortumab Vedotin (ASG-22ME) is an antibody-drug conjugate (ADC) targeting Nectin-4 and is currently clinically investigated for the treatment of patients suffering from solid tumours.

In one embodiment, the second peptide ligand comprises a Nectin-4 binding bicyclic peptide ligand.

Suitable examples of Nectin-4 binding bicyclic peptide ligands are disclosed in GB Patent Application Nos 1810250.9, 1815684.4 and 1818499.4, the peptides of which are incorporated herein by reference.

In one embodiment, the Nectin-4 binding bicyclic peptide ligand comprises an amino acid sequence selected from:

C_iP[1Nal][dD]C_{ii}M[HArg]DWSTP[HyP]WC_{iii} (SEQ ID NO: 15; hereinafter referred to as BCY8116);

$C_iP[1Nal][dD]C_{ii}M[HArg]D[dW]STP[HyP][dW]C_{iii}$ (SEQ ID NO: 16; hereinafter referred to as BCY11415); and

$C_iP[1Nal][dK](Sar_{10}\text{-}(B\text{-}Ala))C_{ii}M[HArg]DWSTP[HyP]WC_{iii}$ (SEQ ID NO: 17);

$C_iPFGC_{ii}M[HArg]DWSTP[HyP]WC_{iii}$ (SEQ ID NO: 18; hereinafter referred to as BCY11414);

wherein C_i, C_{ii} and C_{iii} represent first, second and third cysteine residues, respectively, 1Nal represents 1-naphthylalanine, HArg represents homoarginine, HyP represents hydroxyproline, Sar_{10} represents 10 sarcosine units, B-Ala represents beta-alanine, or a pharmaceutically acceptable salt thereof.

In a further embodiment, the Nectin-4 binding bicyclic peptide ligand optionally comprises N-terminal modifications and comprises:

SEQ ID NO: 15 (hereinafter referred to as BCY8116);

[PYA]-[B-Ala]-[Sar_{10}]-(SEQ ID NO: 15) (hereinafter referred to as BCY8846);

SEQ ID NO: 16 (hereinafter referred to as BCY11415);

[PYA]-[B-Ala]-[Sar_{10}]-(SEQ ID NO: 16) (hereinafter referred to as BCY11942);

Ac-(SEQ ID NO: 17) (hereinafter referred to as BCY8831); and

SEQ ID NO: 18 (hereinafter referred to as BCY11414);

wherein PYA represents 4-pentynoic acid, B-Ala represents beta-alanine, Sar_{10} represents 10 sarcosine units, or a pharmaceutically acceptable salt thereof.

In an alternative embodiment, the component present on a cancer cell is prostate-specific membrane antigen (PSMA).

Prostate-specific membrane antigen (PSMA) (also known as Glutamate carboxypeptidase II (GCPII), N-acetyl-L-aspartyl-L-glutamate peptidase I (NAALADase I) and NAAG peptidase) is an enzyme that in humans is encoded by the *FOLH1* (folate hydrolase 1) gene. Human GCPII contains 750 amino acids and weighs approximately 84 kDa.

Human PSMA is highly expressed in the prostate, roughly a hundred times greater than in most other tissues. In some prostate cancers, PSMA is the second-most upregulated gene product, with an 8- to 12-fold increase over levels in noncancerous prostate cells. Because of this high expression, PSMA is being developed as potential biomarker for therapy and imaging of some cancers. In human prostate cancer, the higher expressing tumors are associated with quicker time to progression and a greater percentage of patients suffering relapse.

In one embodiment, the second peptide ligand comprises a PSMA binding bicyclic peptide ligand.

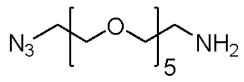
Suitable examples of PSMA binding bicyclic peptide ligands are disclosed in GB Patent Application Nos 1810318.4, 1810325.9 and 1820325.7, the peptides of which are incorporated herein by reference.

Linkers

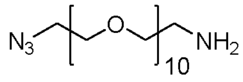
It will be appreciated that the first peptide ligand may be conjugated to the second peptide ligand via any suitable linker. Typically the design of said linker will be such that the two Bicyclic peptides are presented in such a manner that they can bind unencumbered to their respective targets either alone or while simultaneously binding to both target receptors. Additionally, the linker should permit binding to both targets simultaneously while maintaining an appropriate distance between the target cells that would lead to the desired functional outcome. The properties of the linker may be modulated to increase length, rigidity or solubility to optimise the desired functional outcome. The linker may also be designed to permit the attachment of more than one Bicycle to the same target. Increasing the valency of either binding peptide may serve to increase the affinity of the heterotandem for the target cells or may help to induce oligomerisation of one or both of the target receptors.

In one embodiment, the linker is selected from the following sequences: -CH_2-, -PEG_5-, -PEG_{10}-, -PEG_{12}-, -PEG_{23}-, -PEG_{24}-, -PEG_{15}-Sar_5-, -PEG_{10}-Sar_{10}-, -PEG_5-Sar_{15}-, -PEG_5-Sar_5-, -B-Ala-Sar_{20}-, -B-Ala-Sar_{10}-PEG_{10}-, -B-Ala-Sar_5-PEG_{15}- and -B-Ala-Sar_5-PEG_5-.

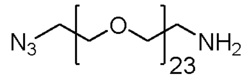
Structural representations of suitable linkers are detailed below:



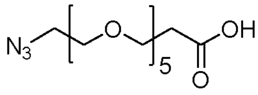
H2N-Peg5-N3
COM00000132



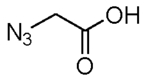
H2N-Peg10-N3
COM00000134



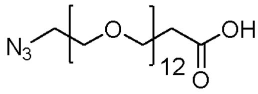
H2N-Peg23-N3
COM00000135



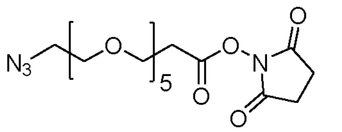
N3-PEG5-COOH
COM00000467



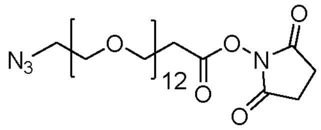
N3-CH2-COOH
COM00000468



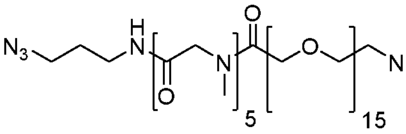
N3-PEG12-COOH
COM00000466



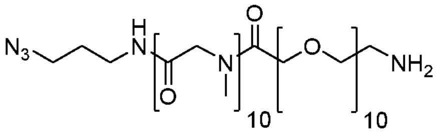
NHS-PEG5-N3



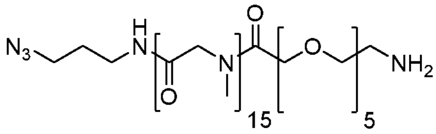
NHS-PEG12-N3



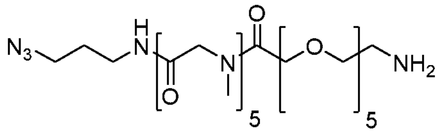
H2N-PEG15-SAR5-N3
COM00000128



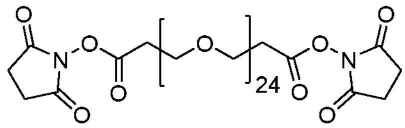
H2N-PEG10-SAR10-N3
COM00000129



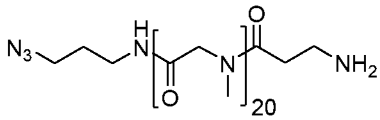
H2N-PEG5-SAR15-N3
COM00000130



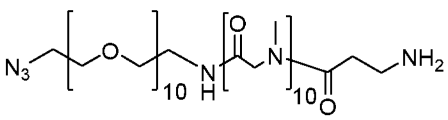
H2N-PEG5-SAR5-N3
COM00000131



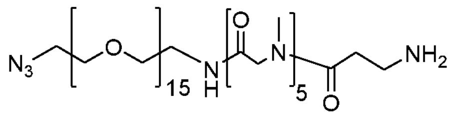
NHS-PEG24-NHS
COM00000469



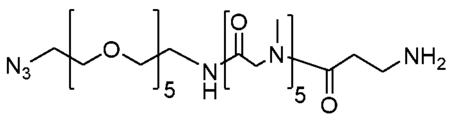
H2N-(B-Ala)-SAR20-N3
COM00000470



H2N-(B-Ala)-SAR10-PEG10-N3
COM00000471



H2N-(B-Ala)-SAR5-PEG15-N3
COM00000472



H2N-(B-Ala)-SAR5-PEG5-N3
COM00000473

Heterotandem Complexes

In one specific embodiment, the first peptide ligand comprises a CD137 binding bicyclic peptide ligand attached to a TATA scaffold, the second peptide ligand comprises an EphA2 binding bicyclic peptide ligand attached to a TATA scaffold and said heterotandem complex is selected from:

Complex No.	***EphA2 BCY No.***	***Attachment Point***	***Linker***	***CD137 BCY No.***	***Attachment Point***
BCY9173	BCY6169	N-terminal PYA	$-PEG_{12}-$	BCY9172	C-terminal Dap
BCY7985	BCY6169	N-terminal PYA	$-PEG_{12}-$	BCY7732	C-terminal Dap

BCY8942	BCY6169	N-terminal PYA	$-PEG_{12}-$	BCY8045	C-terminal Dap
BCY8943	BCY8941	N-terminal PYA	$-PEG_{12}-$	BCY7732	C-terminal Dap
BCY9647	BCY6099	N-terminus	$-PEG_{10}-$	BCY7741	C-terminal Dap(PYA)
BCY9648	BCY6099	N-terminus	$-PEG_{23}-$	BCY7741	C-terminal Dap(PYA)
BCY9655	BCY6099	N-terminus	$-PEG_{15}-Sar_{5}-$	BCY7741	C-terminal Dap(PYA)
BCY9656	BCY6099	N-terminus	$-PEG_{10}-Sar_{10}-$	BCY7741	C-terminal Dap(PYA)
BCY9657	BCY6099	N-terminus	$-PEG_{5}-Sar_{15}-$	BCY7741	C-terminal Dap(PYA)
BCY9658	BCY6099	N-terminus	$-PEG_{5}-Sar_{5}-$	BCY7741	C-terminal Dap(PYA)
BCY9659	BCY6099	N-terminus	$-PEG_{5}-$	BCY7741	C-terminal Dap(PYA)
BCY9758	BCY6099	N-terminus	$-PEG_{24}-$	BCY7732	C-terminal Dap
BCY10568	BCY6169	N-terminal PYA	$-PEG_{12}-$	BCY8919	Lys3
BCY10570	BCY6169	N-terminal PYA	$-PEG_{12}-$	BCY8920	dLys4
BCY10574	BCY9594	N-terminus	$-PEG_{5}-$	BCY8927	Lys (PYA)3
BCY10575	BCY9594	N-terminus	$-PEG_{5}-$	BCY8928	dLys (PYA)4
BCY10576	BCY9594	N-terminus	$-PEG_{5}-$	BCY11014	C-terminal Dap(PYA)
BCY10577	BCY6169	N-terminus	$-CH_{2}-$	BCY9172	C-terminal Dap

The heterotandem bicyclic peptide complex BCY7985 consists of a CD137-specific peptide BCY7859 linked to the N-terminal PYA group of an EphA2-specific peptide BCY6169 via PEG_{12} (shown pictorially in Figure 2).

CD137 is a homotrimeric protein and the natural ligand CD137L exists as a homotrimer either expressed on immune cells or secreted. The biology of CD137 is highly dependent on multimerization to induce CD137 activity in immune cells. One way to generate CD137 multimerization is through cellular cross-linking of the CD137 specific agonist through interaction with a specific receptor present on another cell.

EphA2 is highly expressed on tumour cells and oligomerization of this receptor tyrosine kinase by Ephrin-A ligands drives its activation. Without being bound by theory, the inventors believe that a EphA2-CD137 heterotandem consisting of one EphA2-specific peptide coupled to one CD137-specific peptide acts to cross-link CD137. The implication is that CD137 would be multimerized and activated in the presence of EphA2 on cells such as tumour cells. This would drive CD137 immune cell activation in the local tumour environment (Figure 1).

This hypothesis was tested in the CD137 cellular activity reporter assay described herein and the results are shown herein in Figure 3 wherein it can be seen that BCY7985 showed strong induction of CD137 cell activity in the Promega CD137 luciferase reporter assay (CS196008) in the presence of EphA2-expressing HT1080 cells.

In one alternative specific embodiment, the first peptide ligand comprises a CD137 binding bicyclic peptide ligand attached to a TATA scaffold, the second peptide ligand comprises a Nectin-4 binding bicyclic peptide ligand attached to a TATA scaffold and said heterotandem complex is selected from:

Complex No.	*Nectin-4 BCY No.*	*Attachment Point*	*Linker*	*CD137 BCY No.*	*Attachment Point*
BCY8854	BCY8846	N-terminal PYA	-PEG_{12}-	BCY7732	C-terminal Dap
BCY9350	BCY11942	N-terminal PYA	-PEG_{12}-	BCY7732	C-terminal Dap
BCY9351	BCY8846	N-terminal PYA	-PEG_{12}-	BCY8045	C-terminal Dap
BCY9399	BCY8116	N-terminus	-PEG_{10}-	BCY7741	C-terminal Dap(PYA)
BCY9400	BCY8116	N-terminus	-PEG_{23}-	BCY7741	C-terminal Dap(PYA)
BCY9401	BCY8116	N-terminus	-B-Ala-Sar_{20}-	BCY7741	C-terminal Dap(PYA)
BCY9403	BCY8116	N-terminus	-B-Ala-Sar_{10}-PEG_{10}-	BCY7741	C-terminal Dap(PYA)
BCY9405	BCY8116	N-terminus	-B-Ala-Sar_{5}-PEG_{15}-	BCY7741	C-terminal Dap(PYA)
BCY9406	BCY8116	N-terminus	-B-Ala-Sar_{5}-PEG_{5}-	BCY7741	C-terminal Dap(PYA)
BCY9407	BCY8116	N-terminus	-PEG_{15}-Sar_{5}-	BCY7741	C-terminal Dap(PYA)
BCY9408	BCY8116	N-terminus	-PEG_{10}-Sar_{10}-	BCY7741	C-terminal Dap(PYA)

BCY9409	BCY8116	N-terminus	-PEG_5-Sar_{15}-	BCY7741	C-terminal Dap(PYA)
BCY9410	BCY8116	N-terminus	-PEG_5-Sar_5-	BCY7741	C-terminal Dap(PYA)
BCY9411	BCY8116	N-terminus	-PEG_5-	BCY7741	C-terminal Dap(PYA)
BCY9759	BCY8116	N-terminus	-PEG_{24}-	BCY7732	C-terminal Dap
BCY10000	BCY8846	N-terminal PYA	-PEG_{12}-	BCY9172	C-terminal Dap
BCY10567	BCY8846	N-terminal PYA	-PEG_{12}-	BCY8919	Lys3
BCY10569	BCY8846	N-terminal PYA	-PEG_{12}-	BCY8920	dLys4
BCY10571	BCY8116	N-terminus	-PEG_5-	BCY8927	Lys(PYA)3
BCY10572	BCY8116	N-terminus	-PEG_5-	BCY8928	dLys (PYA)4
BCY10573	BCY8116	N-terminus	-PEG_5-	BCY11014	C-terminal Dap(PYA)
BCY10578	BCY8846	N-terminal PYA	-CH_2-	BCY9172	C-terminal Dap
BCY10917	BCY8831	dLys(Sar_{10})-(B-Ala))4	-PEG_{12}-	BCY11014	C-terminal Dap(PYA)
BCY11020	BCY8831	dLys(Sar_{10})-(B-Ala))4	-PEG_5-	BCY11014	C-terminal Dap(PYA)
BCY11373	BCY8116	N-terminus	-CH_2-	BCY8927	Lys(PYA)3
BCY11374	BCY8116	N-terminus	-CH_2-	BCY8928	dLys (PYA)4
BCY11375	BCY8116	N-terminus	-CH_2-	BCY11014	C-terminal Dap(PYA)
BCY11616	BCY8116	N-terminus	-PEG_5-	BCY7744	dLys (PYA)4
BCY11617	BCY8116	N-terminus	-PEG_5-	BCY11506	Lys(PYA)4
BCY11857	BCY11414	N-terminus	-PEG_5-	BCY7744	dLys (PYA)4
BCY11858	BCY11414	N-terminus	-PEG_5-	BCY8928	dLys (PYA)4
BCY11859	BCY11415	N-terminus	-PEG_5-	BCY8928	dLys (PYA)4

Without being bound by theory, the inventors believe that a Nectin-4-CD137 heterotandem consisting of one Nectin-4-specific peptide coupled to one CD137-specific peptide acts to cross-link CD137 in the same manner as described hereinbefore for EphA2.

In one embodiment, the Nectin-4-CD137 heterotandem is other than any one or more of: BCY11857, BCY11858 and/or BCY11859.

In one alternative specific embodiment, the first peptide ligand comprises a CD137 binding bicyclic peptide ligand attached to a TATA scaffold, the second peptide ligand comprises a

PD-L1 binding bicyclic peptide ligand attached to a TATA scaffold and said heterotandem complex is selected from:

Complex No.	*PD-L1 BCY No.*	*Attachment Point*	*Linker*	*CD137 BCY No.*	*Attachment Point*
BCY8939	BCY8938	N-terminal PYA	$-PEG_{12}-$	BCY7732	C-terminal Dap
BCY10580	BCY10043	N-terminal PYA	$-PEG_{12}-$	BCY9172	C-terminal Dap
BCY10581	BCY10044	C-terminal Lys(PYA)	$-PEG_{12}-$	BCY9172	C-terminal Dap
BCY10582	BCY10045	Lys(PYA)9	$-PEG_{12}-$	BCY9172	C-terminal Dap
BCY11017	BCY10861	Lys(PYA)9	$-PEG_{12}-$	BCY8919	Lys3
BCY11018	BCY10861	Lys(PYA)9	$-PEG_{12}-$	BCY8920	dLys4
BCY11019	BCY10861	Lys(PYA)9	$-PEG_{12}-$	BCY9172	C-terminal Dap
BCY11376	BCY10861	Lys(PYA)9	$-CH_2-$	BCY8919	Lys3
BCY11377	BCY10861	Lys(PYA)9	$-CH_2-$	BCY8920	dLys4
BCY11378	BCY10861	Lys(PYA)9	$-CH_2-$	BCY9172	C-terminal Dap
BCY11379	BCY10861	Lys(PYA)9	$-PEG_5-$	BCY8919	Lys3
BCY11380	BCY10861	Lys(PYA)9	$-PEG_5-$	BCY8920	dLys4
BCY11381	BCY10861	Lys(PYA)9	$-PEG_5-$	BCY9172	C-terminal Dap

Without being bound by theory, the inventors believe that a PD-L1-CD137 heterotandem consisting of one PD-L1-specific peptide coupled to one CD137-specific peptide acts to cross-link CD137 in the same manner as described hereinbefore for EphA2.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art, such as in the arts of peptide chemistry, cell culture and phage display, nucleic acid chemistry and biochemistry. Standard techniques are used for molecular biology, genetic and biochemical methods (see Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 3rd ed., 2001, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Ausubel *et al.*, Short Protocols in Molecular Biology (1999) 4^{th} ed., John Wiley & Sons, Inc.), which are incorporated herein by reference.

Nomenclature

Numbering

When referring to amino acid residue positions within compounds of the invention, cysteine residues (C_i, C_{ii} and C_{iii}) are omitted from the numbering as they are invariant, therefore, the numbering of amino acid residues within SEQ ID NO: 1 is referred to as below:

C_i-I_1-E_2-E_3-G_4-Q_5-Y_6-C_{ii}-F_7-A_8-D_9-P_{10}-Y_{11}-[Nle]$_{12}$-C_{iii} (SEQ ID NO: 1).

For the purpose of this description, all bicyclic peptides are assumed to be cyclised with TBMB (1,3,5-tris(bromomethyl)benzene) or 1,1',1''-(1,3,5-triazinane-1,3,5-triyl)triprop-2-en-1-one (TATA) and yielding a tri-substituted structure. Cyclisation with TBMB and TATA occurs on C_i, C_{ii}, and C_{iii}.

Molecular Format

N- or C-terminal extensions to the bicycle core sequence are added to the left or right side of the sequence, separated by a hyphen. For example, an N-terminal βAla-Sar10-Ala tail would be denoted as:

βAla-Sar10-A-(SEQ ID NO: X).

Inversed Peptide Sequences

In light of the disclosure in Nair *et al* (2003) J Immunol 170(3), 1362-1373, it is envisaged that the peptide sequences disclosed herein would also find utility in their retro-inverso form. For example, the sequence is reversed (i.e. N-terminus becomes C-terminus and *vice versa*) and their stereochemistry is likewise also reversed (i.e. D-amino acids become L-amino acids and *vice versa*).

Peptide Ligands

A peptide ligand, 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 comprise at least three cysteine residues (referred to herein as C_i, C_{ii} and C_{iii}), and form at least two loops on the scaffold.

Pharmaceutically Acceptable Salts

It will be appreciated that salt forms are within the scope of this invention, and references to peptide ligands include the salt forms of said ligands.

The salts of the present invention can be synthesized from the parent compound that contains a basic or acidic moiety by conventional chemical methods such as methods described in *Pharmaceutical Salts: Properties, Selection, and Use*, P. Heinrich Stahl (Editor), Camille G. Wermuth (Editor), ISBN: 3-90639-026-8, Hardcover, 388 pages, August 2002. Generally, such salts can be prepared by reacting the free acid or base forms of these compounds with the appropriate base or acid in water or in an organic solvent, or in a mixture of the two.

Acid addition salts (mono- or di-salts) may be formed with a wide variety of acids, both inorganic and organic. Examples of acid addition salts include mono- or di-salts formed with an acid selected from the group consisting of acetic, 2,2-dichloroacetic, adipic, alginic, ascorbic (e.g. L-ascorbic), L-aspartic, benzenesulfonic, benzoic, 4-acetamidobenzoic, butanoic, (+) camphoric, camphor-sulfonic, (+)-(1*S*)-camphor-10-sulfonic, capric, caproic, caprylic, cinnamic, citric, cyclamic, dodecylsulfuric, ethane-1,2-disulfonic, ethanesulfonic, 2-hydroxyethanesulfonic, formic, fumaric, galactaric, gentisic, glucoheptonic, D-gluconic, glucuronic (e.g. D-glucuronic), glutamic (e.g. L-glutamic), α-oxoglutaric, glycolic, hippuric, hydrohalic acids (e.g. hydrobromic, hydrochloric, hydriodic), isethionic, lactic (e.g. (+)-L-lactic, (±)-DL-lactic), lactobionic, maleic, malic, (-)-L-malic, malonic, (±)-DL-mandelic, methanesulfonic, naphthalene-2-sulfonic, naphthalene-1,5-disulfonic, 1-hydroxy-2-naphthoic, nicotinic, nitric, oleic, orotic, oxalic, palmitic, pamoic, phosphoric, propionic, pyruvic, L-pyroglutamic, salicylic, 4-amino-salicylic, sebacic, stearic, succinic, sulfuric, tannic, (+)-L-tartaric, thiocyanic, *p*-toluenesulfonic, undecylenic and valeric acids, as well as acylated amino acids and cation exchange resins.

One particular group of salts consists of salts formed from acetic, hydrochloric, hydriodic, phosphoric, nitric, sulfuric, citric, lactic, succinic, maleic, malic, isethionic, fumaric, benzenesulfonic, toluenesulfonic, sulfuric, methanesulfonic (mesylate), ethanesulfonic, naphthalenesulfonic, valeric, propanoic, butanoic, malonic, glucuronic and lactobionic acids. One particular salt is the hydrochloride salt. Another particular salt is the acetate salt.

If the compound is anionic, or has a functional group which may be anionic (e.g., -COOH may be $-COO^-$), then a salt may be formed with an organic or inorganic base, generating a suitable cation. Examples of suitable inorganic cations include, but are not limited to, alkali metal ions such as Li^+, Na^+ and K^+, alkaline earth metal cations such as Ca^{2+} and Mg^{2+}, and other cations such as Al^{3+} or Zn^+. Examples of suitable organic cations include, but are not limited to, ammonium ion (i.e., NH_4^+) and substituted ammonium ions (e.g., NH_3R^+, $NH_2R_2^+$, NHR_3^+, NR_4^+). Examples of some suitable substituted ammonium ions are those derived from: methylamine, ethylamine, diethylamine, propylamine, dicyclohexylamine, triethylamine, butylamine, ethylenediamine, ethanolamine, diethanolamine, piperazine, benzylamine, phenylbenzylamine, choline, meglumine, and tromethamine, as well as amino acids, such as lysine and arginine. An example of a common quaternary ammonium ion is $N(CH_3)_4^+$.

Where the compounds of the invention contain an amine function, these may form quaternary ammonium salts, for example by reaction with an alkylating agent according to methods well known to the skilled person. Such quaternary ammonium compounds are within the scope of the invention.

Modified Derivatives

It will be appreciated that modified derivatives of the peptide ligands as defined herein are within the scope of the present invention. Examples of such suitable modified derivatives include one or more modifications selected from: N-terminal and/or C-terminal modifications; replacement of one or more amino acid residues with one or more non-natural amino acid residues (such as replacement of one or more polar amino acid residues with one or more isosteric or isoelectronic amino acids; replacement of one or more non-polar amino acid residues with other non-natural isosteric or isoelectronic amino acids); addition of a spacer group; replacement of one or more oxidation sensitive amino acid residues with one or more oxidation resistant amino acid residues; replacement of one or more amino acid residues with an alanine, replacement of one or more L-amino acid residues with one or more D-amino acid residues; N-alkylation of one or more amide bonds within the bicyclic peptide ligand; replacement of one or more peptide bonds with a surrogate bond; peptide backbone length modification; substitution of the hydrogen on the alpha-carbon of one or more amino acid residues with another chemical group, modification of amino acids such as cysteine, lysine, glutamate/aspartate and tyrosine with suitable amine, thiol, carboxylic acid and phenol-reactive reagents so as to functionalise said amino acids, and introduction or replacement of amino acids that introduce orthogonal reactivities that are suitable for functionalisation, for example azide or alkyne-group bearing amino acids that allow functionalisation with alkyne or azide-bearing moieties, respectively.

In one embodiment, the modified derivative comprises an N-terminal and/or C-terminal modification. In a further embodiment, wherein the modified derivative comprises an N-terminal modification using suitable amino-reactive chemistry, and/or C-terminal modification using suitable carboxy-reactive chemistry. In a further embodiment, said N-terminal or C-terminal modification comprises addition of an effector group, including but not limited to a cytotoxic agent, a radiochelator or a chromophore.

In a further embodiment, the modified derivative comprises an N-terminal modification. In a further embodiment, the N-terminal modification comprises an N-terminal acetyl group. In this embodiment, the N-terminal cysteine group (the group referred to herein as C_i) is capped with acetic anhydride or other appropriate reagents during peptide synthesis leading to a molecule which is N-terminally acetylated. This embodiment provides the advantage of removing a potential recognition point for aminopeptidases and avoids the potential for degradation of the bicyclic peptide.

In an alternative embodiment, the N-terminal modification comprises the addition of a molecular spacer group which facilitates the conjugation of effector groups and retention of potency of the bicyclic peptide to its target.

In a further embodiment, the modified derivative comprises a C-terminal modification. In a further embodiment, the C-terminal modification comprises an amide group. In this embodiment, the C-terminal cysteine group (the group referred to herein as C_{iii}) is synthesized as an amide during peptide synthesis leading to a molecule which is C-terminally amidated. This embodiment provides the advantage of removing a potential recognition point for carboxypeptidase and reduces the potential for proteolytic degradation of the bicyclic peptide.

In one embodiment, the modified derivative comprises replacement of one or more amino acid residues with one or more non-natural amino acid residues. In this embodiment, non-natural amino acids may be selected having isosteric/isoelectronic side chains which are neither recognised by degradative proteases nor have any adverse effect upon target potency.

Alternatively, non-natural amino acids may be used having constrained amino acid side chains, such that proteolytic hydrolysis of the nearby peptide bond is conformationally and sterically impeded. In particular, these concern proline analogues, bulky sidechains, $C\alpha$-disubstituted derivatives (for example, aminoisobutyric acid, Aib), and cyclo amino acids, a simple derivative being amino-cyclopropylcarboxylic acid.

In one embodiment, the modified derivative comprises the addition of a spacer group. In a further embodiment, the modified derivative comprises the addition of a spacer group to the N-terminal cysteine (C_i) and/or the C-terminal cysteine (C_{iii}).

In one embodiment, the modified derivative comprises replacement of one or more oxidation sensitive amino acid residues with one or more oxidation resistant amino acid residues. In a further embodiment, the modified derivative comprises replacement of a tryptophan residue with a naphthylalanine or alanine residue. This embodiment provides the advantage of improving the pharmaceutical stability profile of the resultant bicyclic peptide ligand.

In one embodiment, the modified derivative comprises replacement of one or more charged amino acid residues with one or more hydrophobic amino acid residues. In an alternative embodiment, the modified derivative comprises replacement of one or more hydrophobic amino acid residues with one or more charged amino acid residues. The correct balance of charged versus hydrophobic amino acid residues is an important characteristic of the bicyclic peptide ligands. For example, hydrophobic amino acid residues influence the degree of plasma protein binding and thus the concentration of the free available fraction in plasma, while charged amino acid residues (in particular arginine) may influence the interaction of the peptide with the phospholipid membranes on cell surfaces. The two in combination may influence half-life, volume of distribution and exposure of the peptide drug, and can be tailored according to the clinical endpoint. In addition, the correct combination and number of charged versus hydrophobic amino acid residues may reduce irritation at the injection site (if the peptide drug has been administered subcutaneously).

In one embodiment, the modified derivative comprises replacement of one or more L-amino acid residues with one or more D-amino acid residues. This embodiment is believed to increase proteolytic stability by steric hindrance and by a propensity of D-amino acids to stabilise β-turn conformations (Tugyi *et al* (2005) PNAS, 102(2), 413–418).

In one embodiment, the modified derivative comprises removal of any amino acid residues and substitution with alanines. This embodiment provides the advantage of removing potential proteolytic attack site(s).

It should be noted that each of the above mentioned modifications serve to deliberately improve the potency or stability of the peptide. Further potency improvements based on modifications may be achieved through the following mechanisms:

- Incorporating hydrophobic moieties that exploit the hydrophobic effect and lead to lower off rates, such that higher affinities are achieved;

- Incorporating charged groups that exploit long-range ionic interactions, leading to faster on rates and to higher affinities (see for example Schreiber *et al*, *Rapid, electrostatically assisted association of proteins* (1996), Nature Struct. Biol. 3, 427-31); and

- Incorporating additional constraint into the peptide, by for example constraining side chains of amino acids correctly such that loss in entropy is minimal upon target binding, constraining the torsional angles of the backbone such that loss in entropy is minimal upon target binding and introducing additional cyclisations in the molecule for identical reasons.

(for reviews see Gentilucci *et al*, Curr. Pharmaceutical Design, (2010), 16, 3185-203, and Nestor *et al*, Curr. Medicinal Chem (2009), 16, 4399-418).

Isotopic variations

The present invention includes all pharmaceutically acceptable (radio)isotope-labeled peptide ligands of the invention, wherein one or more atoms are replaced by atoms having the same atomic number, but an atomic mass or mass number different from the atomic mass or mass number usually found in nature, and peptide ligands of the invention, wherein metal chelating groups are attached (termed “effector”) that are capable of holding relevant (radio)isotopes, and peptide ligands of the invention, wherein certain functional groups are covalently replaced with relevant (radio)isotopes or isotopically labelled functional groups.

Examples of isotopes suitable for inclusion in the peptide ligands of the invention comprise isotopes of hydrogen, such as ^{2}H (D) and ^{3}H (T), carbon, such as ^{11}C, ^{13}C and ^{14}C, chlorine, such as ^{36}Cl, fluorine, such as ^{18}F, iodine, such as ^{123}I, ^{125}I and ^{131}I, nitrogen, such as ^{13}N and ^{15}N, oxygen, such as ^{15}O, ^{17}O and ^{18}O, phosphorus, such as ^{32}P, sulfur, such as ^{35}S, copper, such as ^{64}Cu, gallium, such as ^{67}Ga or ^{68}Ga, yttrium, such as ^{90}Y and lutetium, such as ^{177}Lu, and Bismuth, such as ^{213}Bi.

Certain isotopically-labelled peptide ligands of the invention, for example, those incorporating a radioactive isotope, are useful in drug and/or substrate tissue distribution studies, and to clinically assess the presence and/or absence of the Nectin-4 target on diseased tissues. The peptide ligands of the invention can further have valuable diagnostic properties in that they can be used for detecting or identifying the formation of a complex between a labelled compound and other molecules, peptides, proteins, enzymes or receptors. The detecting or identifying methods can use compounds that are labelled with labelling agents such as radioisotopes, enzymes, fluorescent substances, luminous substances (for example, luminol, luminol derivatives, luciferin, aequorin and luciferase), etc. The radioactive isotopes tritium, *i.e.* ^{3}H (T), and carbon-14, *i.e.* ^{14}C, are particularly useful for this purpose in view of their ease of incorporation and ready means of detection.

Substitution with heavier isotopes such as deuterium, *i.e.* ^{2}H (D), may afford certain therapeutic advantages resulting from greater metabolic stability, for example, increased *in vivo* half-life or reduced dosage requirements, and hence may be preferred in some circumstances.

Substitution with positron emitting isotopes, such as ^{11}C, ^{18}F, ^{15}O and ^{13}N, can be useful in Positron Emission Topography (PET) studies for examining target occupancy.

Isotopically-labeled compounds of peptide ligands of the invention can generally be prepared by conventional techniques known to those skilled in the art or by processes analogous to those described in the accompanying Examples using an appropriate isotopically-labeled reagent in place of the non-labeled reagent previously employed.

Molecular scaffold

Molecular scaffolds are described in, for example, WO 2009/098450 and references cited therein, particularly WO 2004/077062 and WO 2006/078161.

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, 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.

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.

In one embodiment, the molecular scaffold may comprise or may consist of hexahydro-1,3,5-triazine, especially 1,3,5-Triacryloylhexahydro-1,3,5-triazine (‘TATA’), 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 (TBMB) but contains three additional methyl 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 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.

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) 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 $\alpha\beta$ 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). 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 peptides of the present invention 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 or conjugates 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/conjugate made by chemical synthesis.

Optionally amino acid residues in the polypeptide of interest may be substituted when manufacturing a conjugate or complex.

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. TBMB) 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.

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.

Pharmaceutical Compositions

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

Generally, the present peptide ligands 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.

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).

The peptide ligands 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 cylcosporine, methotrexate, adriamycin or cisplatinum and immunotoxins. Pharmaceutical compositions can include "cocktails" of various cytotoxic or other agents in conjunction with the protein ligands of the present invention, or even combinations of selected polypeptides 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.

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

The peptide ligands 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.

The compositions containing the present peptide ligands 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 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 peptide ligand 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 peptide ligands or cocktails thereof may also be administered in similar or slightly lower dosages.

A composition containing a peptide ligand 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 peptide ligands described herein may be used extracorporeally or *in vitro* selectively to kill, deplete or otherwise effectively remove a target cell population from a heterogeneous collection of cells. Blood from a mammal may be combined extracorporeally with the selected peptide ligands whereby the undesired cells are killed or otherwise removed from the blood for return to the mammal in accordance with standard techniques.

Therapeutic Uses

According to a further aspect of the invention, there is provided a heterotandem bicyclic peptide complex as defined herein for use in preventing, suppressing or treating cancer.

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 myelomonocyticleukemia [CMML], hypereosinophilic syndrome, myeloproliferative disorders such as polycythaemia vera, essential thrombocythaemia and primary myelofibrosis, myeloproliferative syndrome, myelodysplastic syndrome, and promyelocyticleukemia); 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 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. "Treatment" involves administration of the protective composition after disease symptoms become manifest.

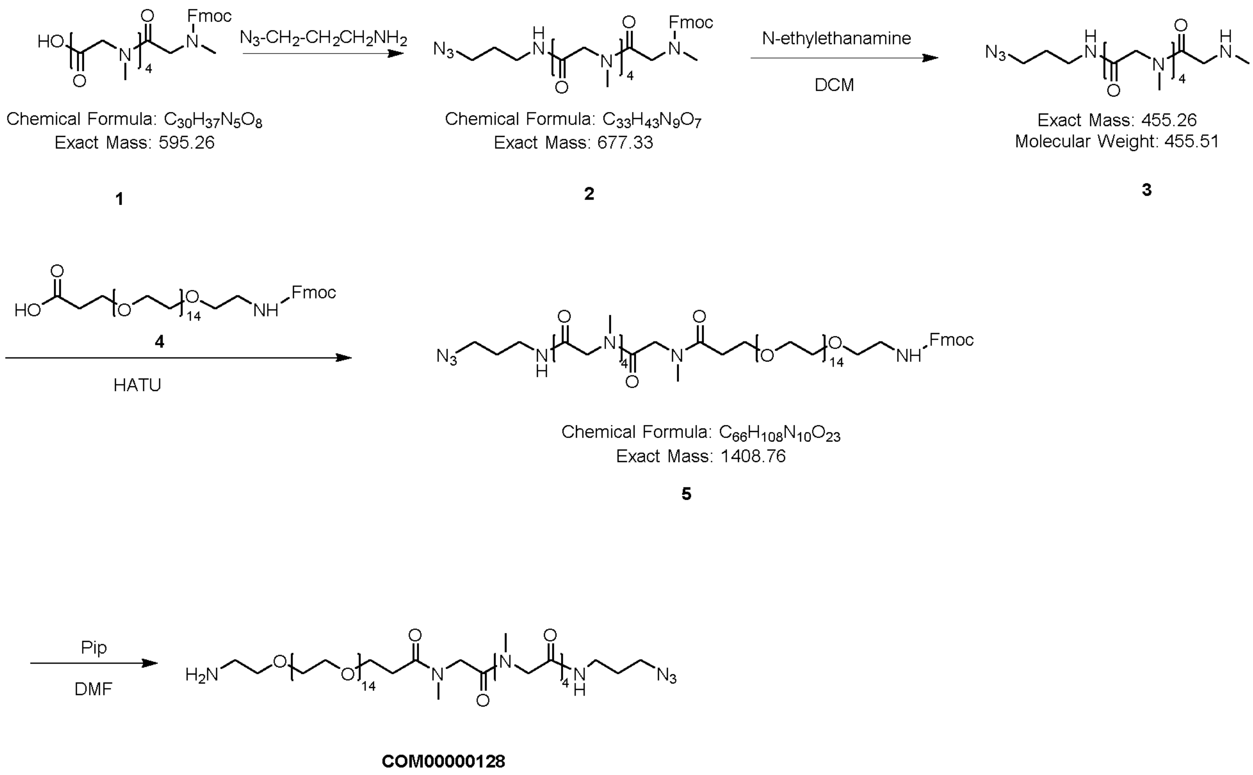
Animal model systems which can be used to screen the effectiveness of the peptide ligands in protecting against or treating the disease are available. The use of animal model systems is facilitated by the present invention, which allows the development of polypeptide ligands which can cross react with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

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

EXAMPLES

Example 1: Synthesis of Linkers

COM128



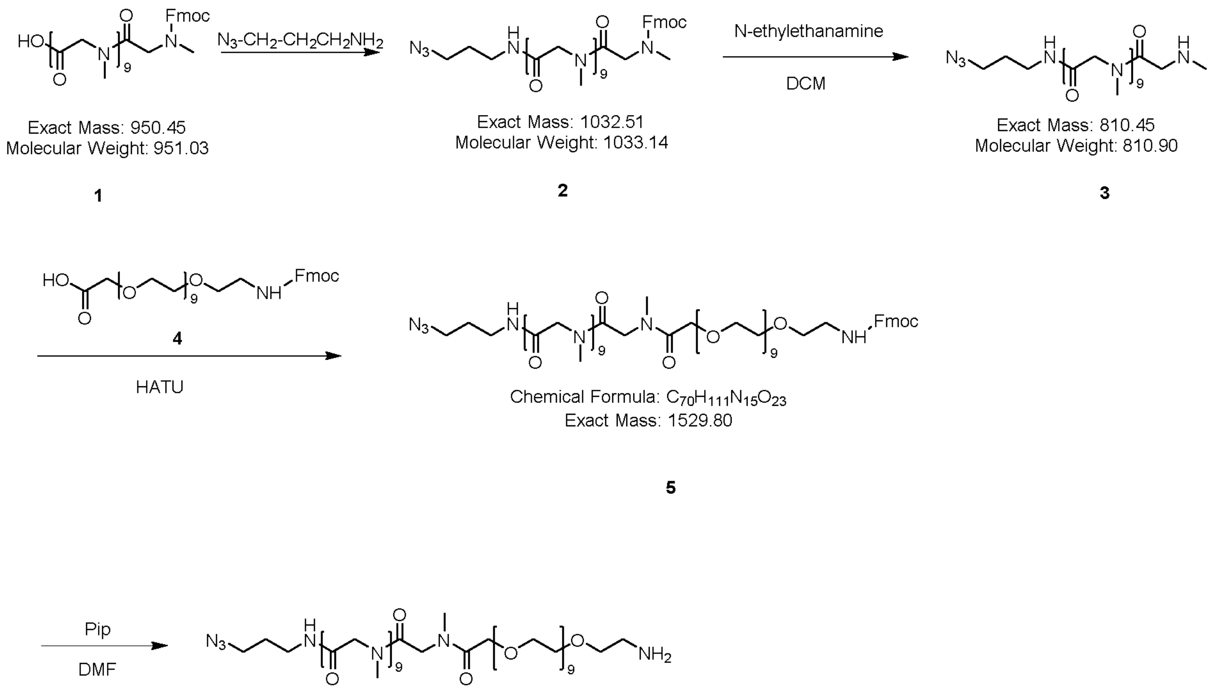
A mixture of compound **1** (700.0 mg, 1.18 mmol, 1.0 eq), 3-azidopropan-1-amine (117.66 mg, 1.18 mmol, 1.0 eq), EDCI (270.4 mg, 1.41 mmol, 1.2 eq), HOBt (190.6 mg, 1.41 mmol, 1.2 eq) was dissolved in DCM (20 mL, pre-degassed and purged with N_2 for 3 times), and then the mixture was stirred at 20-25 °C for 1 hr under N_2 atmosphere. LC-MS showed compound **1** was consumed completely and one main peak with desired m/z (calculated MW: 677.33, observed *m/z*: 678.2 ($[M+H]^+$)) was detected. The solvent was evaporated to produce compound **2** (600 mg, crude) was obtained as a white solid.

A mixture of compound **2** (600.0 mg, 885.3 µmol, 1.0 eq), N-ethylethanamine (1.29 g, 15.19 mmol, 1.50 mL, 17.2 eq) was dissolved in DCM (3 mL, pre-degassed and purged with N_2 for 3 times), and then the mixture was stirred at 25-30 °C for 2 hr under N_2 atmosphere. LC-MS showed compound **2** was consumed completely and one main peak with desired m/z (calculated MW: 455.51, observed *m/z*: 456.3 ($[M+H]^+$)) was detected. The solvent was evaporated to produce compound **3** (400 mg, crude) was obtained as colorless oil.

A mixture of compound **3** (150.0 mg, 329.3 µmol, 1.0 eq), compound **4** (320.1 mg, 329.3 µmol, 1.0 eq), HATU (125.2 mg, 329.3 µmol, 1.0 eq), DIEA (42.6 mg, 329.3 µmol, 57.4 µL, 1.0 eq) was dissolved in DMF (2 mL, pre-degassed and purged with N_2 for 3 times), and then the mixture was stirred at 25-30 °C for 2 under N_2 atmosphere. LC-MS showed compound **3** was consumed completely and one main peak with desired m/z (calculated MW: 1408.76, observed *m/z*: 705.3 ($[M/2+H]^+$)) was detected. The solvent was evaporated to produce compound **5** (400 mg, crude) was obtained as yellow oil.

Compound **5** (400 mg, 283.77 μmol, 1.0 eq) was dissolved in DMF (4 mL, pre-degassed and purged with N_2 for 3 times), following by addition of piperidine (862.2 mg, 10.13 mmol, 1 mL, 35.7 eq), and then the mixture was stirred at 25-30 °C for 15 min under N_2 atmosphere. LC-MS showed compound **5** was consumed completely and one main peak with desired m/z (calculated MW: 1187.37, observed *m/z*: 594.4 ([M/2+H$^+$], 1187.4 ([M+H]$^+$)) was detected. The solvent was evaporated to produce **COM128** (250 mg, crude) was obtained as colorless oil.

<u>COM129</u>



COM00000129

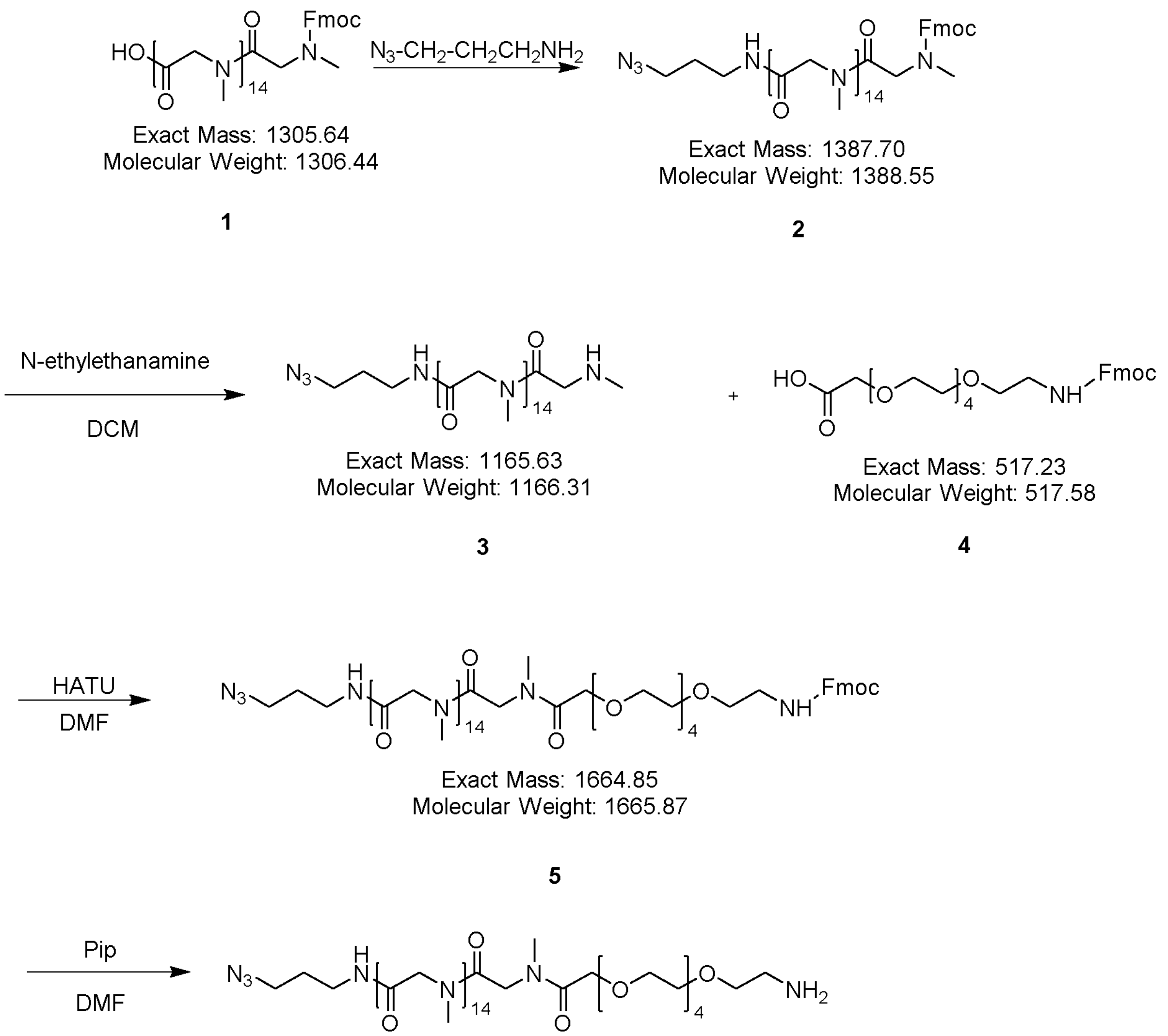
A mixture of compound **1** (1.4 g, 1.47 mmol, 1.0 eq), 3-azidopropan-1-amine (162.1 mg, 1.62 mmol, 1.1 eq), EDCI (338.6 mg, 1.77 mmol, 1.2 eq), HOBt (238.7 mg, 1.77 mmol, 1.2 eq) was dissolved in DCM (5 mL, pre-degassed and purged with N_2 for 3 times), and then the mixture was stirred at 20-25 °C for 1 hr under N_2 atmosphere. LC-MS showed compound **1** was consumed completely and one main peak with desired m/z (calculated MW: 1033.14, observed *m/z*: 1033.2 ([M+H]$^+$)) was detected. The reaction mixture was treated with a few drops of 1 M HCl, and the organic layer was evaporated under reduced pressure to remove solvent. Compound **2** (1.1 g, crude) was obtained as yellow oil.

A mixture of compound **2** (1.1 g, 1.06 mmol, 1 eq), N-ethylethanamine (3.89 g, 53.24 mmol, 5.48 mL, 50 eq) was dissolved in DCM (5 mL, pre-degassed and purged with N_2 for 3 times), and then the mixture was stirred at 20-25 °C for 1 hr under N_2 atmosphere. LC-MS showed compound **2** was consumed completely and one main peak with desired m/z (calculated MW: 810.90, observed *m/z*: 810.9 ([M+H]$^+$)) was detected. The reaction mixture was evaporated under reduced pressure and compound **3** (810 mg, crude) was obtained as a white solid.

A mixture of compound **3** (810.0 mg, 998.9 μmol, 1.0 eq), compound **4** (810.7 mg, 1.10 mmol, 1.1 eq), HATU (455.8 mg, 1.20 mmol, 1.2 eq), DIEA (258.2 mg, 2.00 mmol, 348.0 μL, 2.0 eq) was dissolved in DMF (2 mL, pre-degassed and purged with N_2 for 3 times), and then the mixture was stirred at 25-30 °C for 2 under N_2 atmosphere. LC-MS showed compound **3** was consumed completely and one main peak with desired m/z (calculated MW: 1530.72, observed *m/z*: 765.5 ([M/2+H]$^+$)) was detected. The reaction mixture was treated with a few drops of 1 M HCl, and the organic layer was collected and evaporated under reduced pressure to remove solvent. Compound **5** (1.1 g, crude) was obtained as a yellow solid.

Compound **5** (1 g, 653.29 μmol, 1 eq) was dissolved in DCM (10 mL, pre-degassed and purged with N_2 for 3 times), following by addition of piperidine (2.39 g, 32.66 mmol, 3.36 mL, 50 eq), and then the mixture was stirred at 25-30 °C for 2 hr under N_2 atmosphere. LC-MS showed Compound **5** was consumed completely and one main peak with desired m/z (calculated MW: 1308.47, observed *m/z*: 1308.4 ([M+H]$^+$)) was detected. The residue was purified by prep-HPLC (TFA condition: Phase A：0.075%TFA in H_2O, phase B : MeCN, Column: Luna 200*25 mm 10 um, C18, 110A and Gemin150*30 mm, C18, 5 um, 110A, connection, 50 ℃). **COM129** (700 mg, 463.72 μmol, 70.98% yield) was obtained as a yellow solid.

<u>**COM130**</u>



COM00000130

A mixture of Compound **1** (291 mg, 222.75 µmol, 1.0 eq), 3-azidopropan-1-amine (24.53 mg, 245.02 µmol, 1.1 eq), EDCI (51.24 mg, 267.30 µmol, 1.2 eq), HOBt (36.12 mg, 267.30 µmol, 1.2 eq) was dissolved in DCM (3 mL, pre-degassed and purged with N_2 for 3 times), and then the mixture was stirred at 20-25 °C for 1 hr under N_2 atmosphere. LC-MS showed Compound **1** was consumed completely and one main peak with desired m/z (MW: 1388.53, observed *m/z*: 694.7 ($[M/2+H]^+$)) was detected. The residue was purified by prep-HPLC (neutral condition). Compound **2** (200 mg, 144.04 µmol, 64.66% yield) was obtained as a white solid.

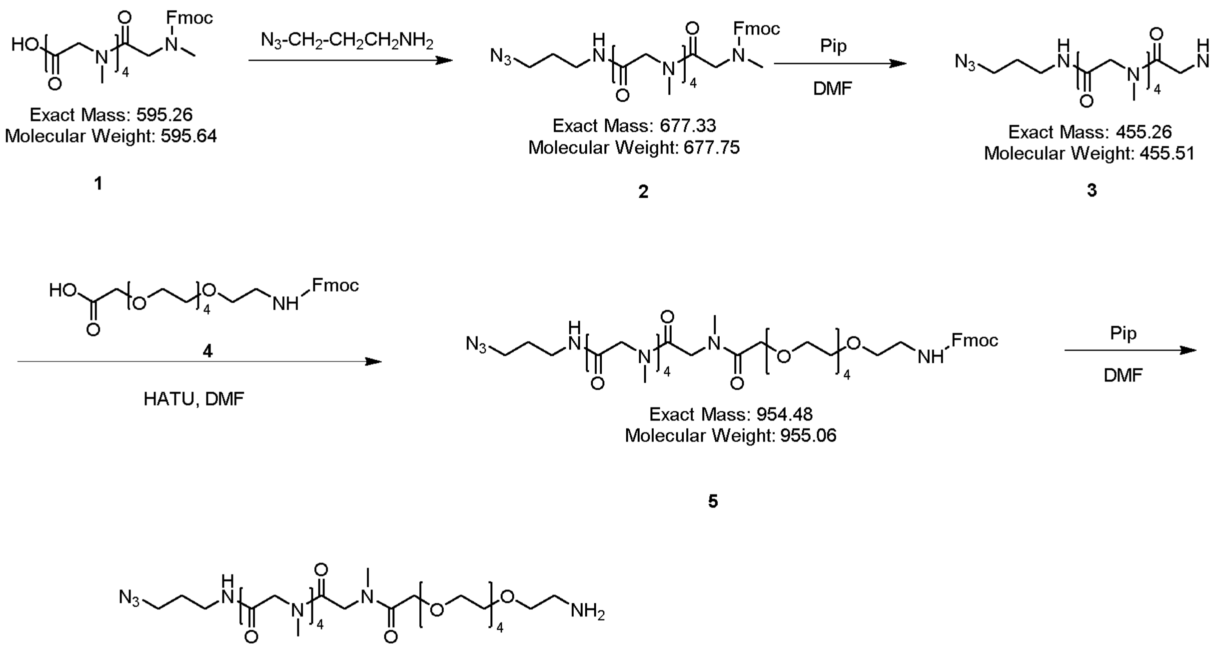
A mixture of Compound **2** (200 mg, 144.04 µmol, 1.0 eq) , N-ethylethanamine (210.7 mg, 2.88 mmol, 297 µL, 20.0 eq) was dissolved in DCM (3 mL, pre-degassed and purged with N_2 for 3 times), and then the mixture was stirred at 20-25 °C for 1 hr under N_2 atmosphere. LC-MS showed Compound **2** was consumed completely and one main peak with desired m/z

(MW: 1166.29, observed *m/z*: 1166.3 ([M+H]$^+$)) was detected. The reaction mixture was evaporated and Compound **3** (150 mg, crude) was obtained as yellow oil.

A mixture of compound **3** (150 mg, 128.61 μmol, 1.0 eq) , compound **4** (75 mg, 144.91 μmol, 1.13 eq), HATU (58.7 mg, 154.34 μmol, 1.2 eq) and DIEA (33.24 mg, 257.23 μmol, 44.80 μL, 2.0 eq) was dissolved in DMF (5 mL, pre-degassed and purged with N_2 for 3 times), and then the mixture was stirred at 20-25 °C for 2 hr under N_2 atmosphere. LC-MS showed compound **3** was consumed completely and one main peak with desired m/z (MW: 1665.84, observed *m/z*: 833.2 ([M/2+H]$^+$)) was detected. The solvent was removed under reduced pressure and compound **5** (300 mg, crude) was obtained as yellow oil.

To crude compound 5 (300 mg, dissolved in 10 mL DMF) was added piperidine (2 mL), and the mixture was stirred at 30 °C for 2 hr. LCMS indicated one main peak with desired m/z (MW: 1443.60 observed *m/z*: 722.7 ([M/2+H]$^+$)) was detected. The residue was purified by prep-HPLC (neutral condition). **COM130** (140 mg, 58.19 μmol, 32.31% yield, 60% purity) was obtained as a white solid.

COM131



COM00000131

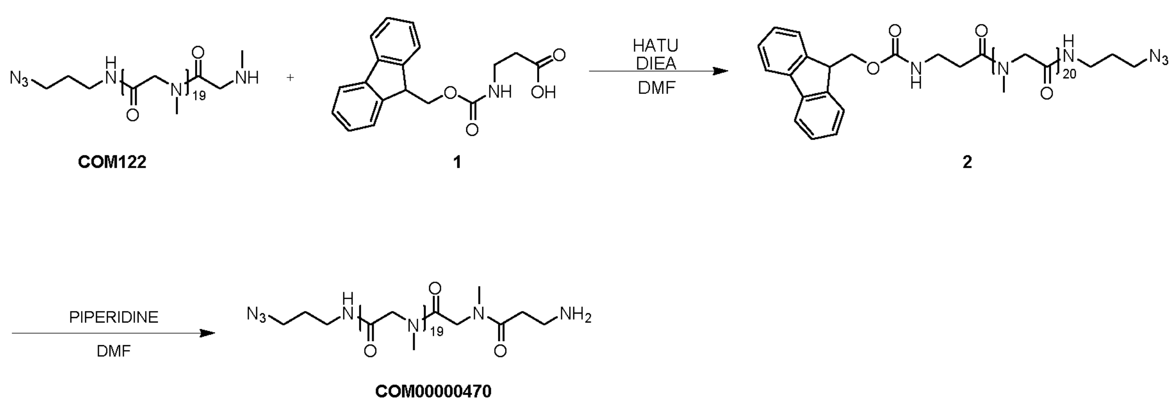
A mixture of compound **1** (700.0 mg, 1.18 mmol, 1.0 eq), 3-azidopropan-1-amine (117.7 mg, 1.18 mmol, 1.0 eq), HOBt (190.6 mg, 1.41 mmol, 1.2 eq), EDCI (270.4 mg, 1.41 mmol, 1.2 eq) was dissolved in DCM (20 mL, pre-degassed and purged with N_2 for 3 times), and then the mixture was stirred at 25-30 °C for 2 hr under N_2 atmosphere. LC-MS showed compound

1 was consumed completely and one main peak with desired m/z (calculated MW: 677.75, observed *m/z*: 678.2 ($[M+H]^+$)) was detected. The reaction mixture was treatment with a few drops of 1 M HCl, and the organic layer was collected and evaporated under reduced pressure. Compound **2** (600.0 mg, crude) was obtained as a white solid.

Compound **2** (600.0 mg, 885.2 µmol, 1.0 eq) was dissolved in DMF (3 mL, pre-degassed and purged with N_2 for 3 times), and then piperidine (1.29 g, 15.19 mmol, 1.50 mL, 17.2 eq) was added and the mixture was stirred at 25-30 °C for 2 hr under N_2 atmosphere. LC-MS showed compound **2** was consumed completely and one main peak with desired m/z (calculated MW: 455.51 observed *m/z*: 456.3 ($[M+H]^+$)) was detected. The reaction mixture was purified by prep-HPLC (TFA condition), and compound **3** (400.0 mg, 879.1 µmol) was obtained as colorless oil.

A mixture of compound **3** (250.0 mg, 548.83 µmol, 1.0 eq), compound **4** (284.1 mg, 548.83 µmol, 1 eq), HATU (229.6 mg, 603.72 µmol, 1.1 eq), DIEA (141.9 mg, 1.10 mmol, 191.19 µL, 2.0 eq) in DCM (20 mL, pre-degassed and purged with N_2 for 3 times), and then the mixture was stirred at 25-30 °C for 2 hr under N_2 atmosphere. LC-MS showed compound **3** was consumed completely and one main peak with desired m/z (calculated MW: 955.06, observed *m/z*: 955.6 ($[M+H]^+$)) was detected. The residue was purified by prep-HPLC (TFA condition). Compound **5** (400.0 mg, 419.1 µmol) was obtained as a white solid
A mixture of Compound **5** (400.0 mg, 418.82 µmol, 1.0 eq) was dissolved in DMF (4 mL, pre-degassed and purged with N_2 for 3 times), and then piperidine (862.2 mg, 10.13 mmol, 1 mL, 24.2 eq) was added and the mixture was stirred at 25-30 °C for 2 hr under N_2 atmosphere. LC-MS showed Compound **5** was consumed completely and one main peak with desired m/z (MW: 732.83 observed *m/z*: 733.3 ($[M+H]^+$)) was detected. The residue was purified by prep-HPLC (TFA condition). **COM131** (200 mg, 272.9 µmol) was obtained as colorless oil.

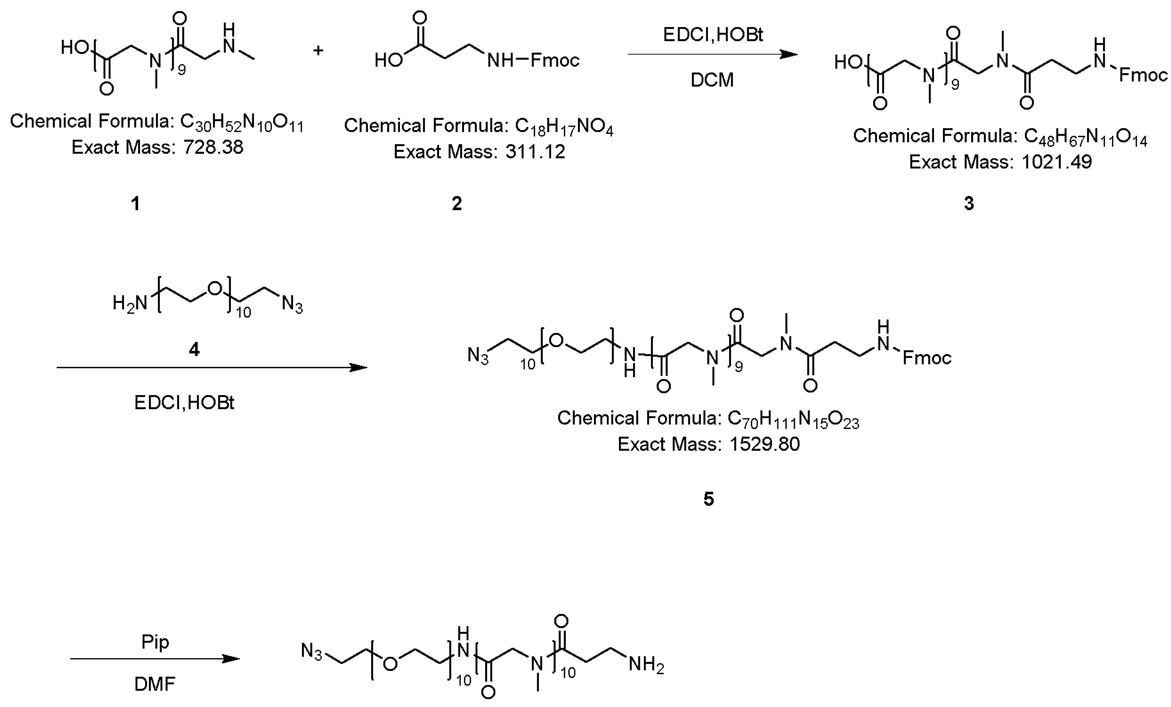
<u>COM470</u>



To a solution of **COM122** (228 mg, 149.83 μmol, 1.0 eq), Compound **1** (51.31 mg, 164.82 μmol, 1.1 eq) in DMF (6 mL) was added HATU (85.40 mg, 224.75 μmol, 1.5 eq) and DIEA (19.37 mg, 149.83 μmol, 26.10 μL, 1.0 eq). The mixture was stirred at 25-30 °C for 2 hr. LC-MS showed Compound **1** was consumed completely and one main peak with desired m/z (MW: 1814.99, observed m/z: 908.2([M/2+H]$^+$)) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent to give a residue. The residue was purified by prep-HPLC (neutral condition). Compound **2** (54 mg, 29.75 μmol, 19.86% yield) was obtained as a white solid.

To a solution of Compound **2** (54 mg, 29.8 μmol, 1.0 eq) in DMF (2 mL) was added piperidine (61 mg, 715 μmol, 71 μL, 24.0 eq). The mixture was stirred at 25-30 °C for 2 hr. LC-MS showed Compound 2 was consumed completely and one main peak with desired m/z (MW: 1592.75 observed m/z: 796.27([M/2+H]$^+$)) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent to give a residue. The residue was purified by prep-HPLC (TFA condition). **COM470** (40 mg, 25.11 μmol, 84.41% yield) was obtained as a white solid.

<u>**COM471**</u>



H2N-(B-Ala)-Sar10-Peg10-N3

COM00000471

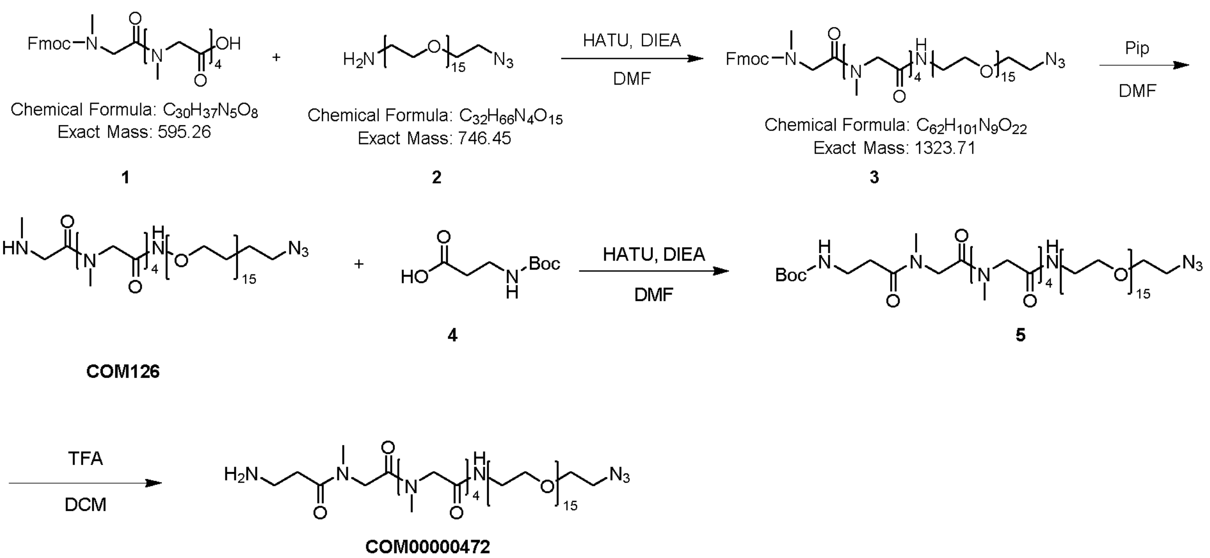
A mixture of compound **1** (900 mg, 1.23 mmol, 1.0 eq) and compound **2** (1.0 g, 3.21 mmol, 2.6 eq) was dissolved in DCM (20 mL), following by addition of (284.0 mg, 1.48 mmol, 1.2

eq), HOBt (200.2 mg, 1.48 mmol, 1.2 eq).The mixture was stirred at 25 °C for 2 hr. LC-MS showed compound **1** was consumed completely and one peak with desired m/z (calculated MW: 1021.49, observed *m/z*: 1022.2 ([M+H]$^+$)) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent. The residue was purified by prep-HPLC (TFA condition). Compound **3** (0.900 g, 880.53 µmol, 71.30% yield) was obtained as a white solid.

A mixture of compound **3** (500.0 mg, 489.19 µmol, 1.0 eq), compound **4** (257.6 mg, 489.19 µmol, 1.0 eq) was dissolved in DCM (5 mL), following by addition of HOBt (132.2 mg, 978.37 µmol, 2.0 eq), EDCI (187.6 mg, 978.37 µmol, 2.0 eq). The mixture was stirred at 25-30 °C for 2 hrs. LC-MS showed compound **3** was consumed completely and one main peak with desired m/z (MW: 1529.80 observed *m/z*: 765.9 ([M/2+H]$^+$) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent to give a residue. The residue was purified by prep-HPLC (neutral condition). Compound **3** (420 mg, 246.94 µmol, 50.48% yield) was obtained as colorless oil.

Compound **5** (420 mg, 274.38 µmol, 1.0 eq) was dissolved in DMF (4 mL), following by addition of piperidine (865.2 mg, 10.16 mmol, 1 mL, 37 eq). The mixture was stirred at 25-30 °C for 2 hr. LC-MS showed compound **5** was consumed completely and one main peak with desired m/z (calculated MW: 1308.48, observed *m/z*: 654.8([M/2+H]$^+$) was detected. The crude product was purified by prep-HPLC (TFA condition). **COM471** (386 mg, 265.50 µmol, 96.76% yield) was obtained as colorless oil.

COM472



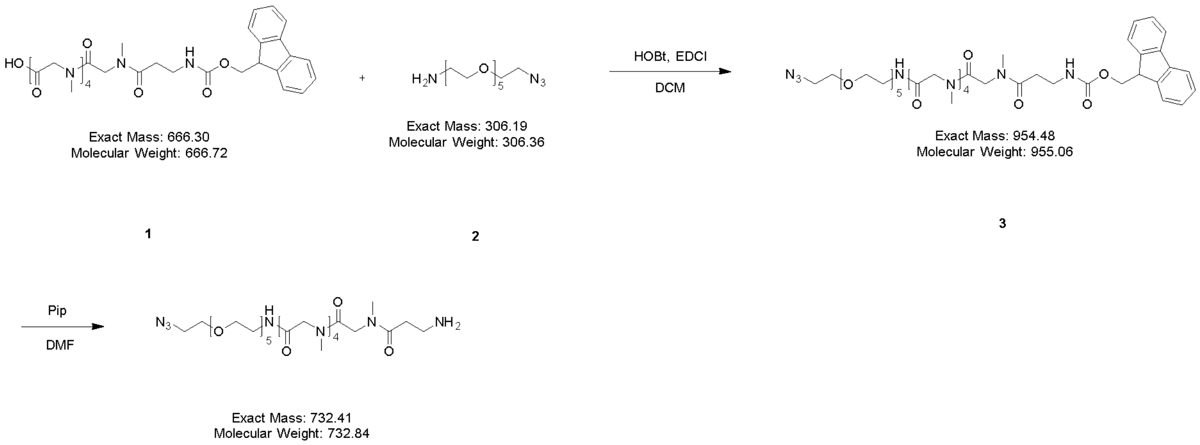
A mixture of compound **1** (0.5 g, 839.43 µmol, 1.0 eq), compound **2** (627.0 mg, 839.43 µmol, 1.0 eq) and DIEA (217.0 mg, 1.68 mmol, 292.4 µL, 2.0 eq) was dissolved in DMF (2 mL), then HATU (319.2 mg, 839.4 µmol, 1.0 eq) was added to the mixture. The mixture was then stirred at 25 °C for 30 min. TLC (DCM: CH_3OH=10:1, R_f=0.24) showed compound **1** was consumed completely and one new spot formed. The solvent was evaporated to produce compound **3** (0.45 g, 339.75 µmol, 40.47% yield, crude) as colorless oil, which was used in next step without further purification.

Compound **3** (450.0 mg, 339.75 µmol, 1.0 eq) was dissolved in DMF (8 mL), following by addition of piperidine (2 mL). The mixture was stirred for 15 mins at 25°C. LC-MS showed compound **3** was consumed completely and one main peak with desired (calculated MW: 1102.27, observed *m/z*: 552.1 ($[M/2+H]^+$)) was detected. The residue was purified by prep-HPLC (TFA condition). Compound **3** (370.0 mg, 335.67 µmol, 98.80% yield) was obtained as colorless oil.

To a solution of COM126 (60 mg, 54.45 µmol, 1.0 *eq*), compound **4** (15.5 mg, 81.68 µmol, 1.5 *eq*) in DMF (5 mL) was added HATU (31 mg, 81.68 µmol, 1.5 *eq*) and DIEA (10.5 mg, 61.68 µmol, 15 µL, 1.5 *eq*) .The mixture was stirred at 30 °C for 2 hr. LC-MS showed **COM126** was consumed completely and one main peak with desired was detected. The mixture was evaporated to remove solvent, and compound **5** (30 mg, crude) was obtained as colorless oil, which was used in next step without further purification.

Compound **5** (30 mg, 23.57 µmol, 1.0 eq) was dissolved DCM (4.5 mL), and then TFA (0.5 mL) was added and the mixture was stirred at 25-30 °C for 2 hr. LC-MS showed compound **5** was consumed completely and one main peak with desired was detected. The residue was purified by prep-HPLC (TFA condition). **COM472** (10 mg, 8.52 µmol) was obtained as white solid.

<u>COM473</u>



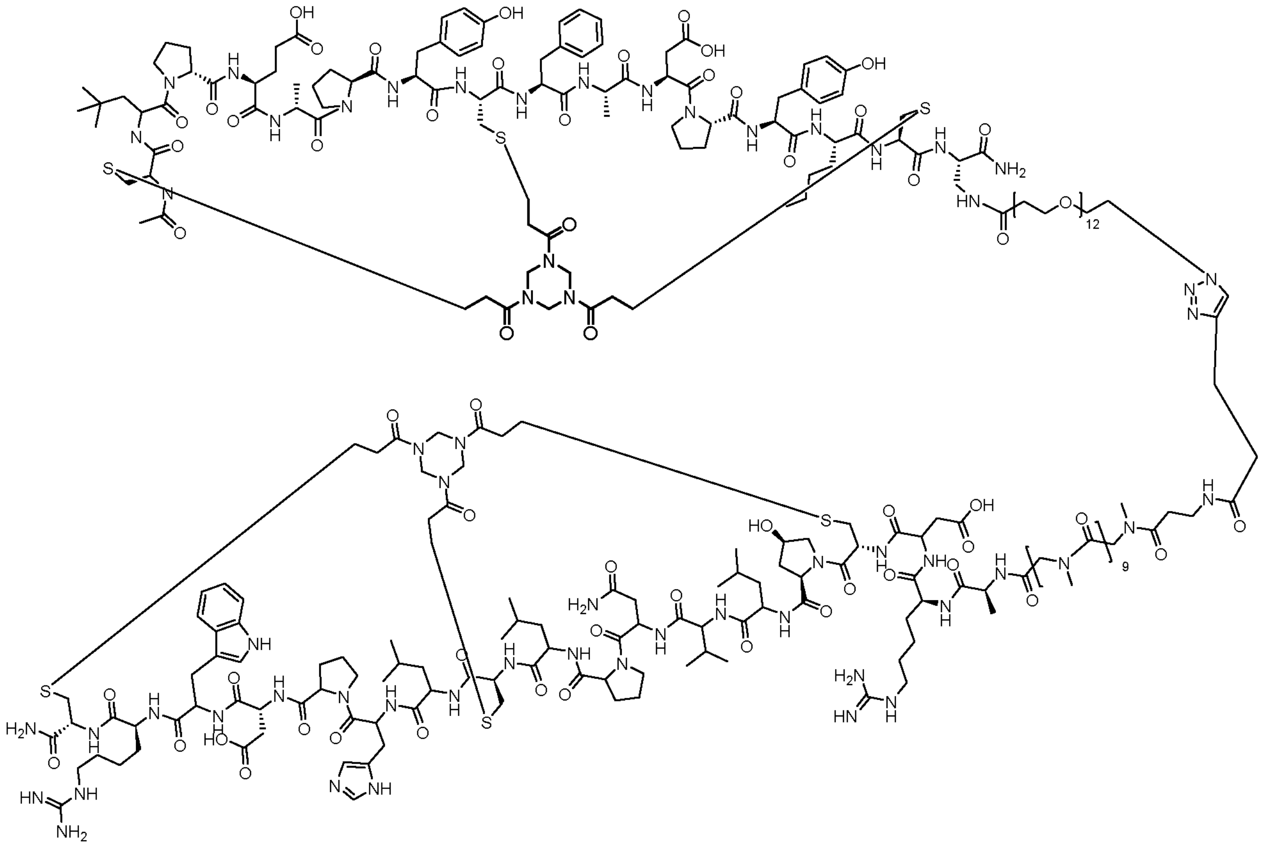
COM00000473

A mixture of compound **1** (300 mg, 449.96 µmol, 1.0 eq), compound **2** (138 mg, 449.96 µmol, 1.0 eq), HOBt (122 mg, 899.93 µmol, 2.0 eq), EDCI (173 mg, 899.93 µmol, 2.0 eq) was dissolved in DCM (10 mL, pre-degassed and purged with N_2 for 3 times), and then the mixture was stirred at 20-25 °C for 1 hr under N_2 atmosphere. LC-MS showed compound **1** was consumed completely and one main peak with desired (MW: 955.06, observed *m/z*: 955.3 ([M+H]$^+$)) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent. The mixture was evaporated under reduced pressure and compound **3** (300 mg, crude) was obtained as yellow oil.

Compound **3** (300 mg, 314.12 µmol, 1.0 eq) was dissolved in DMF (4 mL), and then piperdine (1 mL) was added and the mixture was stirred at 20-25 °C for 1 hr. LC-MS showed compound **3** was consumed completely and one main peak with desired m/z (MW: 732.83 observed *m/z*: 733.2 ([M+H]$^+$)) was detected. The residue was purified by prep-HPLC (neutral condition). **COM473** (160 mg, 218.33 µmol, 69.51% yield) was obtained as a colorless oil.

Example 2: Synthesis of EphA2/CD137 Binding Heterotandem Bicyclic Peptides

<u>**BCY9173**</u>



BCY00009173

Procedure for preparation of BCY9172-PEG12-N$_3$

BCY00009172 + **NHS-PEG12-N$_3$** —($NaHCO_3$; MeCN/H_2O)→ **BCY00009172-PEG12-N$_3$**

1 **2**

BCY9172 (520 mg, 248.16 µmol, 1 eq) and compound **1** (370 mg, 499.47 µmol, 2.01 eq), were dissolved in in DMF (5 mL) was added DIEA (48.11 mg, 372.24 µmol, 64.84 µL, 1.5 eq) and then the mixture was strirred at 30°C for 12 hr. LC-MS showed **BCY9172** was consumed completely and one main peak with desired m/z (calculated MW: 2721.12 observed *m/z*: 1360.9 ([M/2+H]$^+$)) was detected. The reaction mixture was purified by prep-HPLC (TFA condition) and compound **2** (284 mg, 101.10 µmol, 40.74% yield, 96.87% purity) was obtained as a white solid.

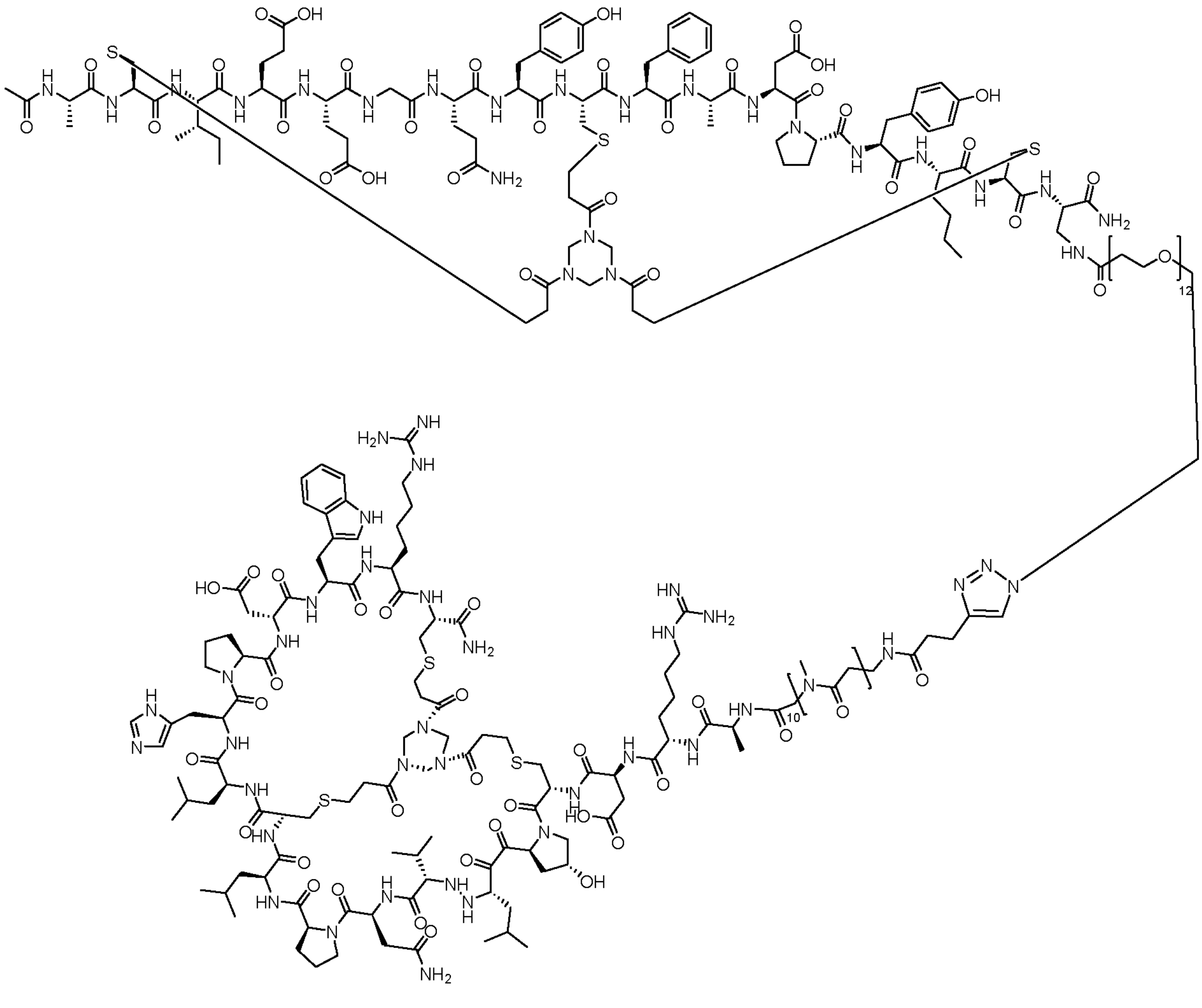
BCY00009172-PEG12-N$_3$ + **BCY00006169** —($CuSO_4$; VcNa; THPTA; t-BuOH/H_2O)→ **BCY00009173**

2

Procedure for preparation of BCY9173

This reaction was performed in two independent containers in parallel. For one container, Compound **2** (100 mg, 36.75 µmol, 1.0 eq) and **BCY6169** (120 mg, 36.78 µmol, 1.0 eq) were first dissolved in 10 mL of t-BuOH/H_2O (1:1), and then $CuSO_4$ (0.4 M, 91.9 µL, 1.0 eq), VcNa (0.4 M, 183.8 µL, 2.0 eq) and THPTA (0.4 M, 91.9 µL, 1.0 eq) was added. Finally 1 M NH_4HCO_3 was added to adjust pH to 8. All solvents here were degassed and purged with N_2 for 3 times. The reaction mixture was stirred at 40 °C for 16 hr under N_2 atmosphere. LC-MS showed compound **2** was consumed completely and one main peak with desired m/z (calculated MW: 5983.85 observed *m/z*: 997.6600 ($[M/6+H]^+$) and 1197.2300 ($[M/5+H]^+$)). The reaction mixture was purified by prep-HPLC (TFA condition) and **BCY9173** (218 mg, 34.97 µmol, 47.58% yield, 96% purity) was obtained as a white solid.

<u>BCY7985</u>



BCY00007985

General procedure for preparation of BCY7859

N3-PEG12-COOH —[1) HOSu, EDCI, DMA/DCM; 2) **BCY00007732,** DIEA, DMF]→ **BCY00007859**

To a solution of **N3-PEG12-COOH** (250 mg, 388 μmol) and HOSu (67.0 mg, 583 μmol) in DMA (4.5 mL) and DCM (1.5 mL) was added EDCI (89.3 mg, 466 μmol) with stirring at 20 °C for 16 hr. To another 50 mL of round flask containing a mixture of **BCY7732** (855 mg, 388 μmol) in 5 mL of DMA was added DIEA (186 mg, 1.44 mmol, 250 μL) with stirring for 10 min. Then the initial reaction mixture was added to the flask with further stirring at 20 °C for additional 5 hr. LC-MS (ES8396-307-P1B1) showed **BCY7732** was consumed completely and one main peak with desired mass was detected. The resulting reaction mixture was purified directly by prep-HPLC (TFA condition) to give compound **BCY7859** (621 mg, 200 μmol, 51.6% yield, TFA salt) as a white solid.

General procedure for preparation of BCY6169

BCY00006099 —[**PYA-NHS**; DIEA, DMA]→ **BCY00006169**

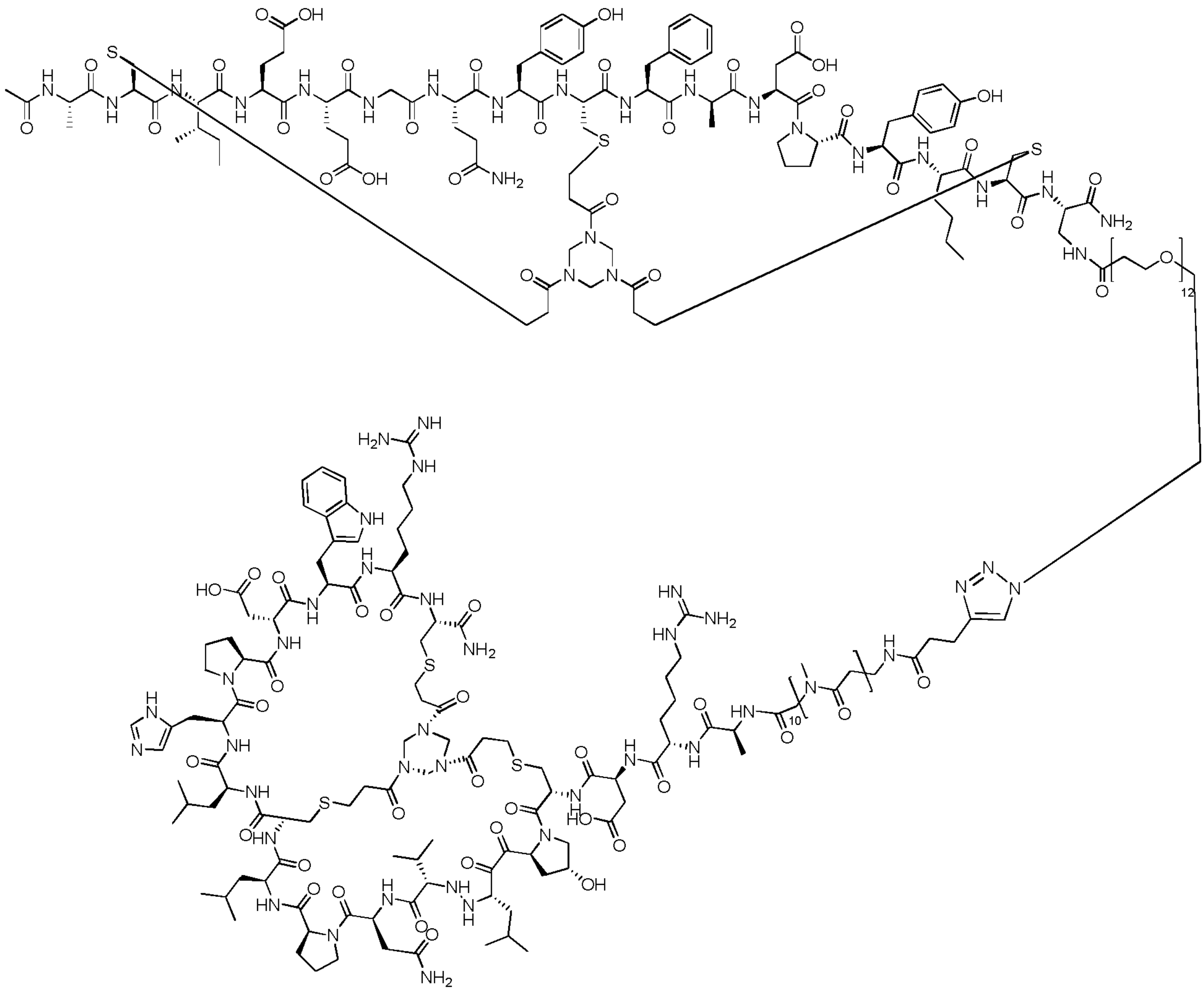
To a solution of **BCY6099** (300 mg, 94.3 μmol) in DMA (2 mL) was added DIEA (36.6 mg, 283 μmol, 49.3 μL) with stirring for 10 min. After, **PYA-NHS** (36.8 mg, 189 μmol) was added with further stirring at 20 °C for additional 15 hr. LC-MS showed **BCY6099** was consumed completely and one main peak with desired mass was detected. The reaction mixture was purified by prep-HPLC (neutral condition) to give compound **BCY6169** (299 mg, 86.2 μmol, 91.5% yield) as a white solid.

General procedure for preparation of BCY7985

BCY00006169 + **BCY00007859** —[Vc, $CuSO_4$; DMF, H_2O]→ **BCY00007985**

To a solution of **BCY7859** (220 mg, 77.8 μmol) and **BCY6169** (251 mg, 77.1 μmol) in DMF (5 mL) purged by nitrogen for 2 hr was added aqueous ascorbic acid solution (0.8 M, 963 μL) follwed by adding aqueous $CuSO_4$ (0.8 M, 289 μL) under nitrogen atmosphere. Then the mixture was stirred at 20 °C for 2 hr. LC-MS showed **BCY6169** was consumed completely and one main peak with desired mass was detected. The reaction mixture was directly purified by prep-HPLC (TFA condition) to give compound **BCY7985** (283 mg, 43.4 μmol, 56.3% yield, TFA) as a white solid.

BCY8942



BCY00008942

General procedure for preparation of BCY8940

BCY00008045 + **N3-PEG12-COOH** —(EDCI, HOSu / DMA. DCM)→ **BCY00008940**

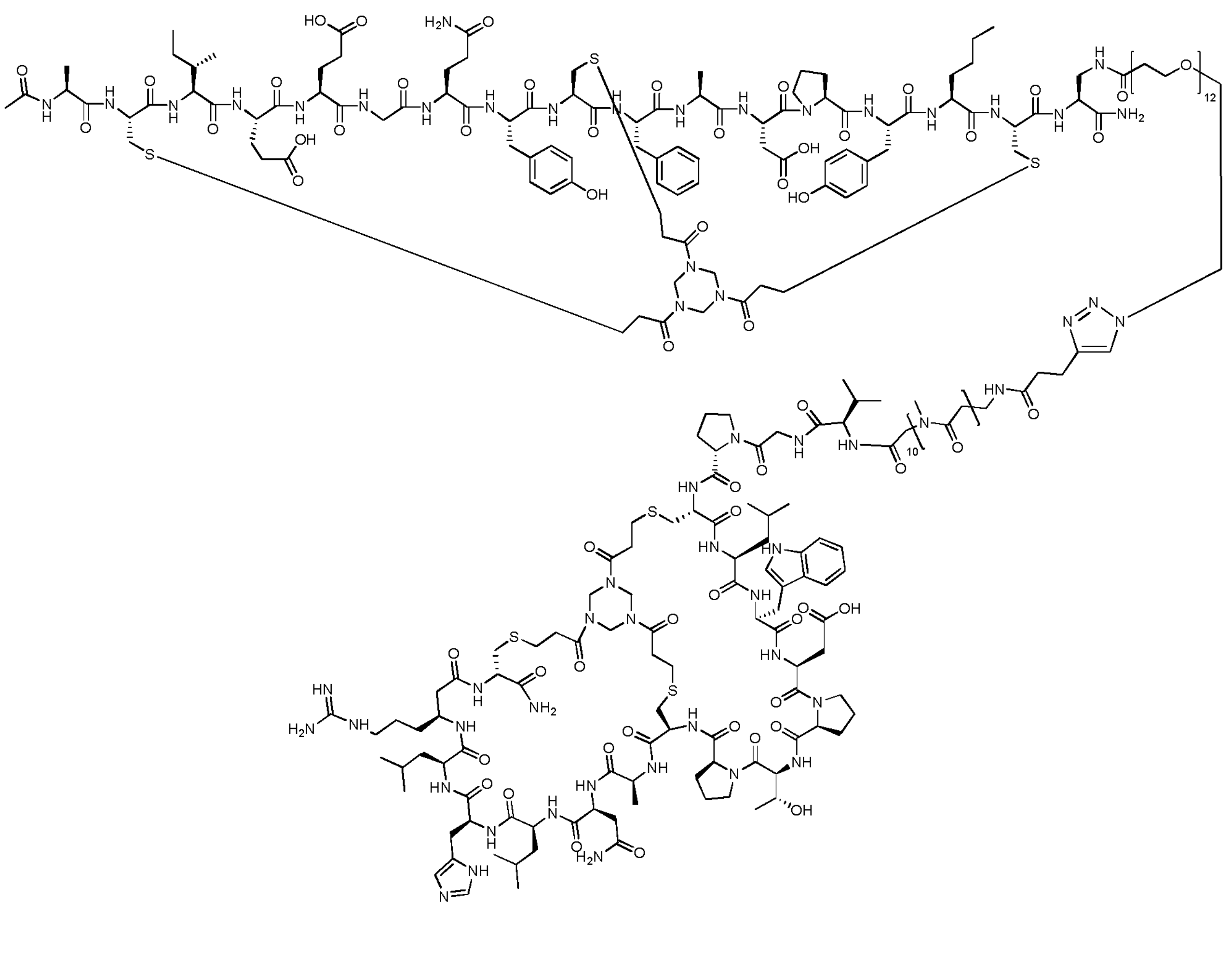
To a solution of N3-PEG12-COOH (120 mg, 186 µmol, 1.0 eq) in DMA (3 mL) and DCM (1 mL) was added HOSu (32.2 mg, 280 µmol, 1.5 eq) with stirring. Then EDCI (42.9 mg, 224 µmol, 1.2 eq) was added to the mixture with further stirring for additional 7 hr at 20 °C. LCMS showed the activated ester was formed completely. To another flask with **BCY8045** (410 mg, 186 µmol, 1.0 eq) in DMA (3 mL) was added DIEA (120 mg, 932 µmol, 162 µL, 5.0 eq) with stirring, then the activated ester was added and the mixture was stirred for 18 hr at 20 °C. LC-MS showed one main peak with desired m/z was detected. The reaction mixture was concentrated in vacuum to remove the DCM. The resulting mixture was purified by prep-HPLC (TFA condition) to give **BCY8940** (190 mg, 67.2 µmol, 36.1% yield) as a white solid.

General procedure for preparation of BCY8942

BCY00006169 —(**BCY00008940**; Vc, $CuSO_4$, DMF, H_2O)→ **BCY00008942**

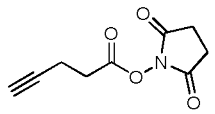
To a solution of **BCY8940** (28.6 mg, 10.1 µmol, 1.1 eq) and **BCY6169** (30.0 mg, 9.19 µmol, 1.0 eq) in DMF (2.0 mL) was added (2R)-2-[(1S)-1,2–dihydroxyethyl]-3, 4-dihydroxy-2H-furan-5-one (1.0 M, 92.0 µL) and $CuSO_4$ (1.0 M, 27.6 µL) with stirring under nitrogen atmosphere for 2 hr at 20 °C. LC-MS showed **BCY6169** was consumed completely and one main peak with desired m/z (*calculated* MW: 6089.91 observed *m/z*: 1218.4($[M/5+H]^+$), 1016.0($[M/6+H]^+$), 870.7($[M/7+H]^+$) was detected. The reaction mixture was purified by prep-HPLC (TFA condition) to give compound **BCY8942** (15.4 mg, 2.46 µmol, 26.8% yield, 97.3% purity) as a white solid.

<u>**BCY8943**</u>



BCY00008943

General procedure for preparation of BCY8941

BCY00006015 +  —DIEA, DMA→ **BCY00008941**

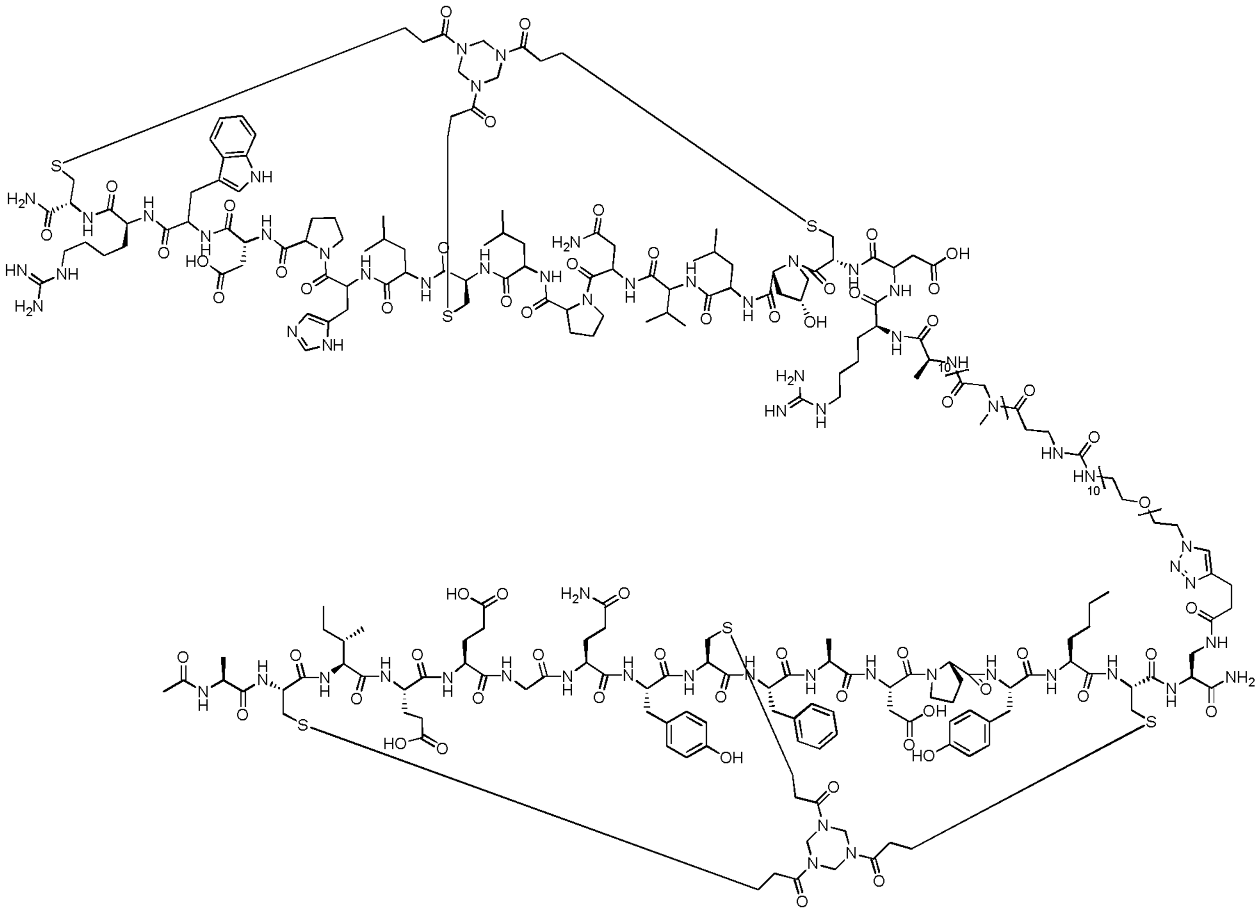
To a solution of **BCY6015** (a peptide identical to BCY8941 except for the absence of a PYA moiety; 100 mg, 32.9 μmol) in DMA (2 mL) was added DIEA (12.8 mg, 98.7 μmol, 17.2 μL) with stirring for 10 min. Then (2,5-dioxopyrrolidin-1-yl) pent-4-ynoate (12.8 mg, 65.8 μmol) was added to the mixture, following with further stirring at 20 °C for 16 hr. LC-MS showed compound **1** was consumed completely and one main peak with desired m/z (*calculated* MW: 3119.60, observed *m/z*: 1040.5($[M/3+H]^+$) was detected. The mixture was purified by prep-HPLC (neutral condition) to give compound **BCY8941** (90.0 mg, 28.9 μmol, 87.7% yield) as a white solid.

General procedure for preparation of BCY8943

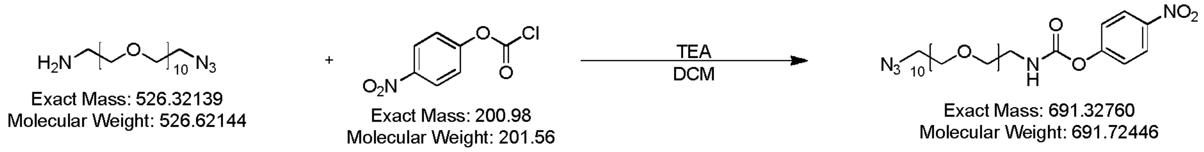
BCY00008941 —**BCY00007859**; Vc, $CuSO_4$, DMF, H_2O→ **BCY00008943**

To a solution of **BCY7859** (which may be prepared as described in BCY7985; 40.0 mg, 14.2 μmol) and **BCY8941** (42.0 mg, 13.5 μmol) in DMSO (2 mL, pre-purged by nitrogen for 1 hr) was added (2R)-2-[(1S)-1,2-dihydroxyethyl]-3,4-dihydroxy-2H-furan-5-one (1.0 M, 270 μL) and $CuSO_4$ (1.0 M, 80.9 μL). The mixture was purged with nitrogen for 3 times and stirred at 15 °C for 2 hr. LC-MS showed **BCY8941** was consumed completely and one main peak with desired m/z (*calculated* MW: 5946.77, observed *m/z*: 1190.2 ($[M/5+H]^+$), 991.5($[M/6+H]^+$), 849.9($[M/7+H]^+$) was detected. The reaction mixture was purified by prep-HPLC (A: 0.075% TFA in H_2O, B: ACN) to give compound **BCY8943** (11.5 mg, 1.90 μmol, 14.1% yield, 98.1% purity) as a white solid.

<u>**BCY9647**</u>



Procedure for preparation of compound 2



COM00000134 **1** **2**

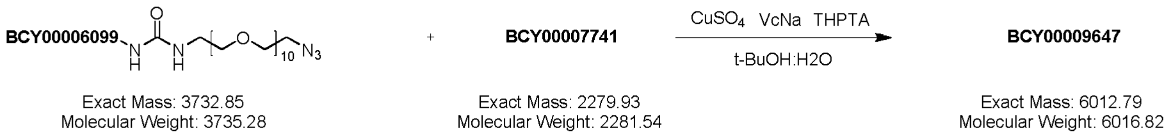
To a solution of **COM134** (30.0 mg, 57.0 µmol, 1.0 eq), compound **1** (17.2 mg, 85.3 µmol, 1.5 eq) in DCM (0.5 mL) was added TEA (8.65 mg, 11.9 µL, 1.5 eq). The mixture was stirred at 25 °C for 1 hr. LC-MS showed **COM134** was consumed completely and one main peak with desired mass (calculated MW: 691.72, observed *m/z*: 692.3($[M+H]^+$) and 709.3($[M+NH4]^+$)) was detected. The reaction mixture was concentrated under reduced pressure, and then lyophilized to produce crude compound **2** (30.5 mg, crude) as a white

Procedure for preparation of compound 3

2 **3**

To a solution of compound **2** (10 mg, 1.0 eq) in DMF (1 mL) was added **BCY6099** (46 mg, 1.0 eq) and DIEA (5.61 mg, 7.55 µL, 3.0 eq). The mixture was stirred at 30 °C for 2 hr. LC-MS showed compound **2** was consumed completely and one main peak with desired m/z (calculated MW: 3735.28 observed *m/z*: 1245.9($[M/3+H]^+$) and 934.5($[M/4+H]^+$)) was detected. The reaction mixture was filtered and concentrated under reduced pressure to give a residue. The crude product was purified by reversed-phase HPLC (TFA condition). Compound **3** (34 mg, 62.96% yield, 100% purity) was obtained as a white solid.

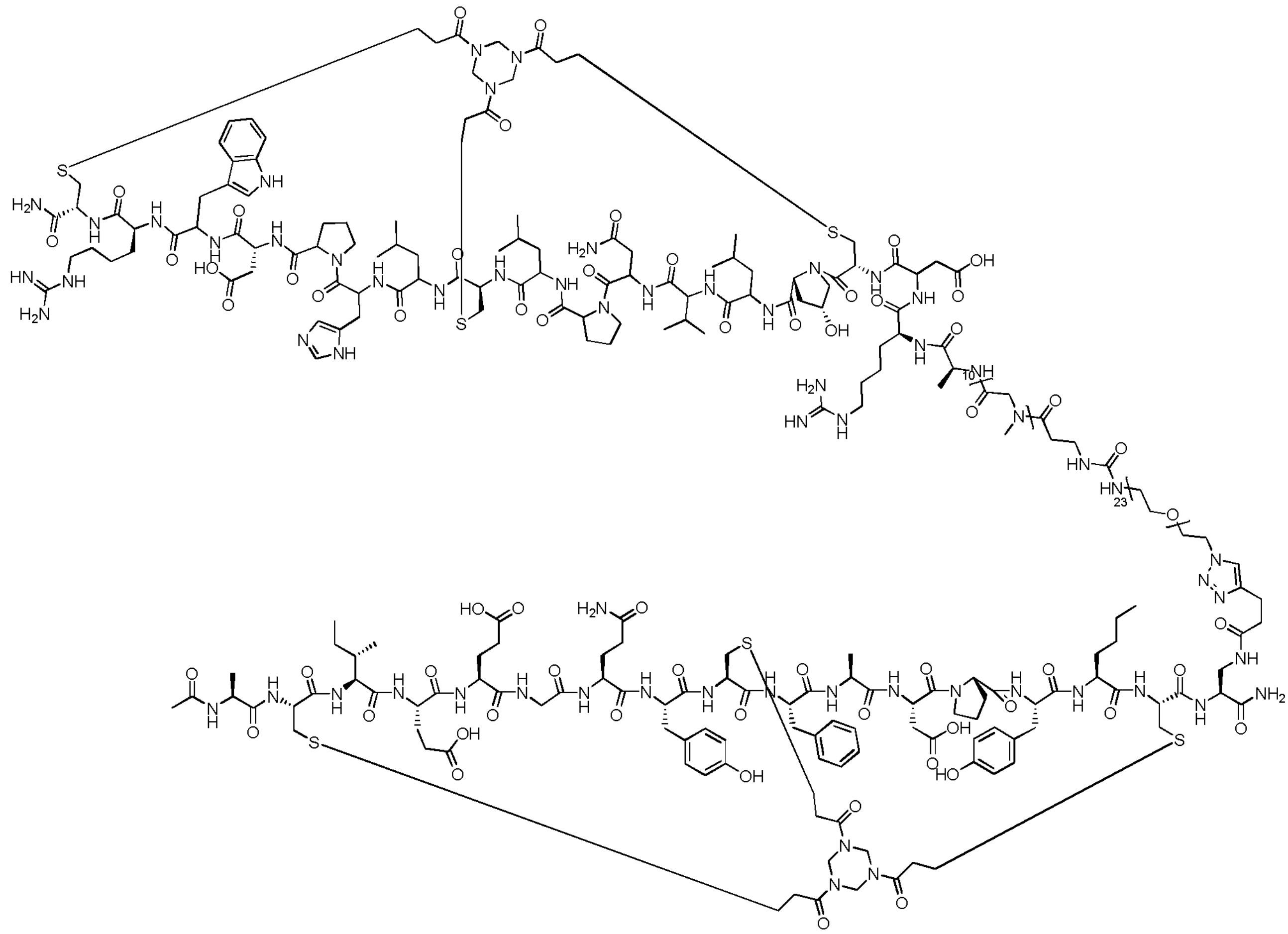
Procedure for preparation of BCY9647



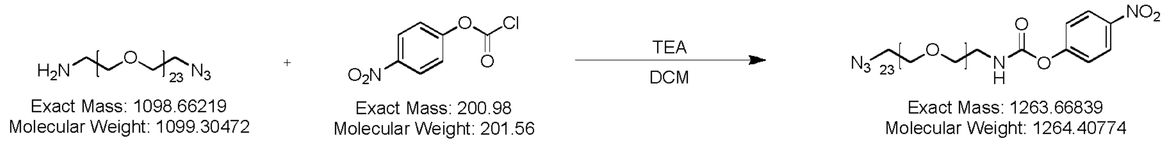
3

A mixture of Compound **3** (34 mg, 9.10 µmol, 1.0 eq), **BCY7741** (23 mg, 10.08 µmol, 1.11 eq), and THPTA (0.4 M, 11.4 µL, 0.5 eq) was dissolved in t-BuOH/H_2O (1:1, 2 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 11.4 µL, 0.5 eq) and VcNa (0.4 M, 22.8 µL, 1 eq) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 25-30 °C for 12 hr under N_2 atmosphere. LC-MS showed compound **3** was consumed completely and one main peak with desired m/z (calculated MW: 6016.82, observed m/z: 1204.1($[M/5+H]^+$), 1003.5($[M/6+H]^+$), 860.3($[M/7+H]^+$)). The reaction mixture was directly purified by prep-HPLC (TFA condition). **BCY9647** (31.2 mg, 54.67% yield, 95.96% purity) was obtained as a white solid.

<u>BCY9648</u>



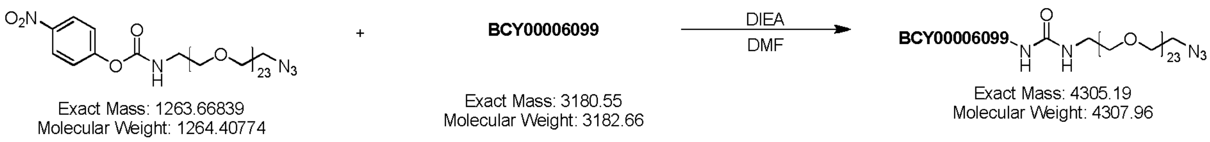
Procedure for preparation of compound 2



COM00000135 **1** **2**

To a solution of **COM135** (30 mg, 27.29 µmol, 1.0 *eq*), compound 1 (8.25 mg, 40.94 µmol, 1.5 *eq*) in DCM (0.5 mL) was added TEA (4.14 mg, 40.94 µmol, 5.70 µL, 1.5 *eq*). The mixture was stirred at 25~30°C for 1 hr. LC-MS showed **COM135** was consumed completely and one main peak with desired mass [calculated MW: 1264.41, observed *m/z*: 1281.4([M+NH$_4$]$^+$), 649.8 ([M/2+H]$^+$)] was detected. The reaction mixture was concentrated under reduced pressure to remove solvent to give a residue. The residue was purified by prep-HPLC (TFA condition) to give compound 2 (18 mg, 14.2 µmol, 52.14% yield).

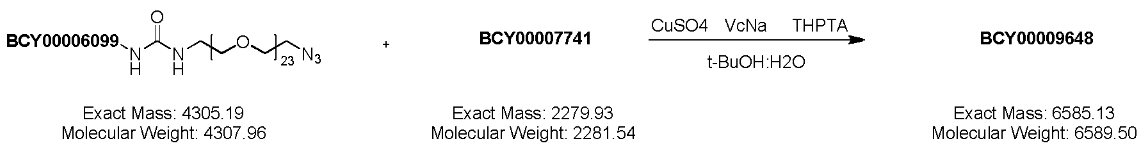
Procedure for preparation of compound 3



2 **3**

To a solution of compound **3** (9 mg, 7.12 µmol, 1 *eq*) in DMF (1 mL) was added **BCY6099** (23 mg, 7.23 µmol, 1.02 *eq*) and DIEA (2.76 mg, 21.35 µmol, 3.72 µL, 3.0 *eq*). The mixture was stirred at 30 °C for 2 hr. LC-MS showed compound **2** was consumed completely and one main peak with desired m/z (calculated MW: 4307.96 observed *m/z*: 1436.9([M/3+H]$^+$), 1077.9([M/4+H]$^+$), 862.5([M/5+H]$^+$)) was detected. The reaction mixture was filtered and concentrated under reduced pressure to give a residue. The crude product was purified by reversed-phase HPLC (TFA condition). Compound **3** (14.6 mg, 47.61% yield, 100% purity) was obtained as a white solid.

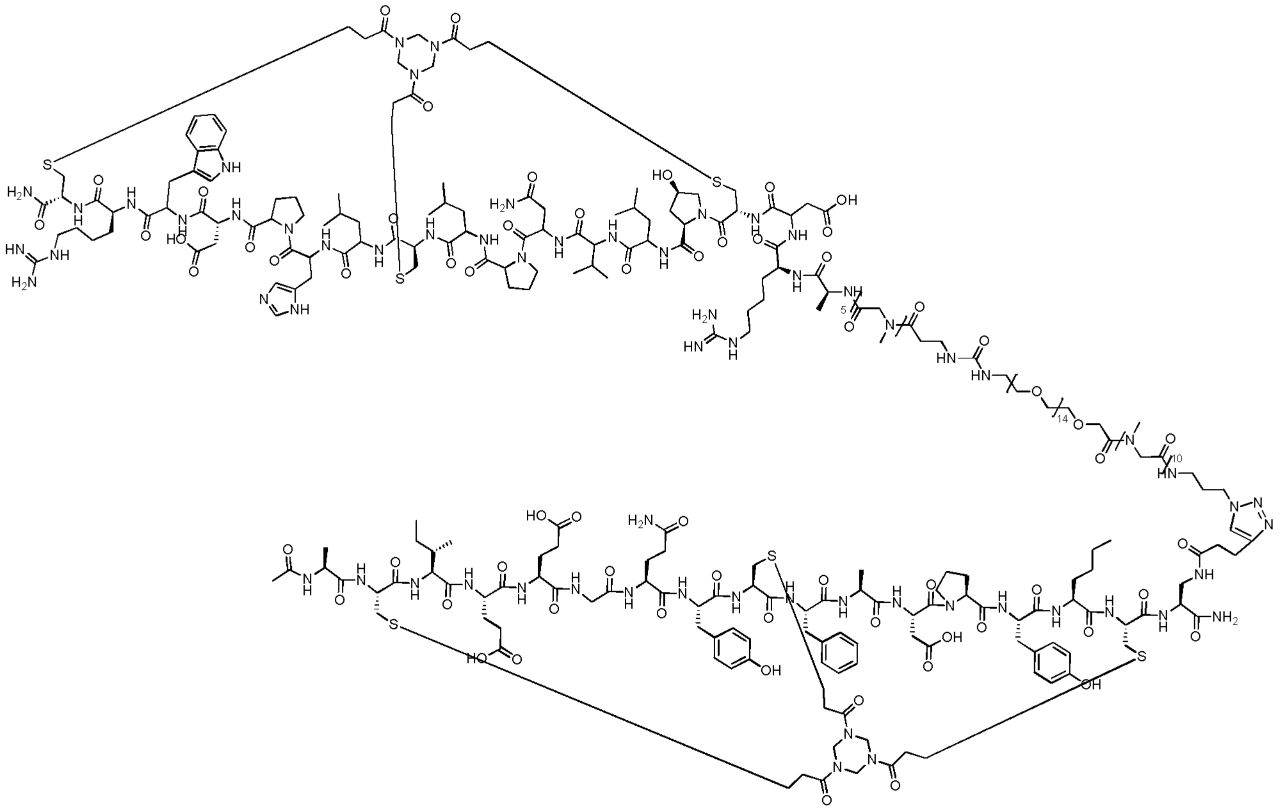
Procedure for preparation of BCY9648



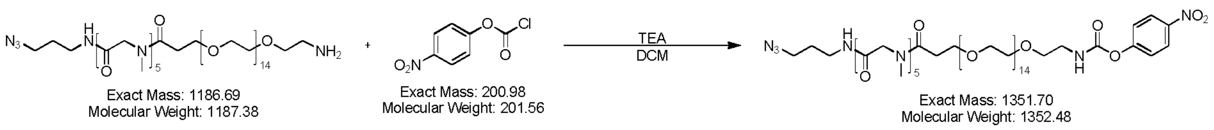
3

A mixture of compound **3** (14.6 mg, 3.39 µmol, 1 *eq*), **BCY7741** (8.5 mg, 3.73 µmol, 1.1 *eq*) and THPTA (0.4 M, 4.3 µL, 0.5 *eq*) was dissolved in t-BuOH/H_2O (1:1, 2 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (.4 M, 4.3 µL, 0.5 *eq*) and VcNa (0.4 M, 8.6 µL, 1.0 *eq*) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 25-30 °C for 12 hr under N_2 atmosphere. LC-MS showed compound **3** was consumed completely and one main peak with desired m/z (MW: 6589.50 observed *m/z*: 1098.8([M/6+H]$^+$), 942.1([M/7+H]$^+$), 824.6([M/8+H]$^+$)). The reaction mixture was directly purified by prep-HPLC (**TFA condition)**. **BCY9648** (14.7 mg, 63.34% yield, 96.22% purity) was obtained as a white solid.

BCY9655



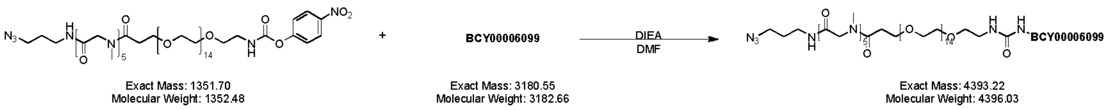
Procedure for preparation of compound 2



COM00000128 1 2

To a solution of **COM128** (120 mg, 101.06 µmol, 1.0 eq), compound **1** (25 mg, 124.03 µmol, 1.25 eq) in DCM (0.5 mL) was added TEA (15.34 mg, 151.59 µmol, 21.10 µL, 1.5 eq). The mixture was stirred at 25 °C for 1 hr. LC-MS showed one new peak with desired m/z (calculated MW: 1352.48, observed *m/z*: 676.8([M/2+H]$^+$), 1369.3([M+NH4]$^+$)) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent to give a residue. The residue was purified by prep-HPLC (neutral condition). Compound **2** (14 mg, 8.99 µmol, 8.90% yield, 86.86% purity) was obtained as colorless oil.

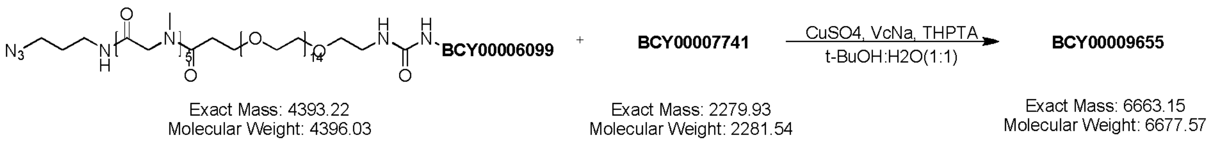
Procedure for preparation of compound 3



2 3

To a solution of compound **2** (7 mg, 5.18 µmol, 1.0 eq) and **BCY6099** (16 mg, 5.03 µmol, 1.0 eq) in DMF (2 mL) was added DIEA (2.01 mg, 15.53 µmol, 2.70 µL). The mixture was stirred at 30 °C for 2 hrs. LC-MS showed compound **2** was consumed completely and one main peak with desired m/z (calculated MW: 4396.02, observed *m/z*: 879.8($[M/5+H]^+$) and 1099.8($[M/4+H]^+$)) was detected. The reaction mixture was filtered and concentrated under reduced pressure to give a residue. The crude product was purified by reversed-phase HPLC (0.1% TFA condition). Compound **3** (11.8 mg, 48.29% yield, 93.11% purity) was obtained as a white solid.

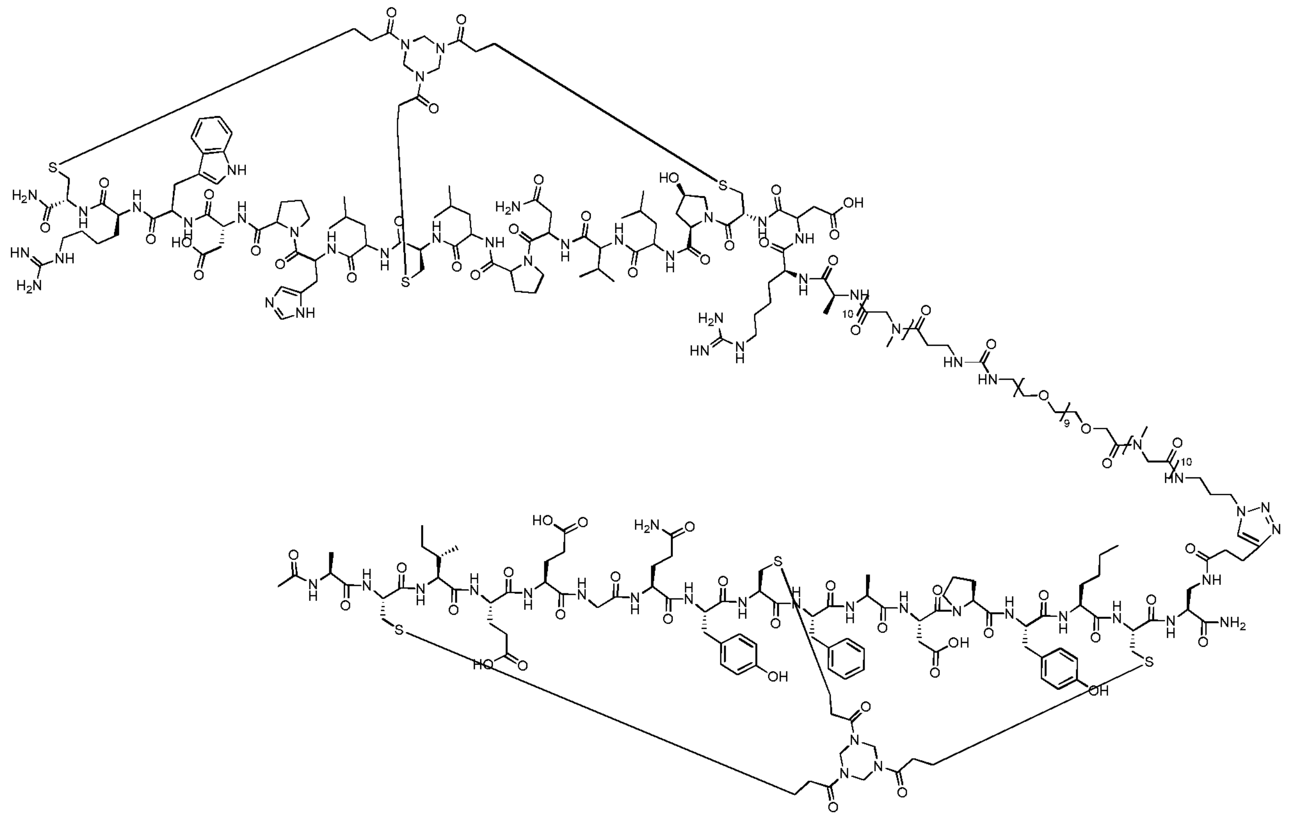
Procedure for preparation of BCY9655



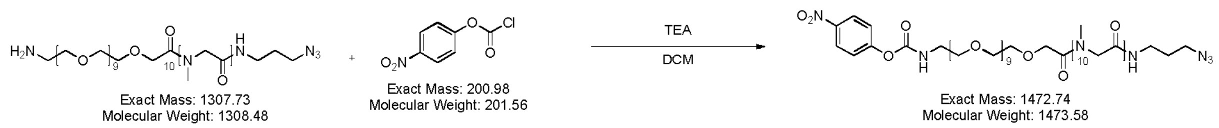
3

A mixture of Compound **3** (11.8 mg, 2.69 µmol, 1.0 eq), **BCY7741** (7.0 mg, 3.07 µmol, 1.14 eq), and THPTA (0.4 M, 6.8 µL, 1 eq) was dissolved in t-BuOH/H_2O (1:1, 2 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 6.8 µL, 1.0 eq) and VcNa (0.4 M, 13.6 µL, 2.0 eq) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 25-30 °C for 12 hr under N_2 atmosphere. LC-MS showed compound **3** was consumed completely and one main peak with desired m/z (calculated MW: 6677.57, observed *m/z*: 1113.7 ($[M/6+H]^+$), 954.7 ($[M/7+H]^+$)). The reaction mixture was directly purified by prep-HPLC (TFA condition). **BCY9655** (1.9 mg, 0.26 µmol, 9.65% yield, 91.15% purity) was obtained as a white solid.

<u>BCY9656</u>



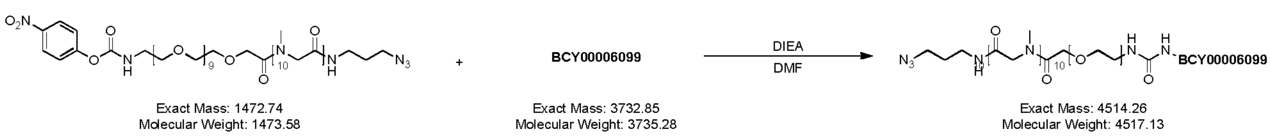
Procedure for preparation of compound 2



COM00000129 1 2

To a solution of **COM129** (30.0 mg, 22.93 µmol, 1.0 eq), compound **1** (6.9 mg, 34.39 µmol, 1.5 eq) in DCM (3 mL) was added TEA (3.5 mg, 34.39 µmol, 4.8 µL, 1.5 eq). The mixture was degassed and purged with N_2 for 3 times, and then the mixture was stirred at 25 °C for 1 hr under N_2 atmosphere. LC-MS showed **COM129** was consumed completely and one main peak with desired m/z (calculated MW: 1473.58, observed *m/z*: 737.3($[M/2+H]^+$)) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent to give a residue. The residue was purified by prep-HPLC (neutral condition) to produce Compound **2** (12.3 mg, 8.35 µmol, 36.41% yield) as a white solid.

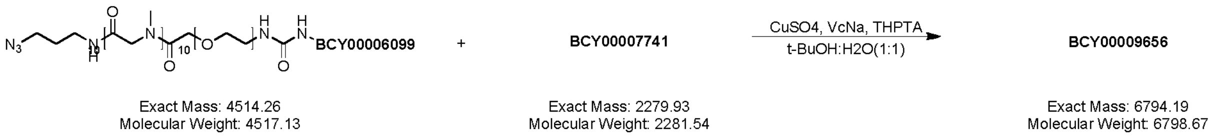
Procedure for preparation of compound 3



2 3

To a solution of compound **2** (9.26 mg, 6.28 µmol, 1.0 eq) and **BCY6099** (10 mg, 3.14 µmol, 0.5 eq) in DMF (3 mL) was added TEA (0.7 mg, 6.93 µmol, 1 µL, 1.1 eq). The mixture was degassed and purged with N_2 for 3 times, and then the mixture was stirred at 25-30 °C for 1 hr under N_2 atmosphere. LC-MS showed compound **2** was consumed completely and one main peak with desired m/z (calculated MW: 4517.12, observed *m/z*: 1129.8 ([M/4+H]$^+$),904.1 ([M/5+H]$^+$), 753.7 ([M/6+H]$^+$)) was detected. The reaction mixture was filtered and concentrated under reduced pressure to give a residue. The crude product was purified by reversed-phase HPLC (TFA condition). Compound **3** (12 mg, 72.36% yield, 85.58% purity) was obtained as a white solid.

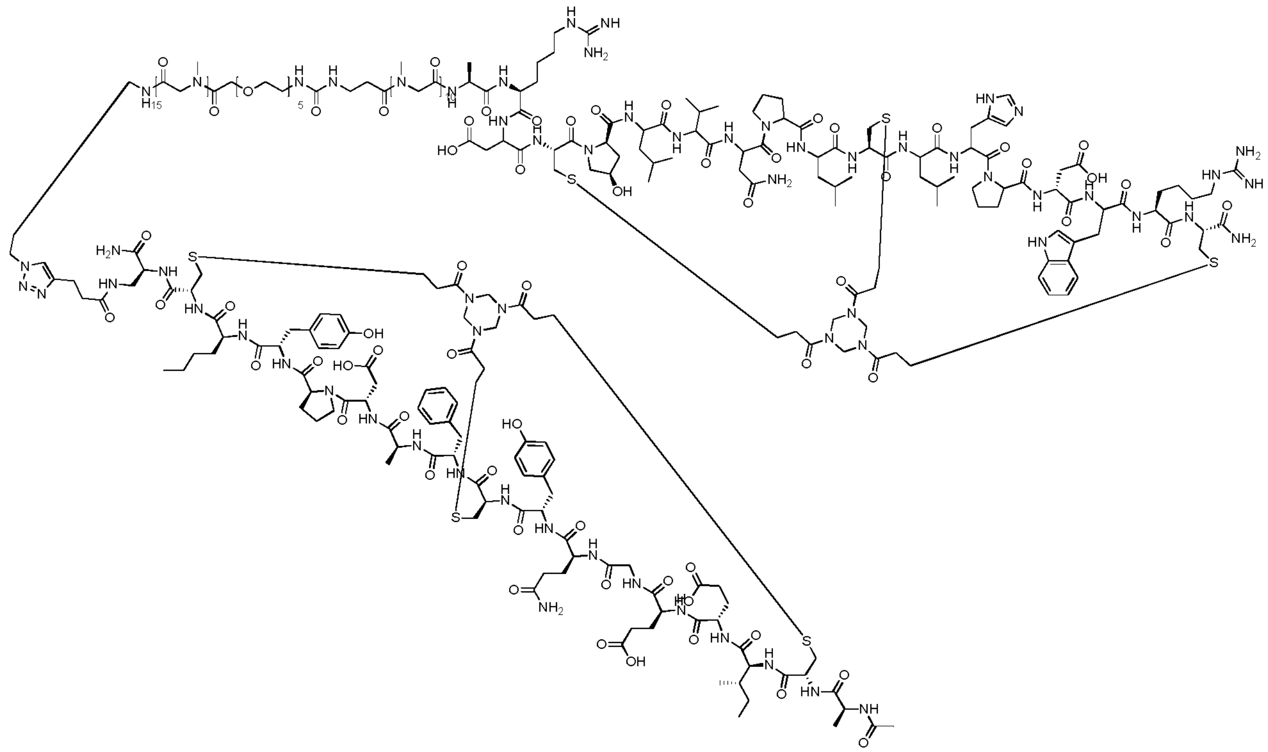
Procedure for preparation of BCY9656



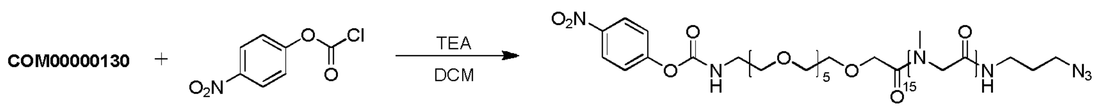
3

A mixture of Compound **3** (11 mg, 2.44 μmol, 1.0 eq), **BCY7741** (6.0 mg, 2.63 μmol, 1.08 eq), and THPTA (0.4 M, 6.1 μL, 1.0 eq) was dissolved in t-BuOH/H_2O (1:1, 2 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 6.1 μL, 1.0 eq) and VcNa (0.4 M, 12.2 μL, 2.0 eq) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 25-30 °C for 12 hr under N_2 atmosphere. LC-MS showed Compound **3** was consumed completely and one main peak with desired m/z (calculated MW: 6798.66, observed m/z: 1133.8 ([M/6+H]$^+$), 971.9 ([M/7+H]$^+$), 850.7 ([M/8+H]$^+$)) was detected. The reaction mixture was directly purified by prep-HPLC (TFA condition). **BCY9656** (6.8 mg, 37.36% yield, 90.97% purity) was obtained as a white solid.

<u>BCY9657</u>



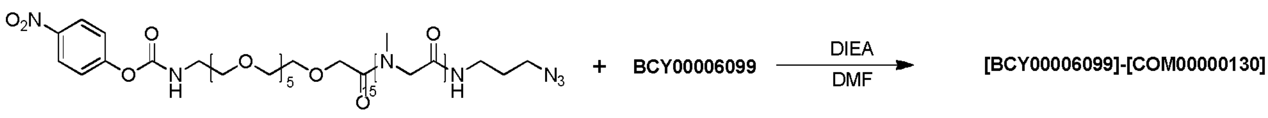
Procedure for preparation of compound 2



1 2

To a solution of **COM130** (30.0 mg, 20.78 µmol, 1.0 eq), compound **1** (6.3 mg, 31.17 µmol, 1.5 eq) in DCM (3 mL) was added TEA (3.2 mg, 31.17 µmol, 4.4 µL, 1.5 eq). The mixture was stirred at 25-30 °C for 1 hr. LC-MS showed **COM130** was consumed completely and one main peak with desired m/z (calculated MW: 1608.7, observed *m/z*: 804.8([M/2+H]$^+$)) was detected. The reaction mixture was concentrated under reduced pressure and lyophilized to produce compound **2** (7.9 mg, crude) as a white solid.

Procedure for preparation of compound 3



2 3

To a solution of compound **2** (7.9 mg, 4.91 µmol, 1.0 eq) and **BCY6099** (16 mg, 5.03 µmol, 1.02 eq) in DMF (1 mL) was added DIEA (1.9 mg, 14.73 µmol, 2.6 µL, 3.0 eq). The mixture was stirred at 30 °C for 2 hrs. LC-MS showed compound **2** was consumed completely and one main peak with desired m/z (calculated MW: 4652.25, observed *m/z*: 1551.3([M/3+H]$^+$), 1163.6([M/4]$^+$), 931.1([M/5+H]$^+$), 776.1([M/6+H]$^+$))was detected. The reaction mixture was filtered and concentrated under reduced pressure to give a residue. The crude product was purified by reversed-phase HPLC (TFA condition). Compound **3** (13.3 mg, 2.86 µmol, 53.22% yield, 91.42% purity) was obtained as a white solid.

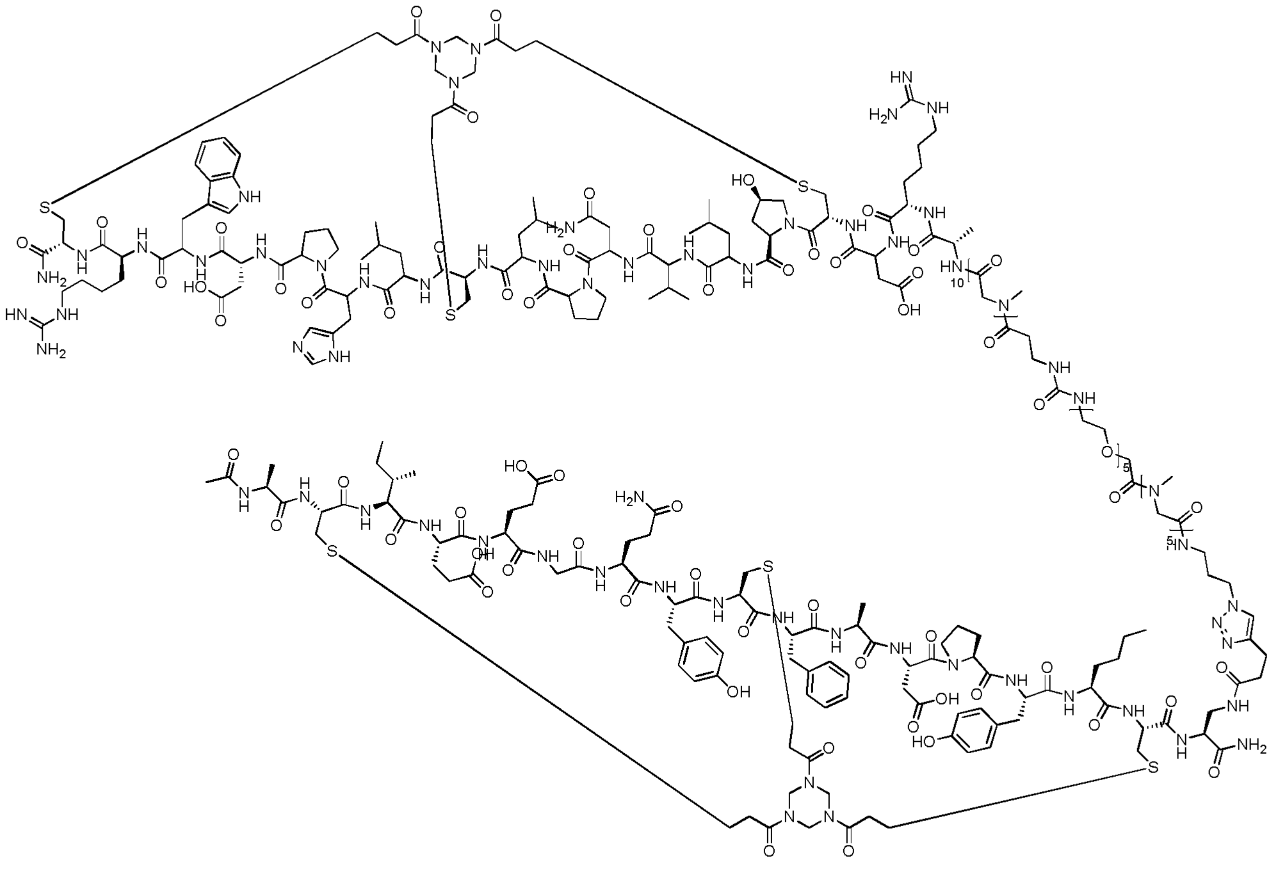
Procedure for preparation of BCY9657

[BCY00006099]-[COM00000130] + **BCY00007741** $\xrightarrow[\text{t-BuOH/H}_2\text{O}]{\text{CuSO}_4\ \text{VcNa}\ \text{THPTA}}$ **BCY00009657**

3

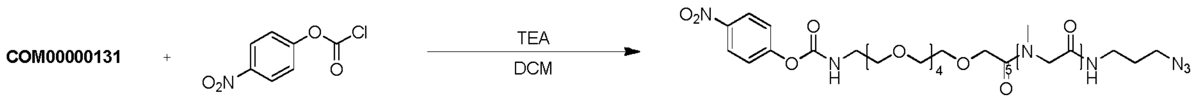
A mixture of Compound **3** (13.3 mg, 2.86 µmol, 1.0 eq), **BCY7741** (7.0 mg, 3.07 µmol, 1.03 eq), and THPTA (0.4 M, 7.5 µL, 1.0 eq) was dissolved in t-BuOH/H_2O (1:1, 2 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 7.5 µL, 1 eq) and VcNa (0.4 M, 15 µL, 2.0 eq) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 25-30 °C for 12 hr under N_2 atmosphere. LC-MS showed Compound **3** was consumed completely and one main peak with desired m/z [MW: 6933.78, observed *m/z*: 1156.7([M/6+H]$^+$), 991.4([M/7+H]$^+$), 867.4([M/8+H]$^+$)] was detected. The reaction mixture was directly purified by prep-HPLC (TFA condition). **BCY9657** (8.4 mg, 40.21% yield, 94.9% purity) was obtained as a white solid.

<u>BCY9658</u>



BCY00009658

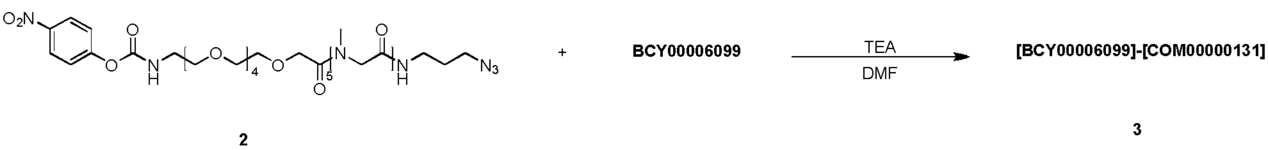
Procedure for preparation of compound 2



1 2

To a solution of **COM131** (167.0 mg, 227.89 μmol, 1.0 eq), compound **1** (55.0 mg, 272.87 μmol, 1.2 eq) in DCM (5 mL) was added TEA (36.4 mg, 359.23 μmol, 50.0 μL, 1.6 eq). The mixture was stirred at 25-30 °C for 1 hr. LC-MS showed one main peak with desired m/z (MW: 897.93 observed 920.3([M+Na$^+$])) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent to give a residue. The residue was purified by prep-HPLC (TFA condition). Compound **2** (35 mg, 33.74 μmol, 14.81% yield, 86.56% purity) was obtained as colorless oil.

Procedure for preparation of compound 3



2 3

To a solution of compound **2** (15 mg, 16.71 μmol, 1.0 eq) and **BCY6099** (53 mg, 16.65 μmol, 1.0 eq) in DMF (2 mL) was added DIEA (6.48 mg, 65.05 μmol, 50.1 μL, 4.0 eq). The mixture was stirred at 30 °C for 2 hrs. LC-MS showed compound **2** was consumed completely and one main peak with desired m/z (MW: 3941.47 observed *m/z*: 986.0([M/4+H]$^+$)) was detected. The reaction mixture was filtered and concentrated under reduced pressure to give a residue. The crude product was purified by reversed-phase HPLC (TFA condition). **[BCY6099]-[COM131] (**5 mg, 50.48% yield, 94.96% purity) was obtained as a white solid.

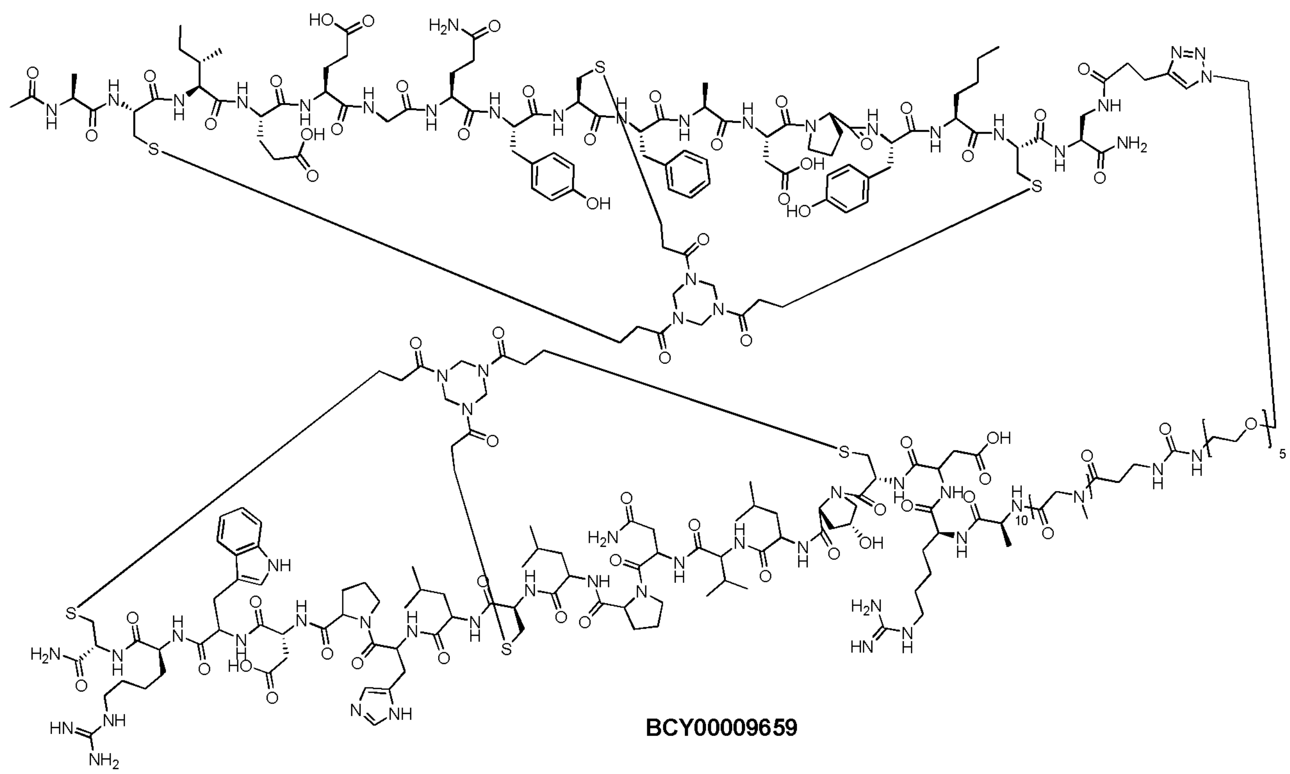
Procedure for preparation of BCY9658

[BCY00006099]-[COM00000131] + **BCY00007741** $\xrightarrow[\text{t-BuOH/H}_2\text{O}]{\text{CuSO}_4\ \text{VcNa}\ \text{THPTA}}$ **BCY00009658**

3

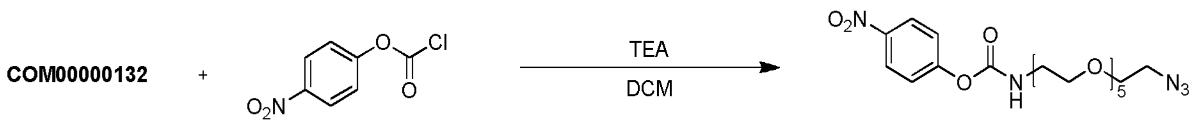
A mixture of Compound **3** (35 mg, 8.88 µmol, 1.0 eq), **BCY7741** (21 mg, 9.20 µmol, 1.03 eq), and THPTA (0.4 M, 22.2 µL, 1.0 eq) was dissolved in t-BuOH/H_2O (1:1, 2 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 22.2 µL, 1.0 eq) and VcNa (0.4 M, 44.4 µL, 2.0 eq) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 25-30 °C for 12 hr under N_2 atmosphere. LC-MS showed Compound **3** was consumed completely and one main peak with desired m/z [MW: 6223.01 observed *m/z*: 1038.0([M/6+H]$^+$) and 889.8([M/8+H]$^+$)] was detected. The reaction mixture was directly purified by prep-HPLC (TFA condition)**. BCY9658** (13.2 mg, 21.54% yield, 90.16% purity) was obtained as a white solid.

<u>BCY9659</u>



BCY00009659

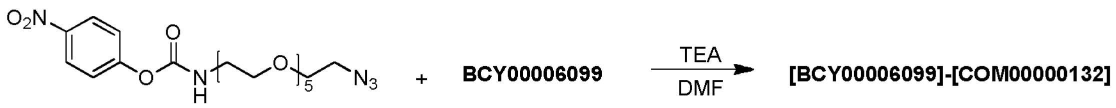
Procedure for preparation of compound 2



1 2

To a solution of **COM132** (20.0 mg, 65.28 µmol, 1.0 *eq*), compound **1** (15.8 mg, 78.34 µmol, 1.2 *eq*) in DCM (5 mL) was added TEA (36.4 mg, 359.23 µmol, 50 µL, 5.5 *eq*). The mixture was stirred at 25 °C for 1 hr. LC-MS one main peak with desired m/z (MW: 471.46, observed *m/z*: 489.2($[M+NH_4]^+$)) was detected. The reaction mixture was concentrated under reduced pressure to give compound **2** (26 mg, crude) colorless oil.

Procedure for preparation of compound 3



2 3

To a solution of compound **2** (15.0 mg, 4.71 µmol, 1.0 *eq*) and **BCY6099** (3.33 mg, 7.07 µmol, 1.5 *eq*) in DMF (3 mL) was added TEA (0.7 mg, 6.93 µmol, 1 µL, 1.5 *eq*). The mixture was stirred at 30 °C for 2 hrs. LC-MS showed compound **2** was consumed completely and one main peak with desired m/z (MW: 3515.01, observed *m/z*: 1172.1($[M/3+H]^+$) 879.5($[M/4+H]^+$)) was detected. The reaction mixture was filtered and concentrated under reduced pressure to give a residue. he crude product was purified by reversed-phase HPLC

(TFA condition). Compound **3** (12.7 mg, 3.26 µmol, 69.23% yield, 90.3% purity) was obtained as a white solid.

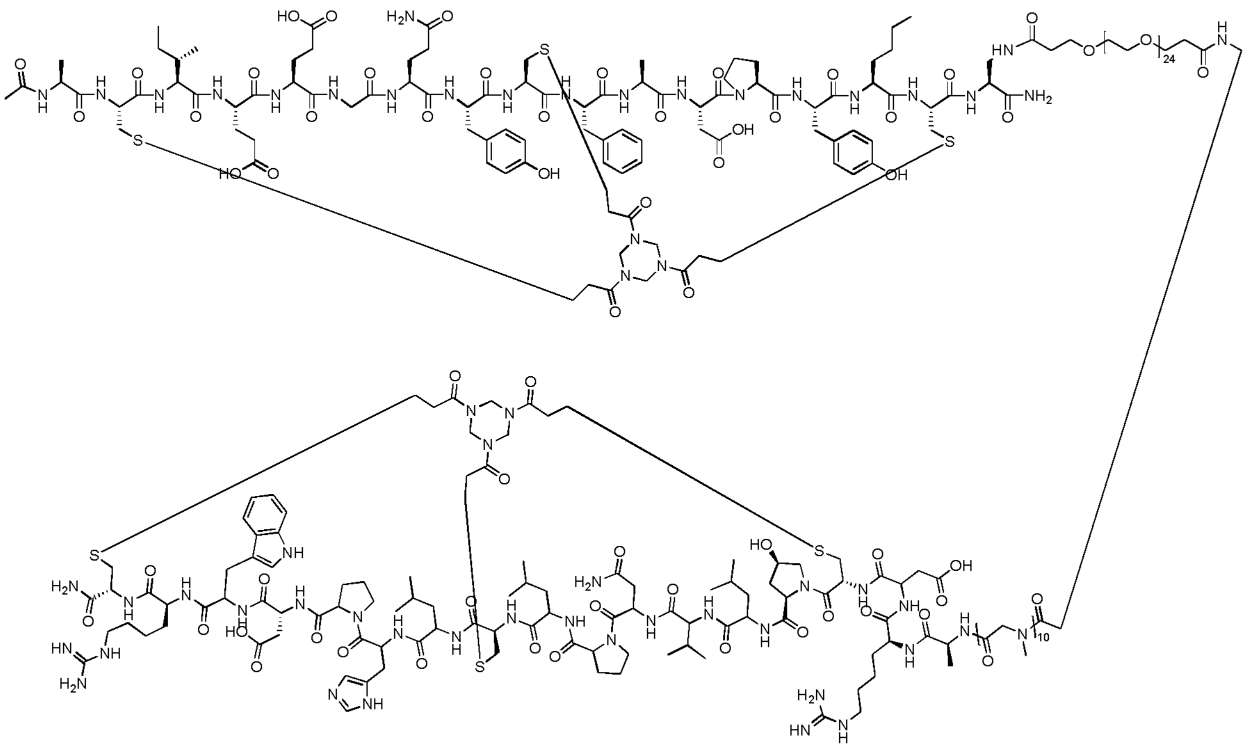
Procedure for preparation of BCY9659

[BCY00006099]-[COM00000132] + **BCY00007741** $\xrightarrow[\text{t-BuOH/H}_2\text{O}]{\text{CuSO}_4\ \ \text{VcNa}\ \ \text{THPTA}}$ **BCY00009659**

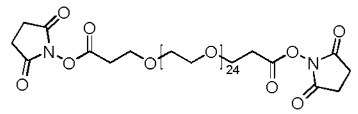
3

A mixture of Compound **3** (12.7 mg, 2.89 µmol, 1.0 *eq*), **BCY7741** (6.80 mg, 2.98 µmol, 1.03 *eq*), and THPTA (1.3 mg, 2.99 µmol, 1.03 *eq*) was dissolved in t-BuOH/H_2O (1:1, 2 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 7.3 µL, 1.0 *eq*) and VcNa (0.4 M, 14.6 µL, 2.0 *eq*) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 25-30 °C for 12 hr under N_2 atmosphere. LC-MS showed Compound **3** was consumed completely and one main peak with desired m/z [MW: 5796.54 observed *m/z*: 1159.8([M/5+H]) 966.7([M/6+H]$^+$)] was detected. The reaction mixture was directly purified by prep-HPLC (TFA condition)**.** **BCY9659** (6.2 mg, 1.06 µmol, 36.58% yield, 98.86% purity) was obtained as a white solid.

<u>**BCY9758**</u>



Procedure for preparation of compound 2



1 + **BCY00006099** —(2.0 eq DIPEA, Dry DMF)→ **BCY00006099-PEG24-NHS ester** 2

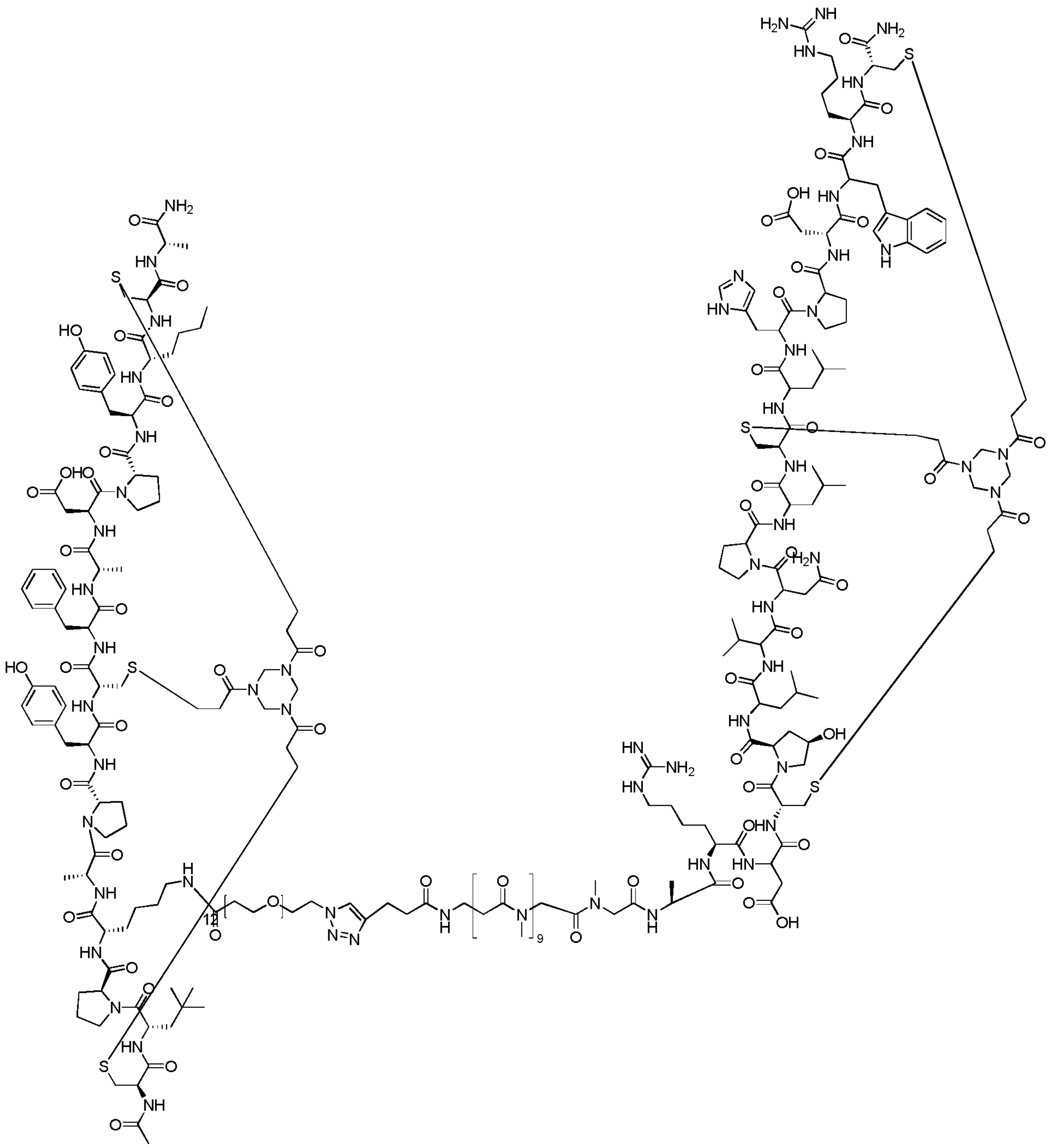
To a solution of compound **1** (5.0 mg, 3.54 μmol, 1.0 eq), **BCY6099** (11.3 mg, 3.54 μmol, 1.0 eq) in DMF (3 mL) was added DIEA (0.9 mg, 7.07 μmol, 1.2 μL, 2.0 eq). The mixture was stirred at 25-30 °C for 20 min. LC-MS showed one peak with desired m/z (MW: 4481.11, observed *m/z*: 1101.3 ([M/4+H]$^+$)) was detected. The reaction mixture was filtered and concentrated under reduced pressure and lyophilized to give compound **2** (15 mg, crude) as a white solid.

Procedure for preparation of BCY9758

BCY00006099-PEG24-NHS ester (2) + **BCY00007732** —(2.0 eq DIPEA, Dry DMF)→ **BCY00009758**

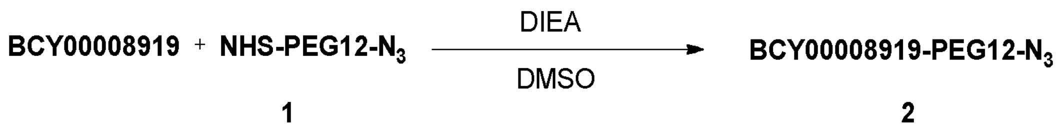
To a solution of compound **2** (15 mg, 3.35 μmol, 1.0 eq) and **BCY7732** (14.74 mg, 6.69 μmol, 2.0 eq) in DMF (3 mL) was added DIEA (0.9 mg, 7.07 μmol, 1.2 μL, 2.1 eq). The mixture was stirred at 25~30 °C for 2 hrs. LC-MS showed compound **2** was consumed completely and one main peak with desired m/z (MW: 6567.48, observed *m/z*: 1095.1([M/6+H]), 938.8([M/7+H]$^+$)) was detected. The reaction mixture was filtered and concentrated under reduced pressure to give a residue. The crude product was purified by reversed-phase HPLC (TFA condition). **BCY9758** (5.8 mg, 24.26% yield, 91.97% purity) was obtained as a white solid.

BCY10568



BCY00010568

Procedure for preparation of BCY8919-PEG12-N₃



BCY8919 (80.0 mg, 38.47 μmol, 1.0 eq) and compound **1** (29.6 mg, 40.01 μmol, 1.04 eq) were dissolved in DMSO (1 mL). The solution was then added with DIPEA (7.46 mg, 55.71 μmol, 10.0 μl, 1.5 eq), and then the mixture was stirred at 25-30°C for 2 hr. LC-MS showed majority of **BCY8919** was consumed and one main peak with desired m/z (calculated MW: 2705.16, observed *m/z*: 1353.1([M/2+H]$^+$)) was detected. The reaction mixture was purified by prep-HPLC (TFA condition) and compound **2** (18.6 mg, 6.86 μmol, 17.83% yield, 99.76% purity) was obtained as a white solid.

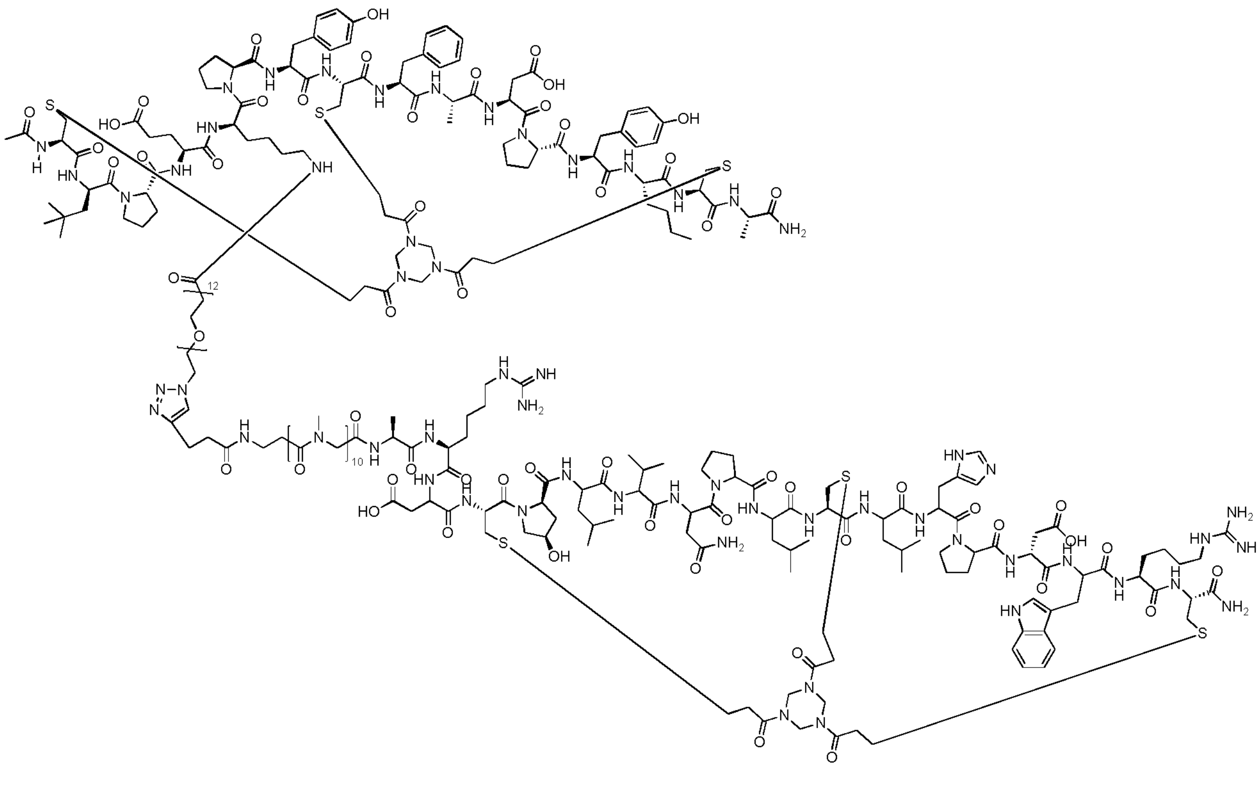
Procedure for preparation of BCY10568

BCY00008919-PEG12-N_3 + **BCY00006169** —($CuSO_4$, VcNa, THPTA / t-BuOH/H_2O)→ **BCY00010568**

2

Compound **2** (9.0 mg, 3.33 μmol, 1.0 eq) and **BCY6169** (11.0 mg, 3.36 μmol, 1.01 eq) were first dissolved in 2 mL of t-BuOH/H_2O (1:1), and then $CuSO_4$ (0.4 M, 8.3 μL, 1.0 eq), VcNa (1.4 mg, 7.06 μmol, 2.1 eq) and THPTA (1.4 mg, 3.22 μmol, 1.0 eq) were added. Finally 0.4 M NH_4HCO_3 was added to adjust pH to 8. All solvents here were degassed and purged with N_2 for 3 times. The reaction mixture was stirred at 30°C for 16 hr under N_2 atmosphere. LC-MS showed compound **2** was consumed completely and one main peak with desired m/z (calculated MW: 5967.90, observed *m/z*: 995.00($[M/5+H]^+$) and 1194.70($[M/6+H]^+$)). The reaction mixture was purified by prep-HPLC (TFA condition) and **BCY10568** (13.4 mg, 2.16 μmol, 69.44% yield, 96.3% purity) was obtained as a white solid.

<u>**BCY10570**</u>



BCY00010570

Procedure for preparation of BCY8920-PEG12-N_3

BCY00008920 + **NHS-PEG12-N_3** —(DIEA / DMSO)→ **BCY00008920-PEG12-N_3**

1 **2**

To a solution of **BCY8920** (37 mg, 17.31 μmol, 1.0 eq) and compound **1** (15 mg, 20.25 μmol, 1.2 eq) in DMSO (2 mL) was added DIEA (3.36 mg, 25.96 μmol, 4.5 μL, 1.5 eq). The mixture was stirred at 30 °C for 12 hr. LC-MS showed **BCY8920** was consumed completely and one main peak with desired m/z (calculated MW: 2763.2, observed m/z: 689.07([M/4-H^+])) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent and produced a residue. The residue was then purified by prep-HPLC (neutral condition). Compound **2** (22.8 mg, 8.15 μmol, 47.09% yield, 98.78% purity) was obtained as a white solid.

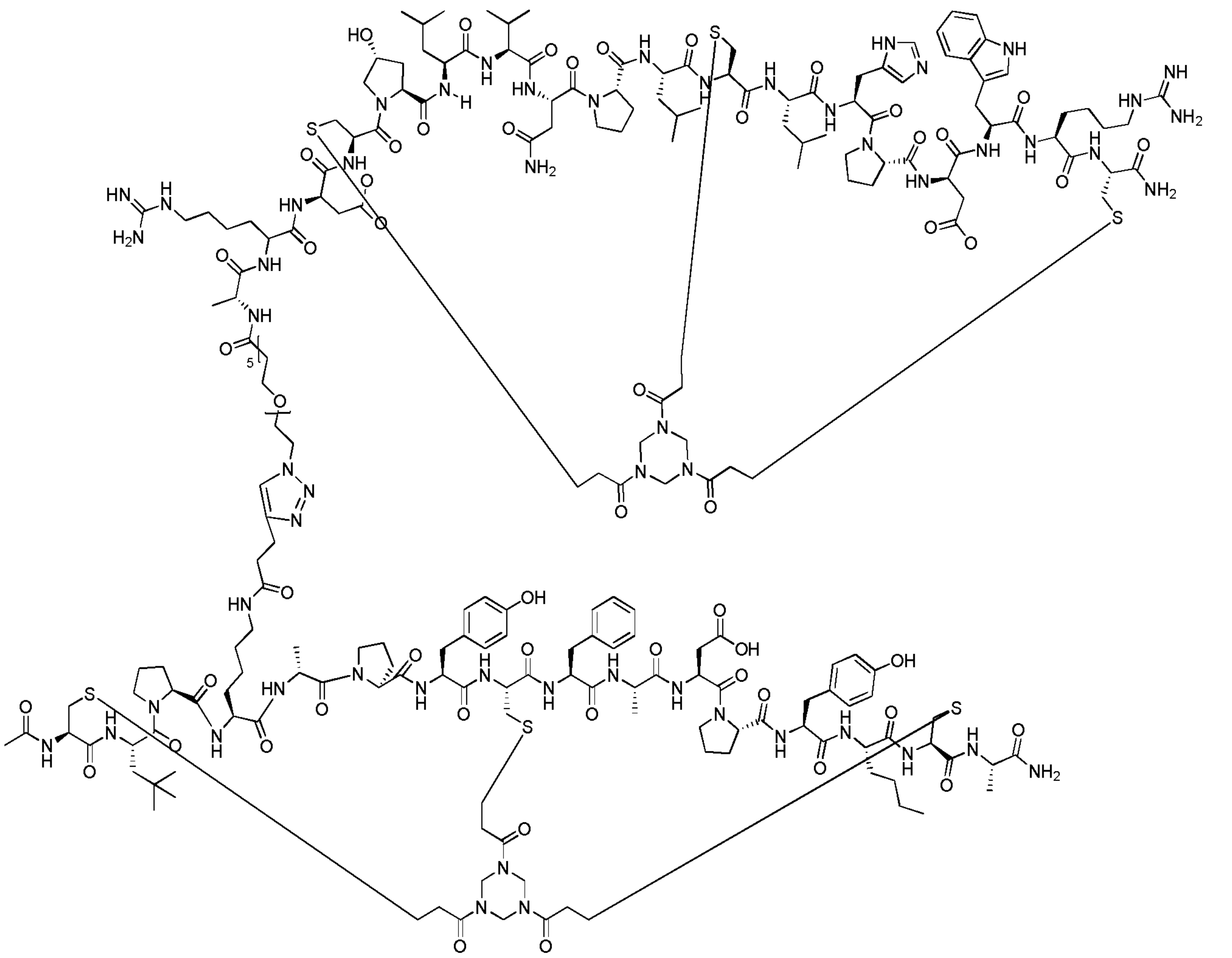
Procedure for preparation of BCY10570

BCY00008920-PEG12-N_3 + **BCY00006169** —($CuSO_4$, VcNa, THPTA / t-BuOH/H_2O)→ **BCY00010570**

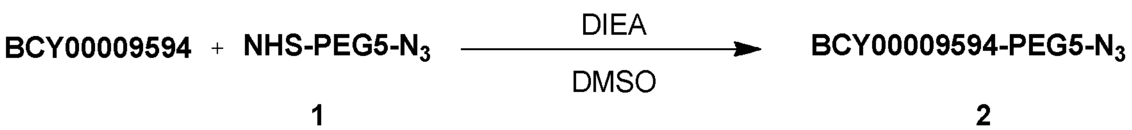
2

Compound **2** (6 mg, 2.17 μmol, 1.0 eq) and **BCY6169** (7.08 mg, 2.17 μmol, 1.0 eq) were first dissolved in 2 mL of t-BuOH/H_2O (1:1), and then $CuSO_4$ (0.4 M, 5.4 μL, 1.0 eq), VcNa (0.4 M, 10.8 μL, 2.0 eq) and THPTA (0.4 M, 5.4 μL, 1.0 eq) was added. Finally 0.2 M NH_4HCO_3 was added to adjust pH to 8. All solvents here were degassed and purged with N_2 for 3 times. The reaction mixture was stirred at 30 °C for 4 hr under N_2 atmosphere. LC-MS showed compound 2 was consumed completely and one main peak with desired m/z (MS: 6025.93, observed m/z: 1004.56([M/6]+H^+) and 861.48([M/7+H^+])) was detected. The reaction mixture was filtered and concentrated under reduced pressure to give a residue. The crude product was purified by prep-HPLC (TFA condition). **BCY10570** (7.2 mg, 1.17 μmol, 53.90% yield, 97.95% purity) was obtained as a white solid.

BCY10574

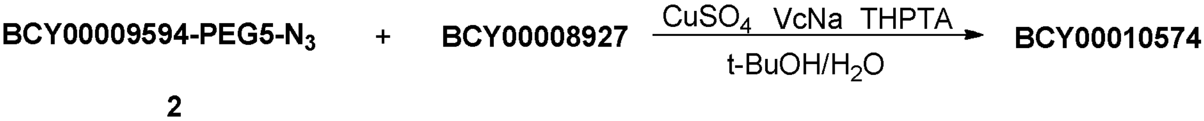


Procedure for preparation of compound 2



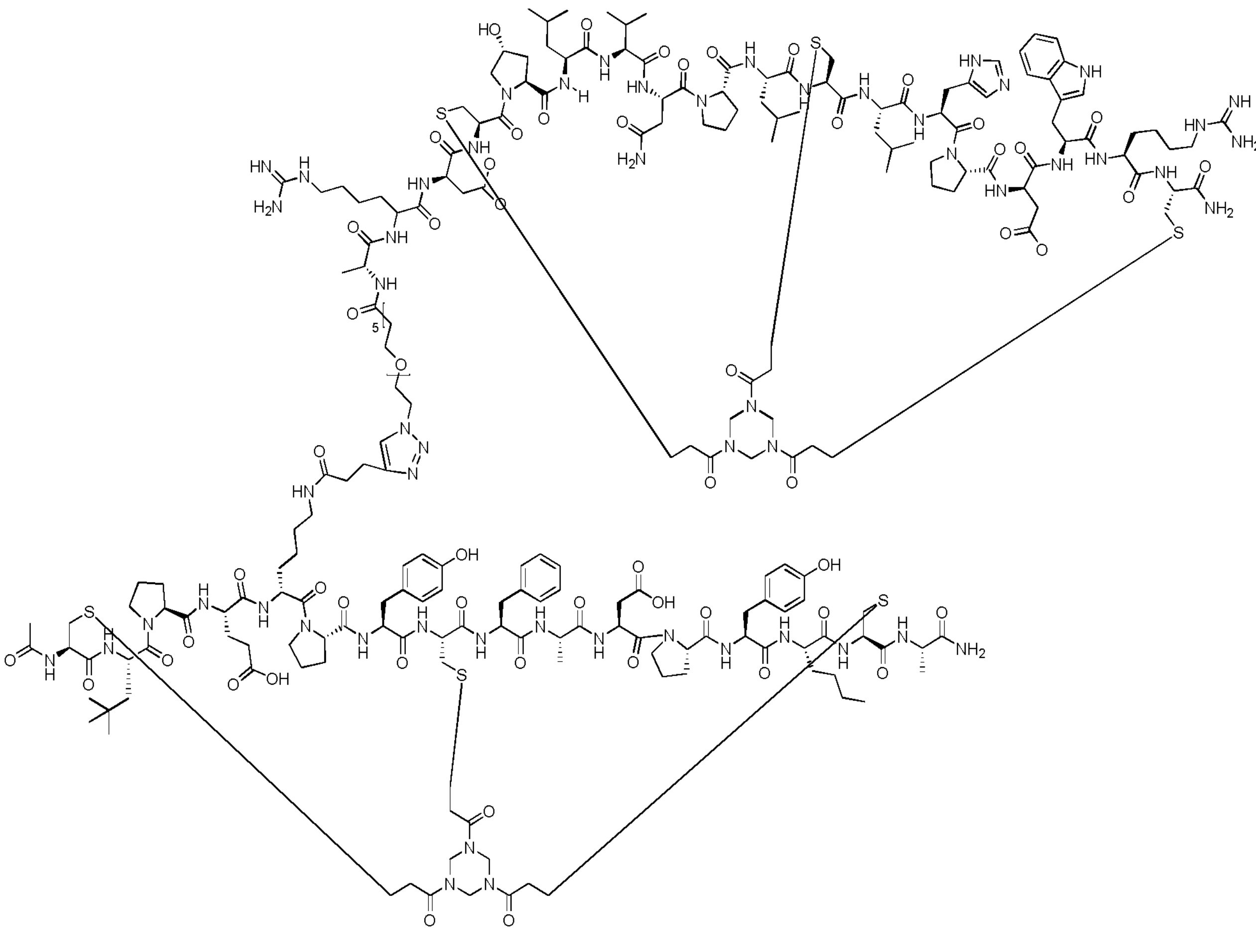
To a solution of **BCY9594** (65 mg, 27.07 µmol, 1 *eq*), Compound **1** (12.00 mg, 27.75 µmol, 1.02 *eq*) in DMSO (1 mL) was added DIEA (5.25 mg, 40.61 µmol, 7.07 µL, 1.5 *eq*). The mixture was stirred at 25-30 °C for 2 hr. LC-MS showed **BCY9594** was consumed completely and one main peak with desired m/z (calculated MW: 2718.13, observed *m/z*: 906.04($[M/3+H]^+$), 1359.07($[M/2+H]^+$)) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent to give a residue. The residue was purified by prep-HPLC (TFA condition). Compound **2** (42.6 mg, 15.67 µmol, 57.89% yield, 100% purity) was obtained as a white solid.

Procedure for preparation of BCY10574

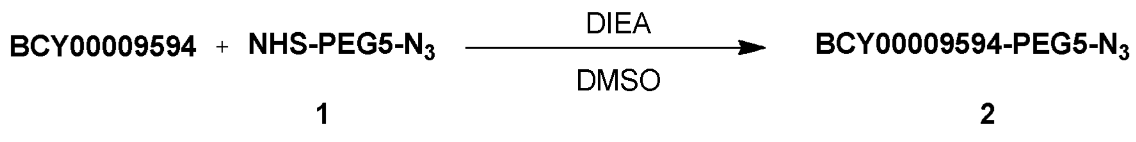


A mixture of Compound **2** (20 mg, 7.36 µmol, 1.0 *eq*), **BCY8927** (17 mg, 7.87 µmol, 1.07 *eq*), and THPTA (0.4 M, 18.4 µL, 1.0 *eq*) was dissolved in t-BuOH/H_2O (1:1, 2 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 18.4 µL, 1.0 *eq*) and VcNa (0.4 M, 36.8 µL, 2.0 *eq*) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 25-30 °C for 12 hr under N_2 atmosphere. LC-MS showed Compound **2** was consumed completely and one main peak with desired m/z (calculated MW: 4877.68, observed *m/z*: 1219.42 ($[M/4+H]^+$) and 975.54($[M/5+H]^+$))was detected. The reaction mixture was directly purified by prep-HPLC (TFA condition). **BCY10574** (17.6 mg, 3.41 µmol, 46.29% yield, 94.40% purity) was obtained as a white solid.

BCY10575



Procedure for preparation of compound 2



To a solution of **BCY9594** (65 mg, 27.07 µmol, 1 *eq*), Compound **1** (12.0 mg, 27.75 µmol, 1.02 *eq*) in DMSO (1 mL) was added DIEA (5.25 mg, 40.61 µmol, 7.07 µL, 1.5 *eq*). The mixture was stirred at 25-30 °C for 2 hr. LC-MS showed Compound **1** was consumed completely and one main peak with desired m/z [calculated MW:2718.13 observed *m/z*: 906.04($[M/3+H]^+$) and 1359.07($[M/2+H]^+$)] was detected. The reaction mixture was concentrated under reduced pressure to remove solvent to give a residue. The residue was purified by prep-HPLC (TFA condition). Compound **2** (42.6 mg, 15.67 μmol, 57.89% yield, 100% purity) was obtained as a white solid.

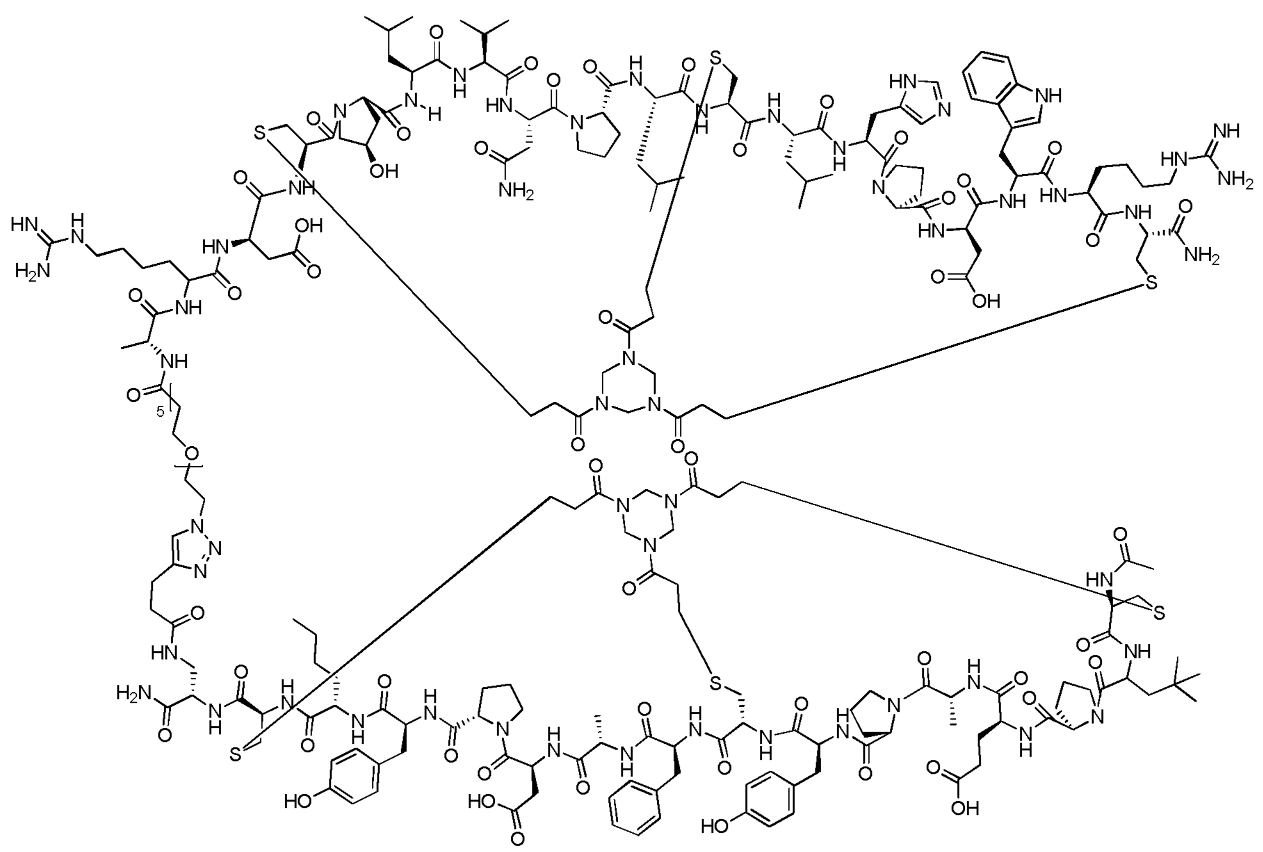
Procedure for preparation of BCY10575

BCY00009594-PEG5-N_3 + **BCY00008928** $\xrightarrow[\text{t-BuOH/H}_2\text{O}]{\text{CuSO}_4\ \text{VcNa}\ \text{THPTA}}$ **BCY00010575**

2

A mixture of Compound **2** (20 mg, 7.36 μmol, 1.0 *eq*), **BCY8928** (17 mg, 7.67 μmol, 1.04 *eq*), and THPTA (0.4 M, 18.4 μL, 1.0 *eq*) was dissolved in t-BuOH/H_2O (1:1, 2 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 18.4 μL, 1.0 *eq*) and VcNa (0.4 M, 36.8 μL, 2.0 *eq*) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 25-30 °C for 12 hr under N_2 atmosphere. LC-MS showed Compound **2** was consumed completely and one main peak with desired m/z [calculated MW: 4935.71, observed *m/z*: 1234.59 ($[M/4+H]^+$) and 987.71($[M/5+H]^+$)] was detected. The reaction mixture was directly purified by prep-HPLC (TFA condition). **BCY10575** (12 mg, 2.37 μmol, 32.27% yield, 97.67% purity) was obtained as a white solid.

BCY10576



Procedure for preparation of compound 2

BCY00009594 + **NHS-PEG5-N_3** —(DIEA, DMSO)→ **BCY00009594-PEG5-N_3**

1 **2**

To a solution of **BCY9594** (30.0 mg, 12.50 μmol, 1.0 *eq*), Compound **1** (5.54 mg, 12.81 μmol, 1.02 *eq*) in DMSO (1 mL) was added DIEA (2.42 mg, 18.74 μmol, 3.3 μL, 1.5 *eq*). The mixture was stirred at 25-30 °C for 2 hr. LC-MS showed Compound **1** was consumed completely and one main peak with desired m/z [calculated MW: 2718.13, observed *m/z*: 906.45($[M/3+H]^+$) and 1359.50($[M/2+H]^+$)] was detected. The reaction mixture was concentrated under reduced pressure to remove solvent to give a residue. The residue was purified by prep-HPLC (TFA condition). Compound **2** (16 mg, 5.80 μmol, 46.42% yield, 98.54% purity) was obtained as a white solid.

Procedure for preparation of BCY10576

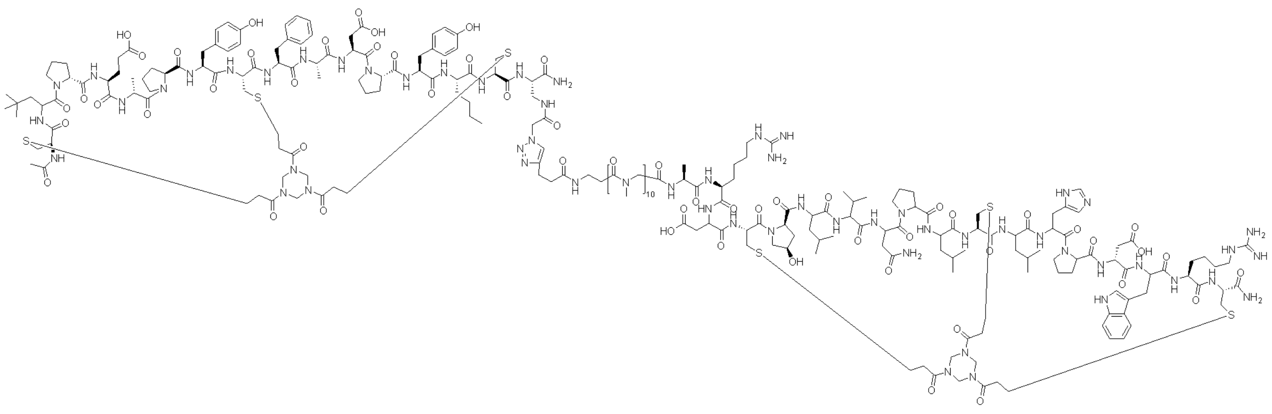
BCY00009594-PEG5-N_3 + **BCY00011014** —($CuSO_4$ VcNa THPTA, t-BuOH/H_2O)→ **BCY00010576**

2

A mixture of compound **2** (17.0 mg, 6.25 μmol, 1.0 *eq*), **BCY11014** (13.6 mg, 6.25 μmol, 1.0 *eq*), and THPTA (0.4 M, 1.8 μL, 2.0 *eq*) was dissolved in t-BuOH/H_2O (1:1, 2 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 15.6 μL, 1.0 *eq*) and

VcNa (0.4 M, 1.84 µL, 2.0 *eq*) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 25-30 °C for 12 hr under N_2 atmosphere. LC-MS showed majority of compound **2** was consumed and one main peak with desired m/z [calculated MW: 4893.63, observed *m/z*: 1224.7 ($[M/4+H]^+$) and 980.0 ($[M/6+H]^+$)] was detected. The reaction mixture was directly purified by prep-HPLC (TFA condition). **BCY10576** (20.5 mg, 4.13 µmol, 66.02% yield, 98.57% purity) was obtained as a white solid.

<u>BCY10577</u>



BCY00010577

Procedure for preparation of compound 2

BCY00009172 + **N_3-CH_2-COOH** (**1**) —(**1. EDCI, HOSu**; **2.DIEA, DMF**)→ **BCY00009172-CH_2-N_3** (**2**)

To a solution of compound 1 (5.0 mg, 49.5 µmol, 1.0 eq) in DMF (1 mL) was added EDCI (8.5 mg, 54.8 µmol, 1.1 eq) and HOSu (5.7 mg, 49.5 µmol, 1.0 eq). The mixture was stirred at 25-30 °C for 30 min. TLC indicated compound **1** was consumed completely and **one new spot** formed. Then **BCY9172** (53 mg, 25.29 µmol, 0.47eq) and DIEA (3.27 mg, 25.29 µmol, 4.4 µL, 0.47 eq) were added to the reaction mixture. The mixture was stirred at 25-30 °C for 2 hr. LC-MS showed **BCY9172** was consumed completely and one main peak with desired m/z (MW: 2178.46, observed *m/z*: 1089.5700 ($[(M/2+H^+])$) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent and produced a residue. The residue was then purified by prep-HPLC (neutral condition). Compound **2** (30 mg, 13.77 µmol, 54.45% yield, 100% purity) was obtained as a white solid.

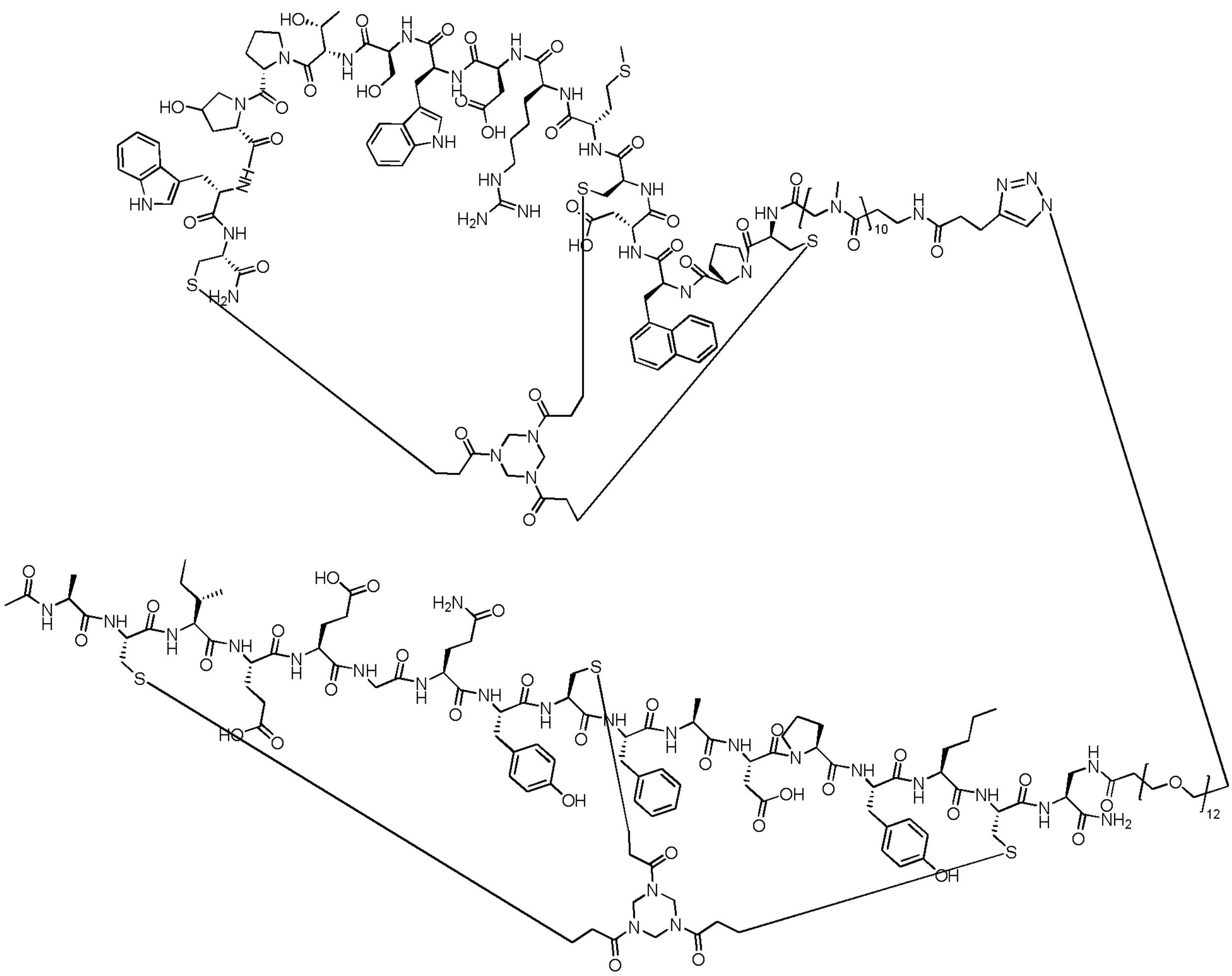
Procedure for preparation of BCY10577

BCY00009172-CH_2-N_3 (**2**) + **BCY00006169** —($CuSO_4$, VcNa, THPTA; t-BuOH/H_2O)→ **BCY00010577**

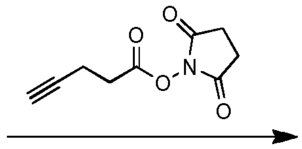
Compound **2** (20 mg, 9.18 µmol, 1.0 eq) and **BCY6169** (32.95 mg, 10.10 µmol, 1.1 eq) were first dissolved in 2 mL of t-BuOH/H_2O (1:1), and then $CuSO_4$ (0.4 M, 23 µL, 1 eq), VcNa (0.4 M, 46 µL, 2.0 eq) and THPTA (0.4 M, 23 µL, 1.0 eq) was added. Finally 1 M NH_4HCO_3 was added to adjust pH to 8. All solvents here were degassed and purged with N_2 for 3 times. The reaction mixture was stirred at 30 °C for 4 hr under N_2 atmosphere. LC-MS showed compound 2 was consumed completely and one main peak with desired m/z (calculated MW: 5441.20, observed m/z: 1361.8 ($[M/4+H]^+$)) was detected. The reaction mixture was filtered and concentrated under reduced pressure to give a residue. The crude product was purified by prep-HPLC (TFA condition). **BCY10577** (16.2 mg, 2.98 µmol, 32.43% yield) was obtained as a white solid.

Example 3: Synthesis of Nectin-4/CD137 Binding Heterotandem Bicyclic Peptides

<u>**BCY8854**</u>



General procedure for preparation of BCY8846

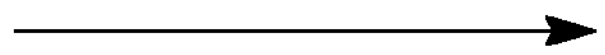


BCY00008234 → BCY00008846

DIEA, DMA

To a solution of **BCY8234** (a peptide identical to BCY8846 except for the absence of a PYA moiety; 300 mg, 102 µmol, 1.0 eq) in DMA (3 mL) was added DIEA (52.5 mg, 406 µmol, 70.8 µL, 4.0 eq) with stirring for 10 min. Then (2,5-dioxopyrrolidin-1-yl) pent-4-ynoate (25.8 mg, 132 µmol, 1.3 eq) was added thereto and the mixture was further stirred at 20 °C for additional 16 hr. LC-MS showed **BCY8234** was consumed completely and one main peak with desired m/z (*calculated* MW: 3034.43, *observed* m/z: 1011.8 ($[M/3+H]^+$), 1517.0 ($[M/2+H]^+$)) was detected. The reaction mixture was purified by prep-HPLC (neutral condition) to give compound **BCY8846** (290 mg, 95.6 µmol, 94.1% yield) as a white solid.

General procedure for preparation of BCY8854



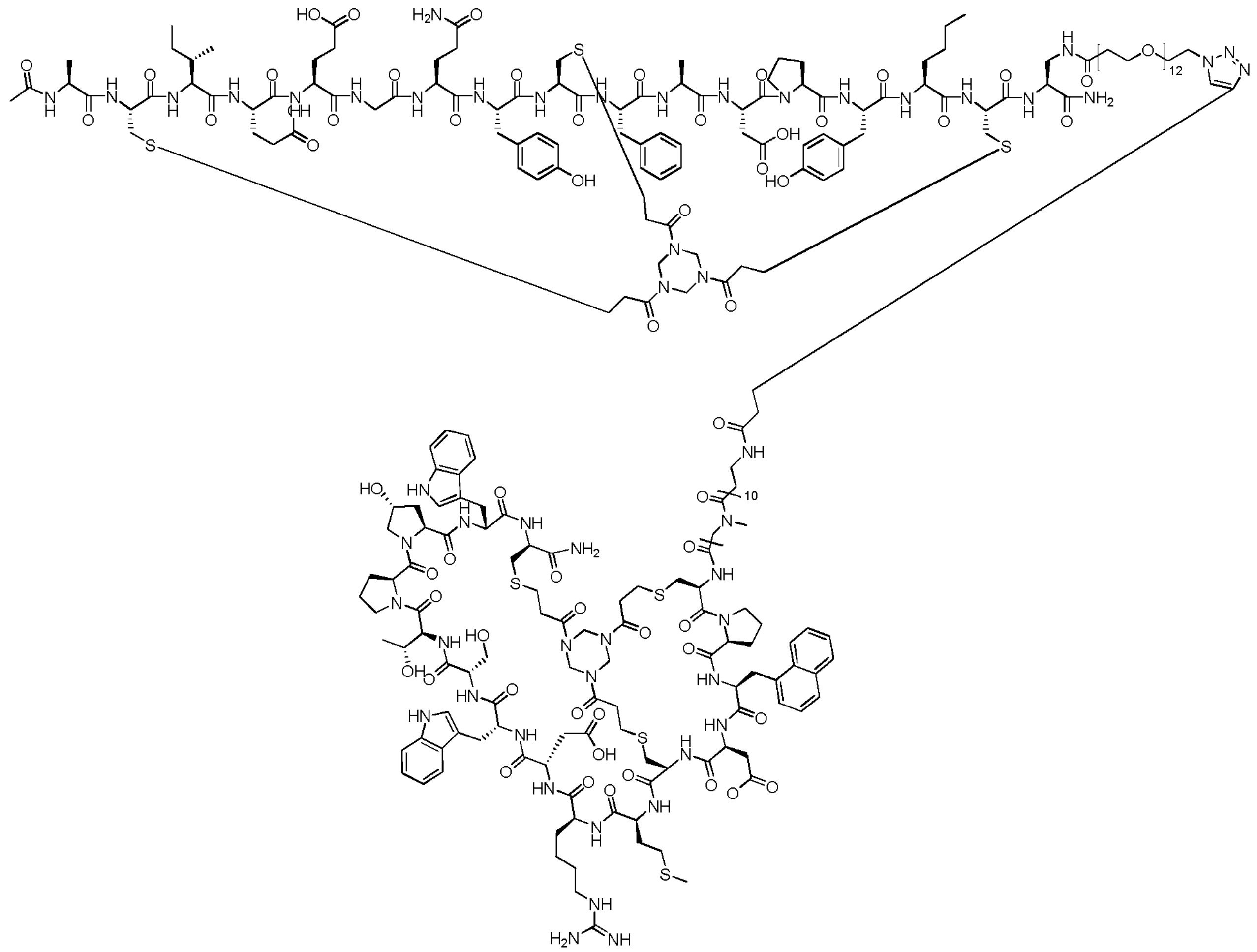
BCY00008846 → BCY00008854

BCY00007859

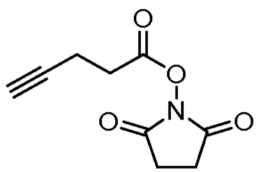
Vc, $CuSO_4$, DMF, H_2O

To a solution of **BCY8846** (234 mg, 77.1 µmol, 1.0 eq) in DMF (5 mL) was added **BCY7859** (which may be prepared as described in BCY7985; 220 mg, 77.8 µmol, 1.0 eq), followed by addition (2R)-2-[(1S)-1,2-dihydroxyethyl]-3,4-dihydroxy-2H-furan-5-one (0.80 M, 963 µL, 1.0 eq) and $CuSO_4$ (0.80 M, 289 µL, 0.3 eq). The mixture was stirred at 20 °C for 2 hr. LC-MS showed **BCY8846** was consumed completely and one main peak with desired m/z (*Calculated* MW: 5861.59, *observed* m/z: 837.9 ($[M/7+H]^+$), 977.6 ($[M/6+H]^+$), 1173.3 ($[M/5+H]^+$)) was detected. The reaction mixture was purified by prep-HPLC (A: 0.075% TFA in H_2O, B: ACN) to give compound **BCY8854** (292 mg, 46.8 µmol, 60.8% yield, 95.9% purity, TFA) as a white solid.

<u>BCY9350</u>



General procedure for preparation of BCY8782-PYA



To a solution of **BCY8782** (a peptide identical to BCY11942 except for the absence of a PYA moiety; 20.0 mg, 6.77 µmol, 1.0 eq) in DMA (1 mL) was added DIEA (4.37 mg, 33.9 µmol, 5.90 µL, 5.0 eq) and (2,5-dioxopyrrolidin-1-yl) pent-4-ynoate (2.64 mg, 13.5 µmol, 2.0 eq) with stirring for 12 hr at 25 °C. LC-MS showed **BCY8782** was consumed completely and one main peak with desired m/z (*calculated* MW: 3034.43, *observed* m/z: 1012.1 $[M/3+H]^+$) was detected. The reaction mixture was purified by prep-HPLC (neutral condition) to give **BCY11942** (20.0 mg, 6.00 µmol, 88.6% yield, 91.0% purity) as a white solid.

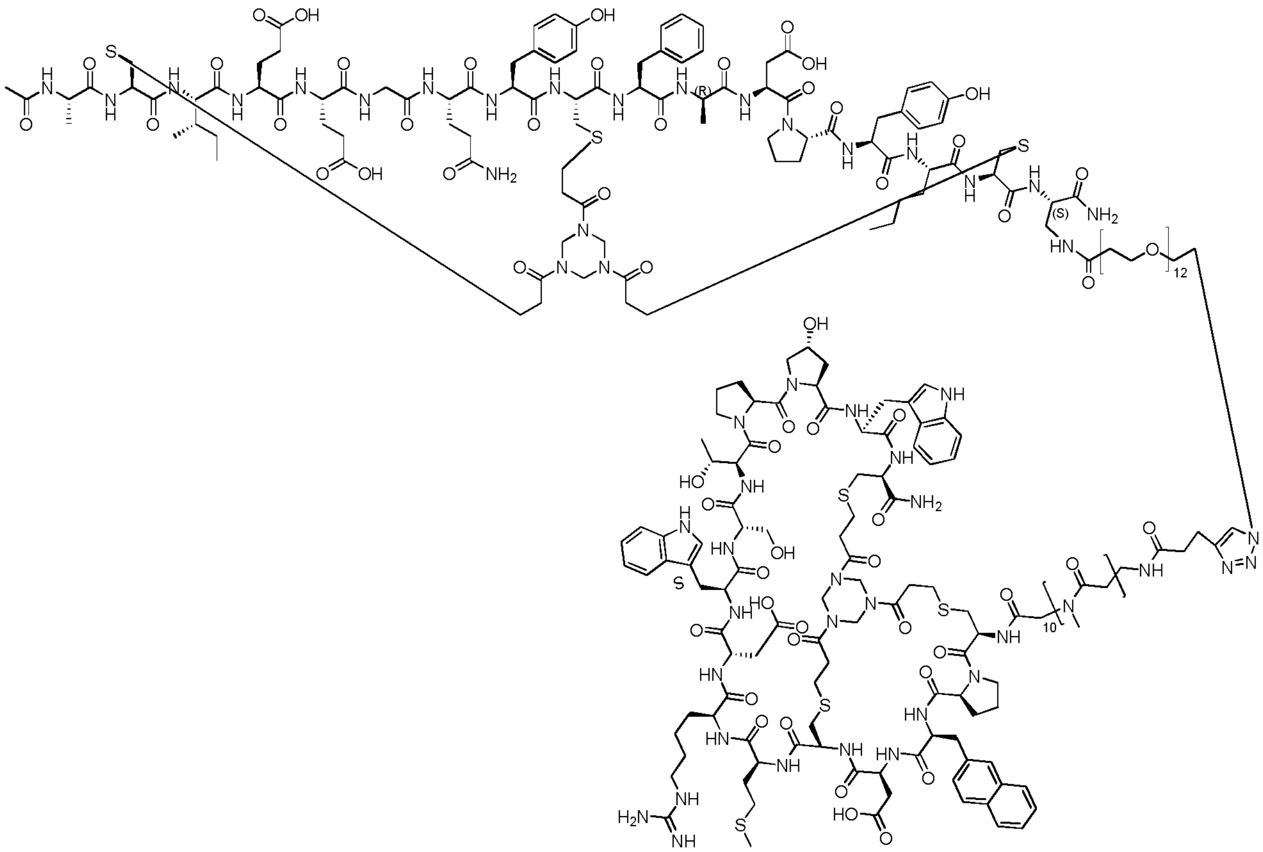
General procedure for preparation of BCY9350

BCY00011942 + **BCY00007859** —(Vc, $CuSO_4$ / DMF)→ **BCY00009350**

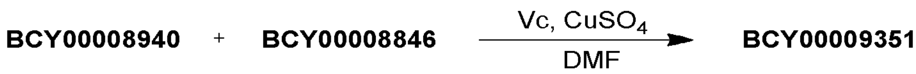
To a solution of **BCY11942** (20 mg, 6.59 µmol, 1.0 eq) and **BCY7859** (which may be prepared as described in BCY7985; 20.5 mg, 7.25 µmol, 1.1 eq) in DMF (1 mL) was added

(2R)-2-[(1S)-1,2-dihydroxyethyl]-3, 4-dihydroxy-2H-furan-5-one (0.4 M, 330 µL, 20.0 eq) and $CuSO_4$ (0.4 M, 98.9 µL, 6.0 eq) were added to the mixture. The mixture was stirred at 25 °C for 2 hr. LC-MS showed **BCY8782-PYA** was consumed completely and one main peak with desired m/z (*calculated* MW: 5861.59, *observed* m/z: 1173.3 [M/5+H]$^+$) was detected. The reaction mixture was purified by prep-HPLC (A: 0.075% TFA in H_2O, B: ACN) to give **BCY9350** (14.5 mg, 2.40 µmol, 36.5% yield, 97.2% purity) as a white solid.

BCY9351

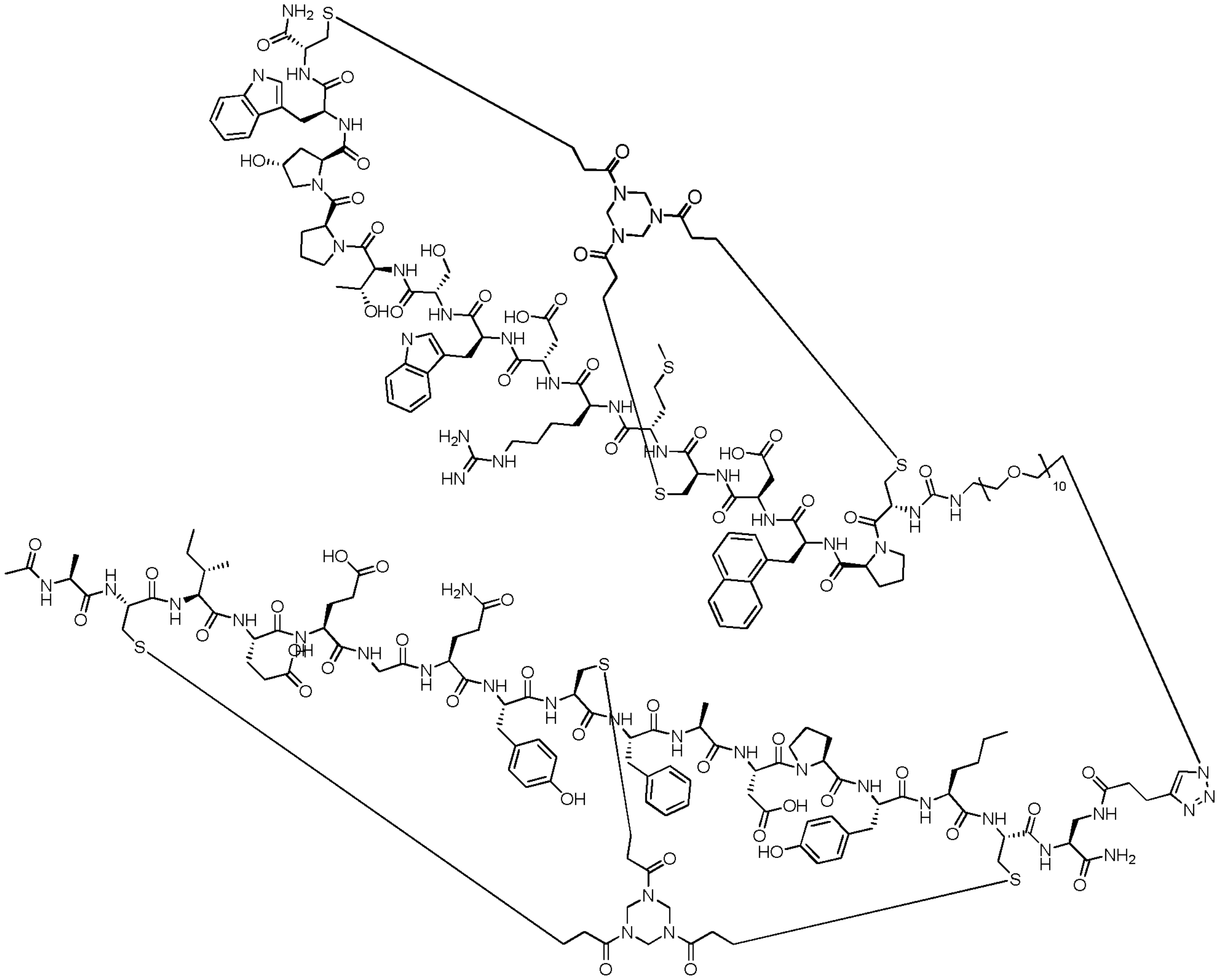


General procedure for preparation of BCY9351

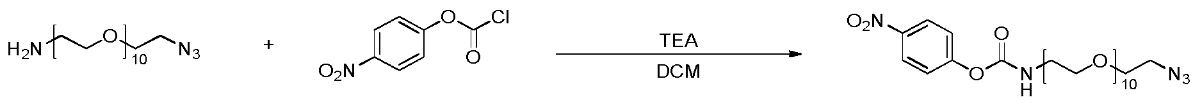


To a solution of **BCY8940** (which may be prepared as described in BCY8942; 9.4 mg, 3.33 µmol, 1.01 eq) and **BCY8846** (10.0 mg, 3.30 µmol, 1.0 eq) in DMF (1 mL) was added Vc (0.4 M, 165 µL, 20.0 eq) and $CuSO_4$ (0.4 M, 49.4 µL, 6.0 eq) under nitrogen atmosphere. The mixture was stirred at 25 °C for 1 hr. LC-MS showed **BCY8940** was consumed completely and one main peak with desired m/z (*calculated* MW: 5861.59, *observed* m/z: 975.4 [M/6+H]$^+$, 1172.3 [M/5+H]$^+$) was detected. The reaction mixture was purified by prep-HPLC (A: 0.075% TFA in H_2O, B: ACN) to give **BCY9351** (5.30 mg, 0.904 µmol, 26.3% yield, 96.0% purity) as a white solid.

BCY9399



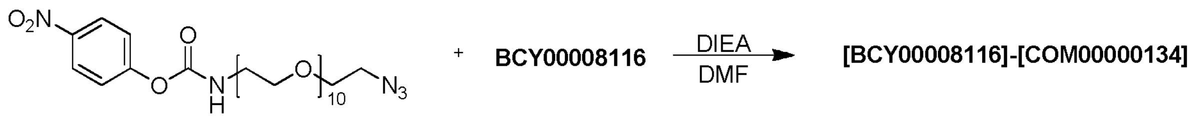
Procedure for preparation of compound 2



COM00000134 **1** **2**

To a solution of **COM134** (30 mg, 56.97 µmol), Compound **1** (17.22 mg, 85.45 µmol) in DCM (0.5 mL) was added TEA (8.65 mg, 85.45 µmol, 11.9 µL). The mixture was stirred at 25 °C for 1 hr. LC-MS showed **COM134** was consumed completely and one main peak with desired m/z (calculated MW: 691.72, observed *m/z*: 692.3([M+H]$^+$) and 709.3 ([M+NH4]$^+$)) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent to give a residue. The residue was purified by prep-HPLC (neutral condition). Compound **2** (30.5 mg) was obtained as a colorless oil.

Procedure for preparation of compound 3



2

3

To a solution of Compound **2** (15 mg, 21.68 μmol) and **BCY8116** (47 mg, 21.68 μmol) in DMF (1 mL) was added DIEA (8.41 mg, 65.05 μmol, 11.33 μL). The mixture was stirred at 30 °C for 2 hrs. LC-MS showed Compound **2** was consumed completely and one main peak with desired m/z (MW: 2725.1 observed *m/z*: 1362.7([M/2+H]$^+$), 909.0([M/3+H]$^+$)) was detected. The reaction mixture was filtered and concentrated under reduced pressure to give a residue. The crude product was purified by reversed-phase HPLC (TFA condition). Compound **3** (20 mg, 33.41% yield, 98.71% purity) was obtained as a white solid.

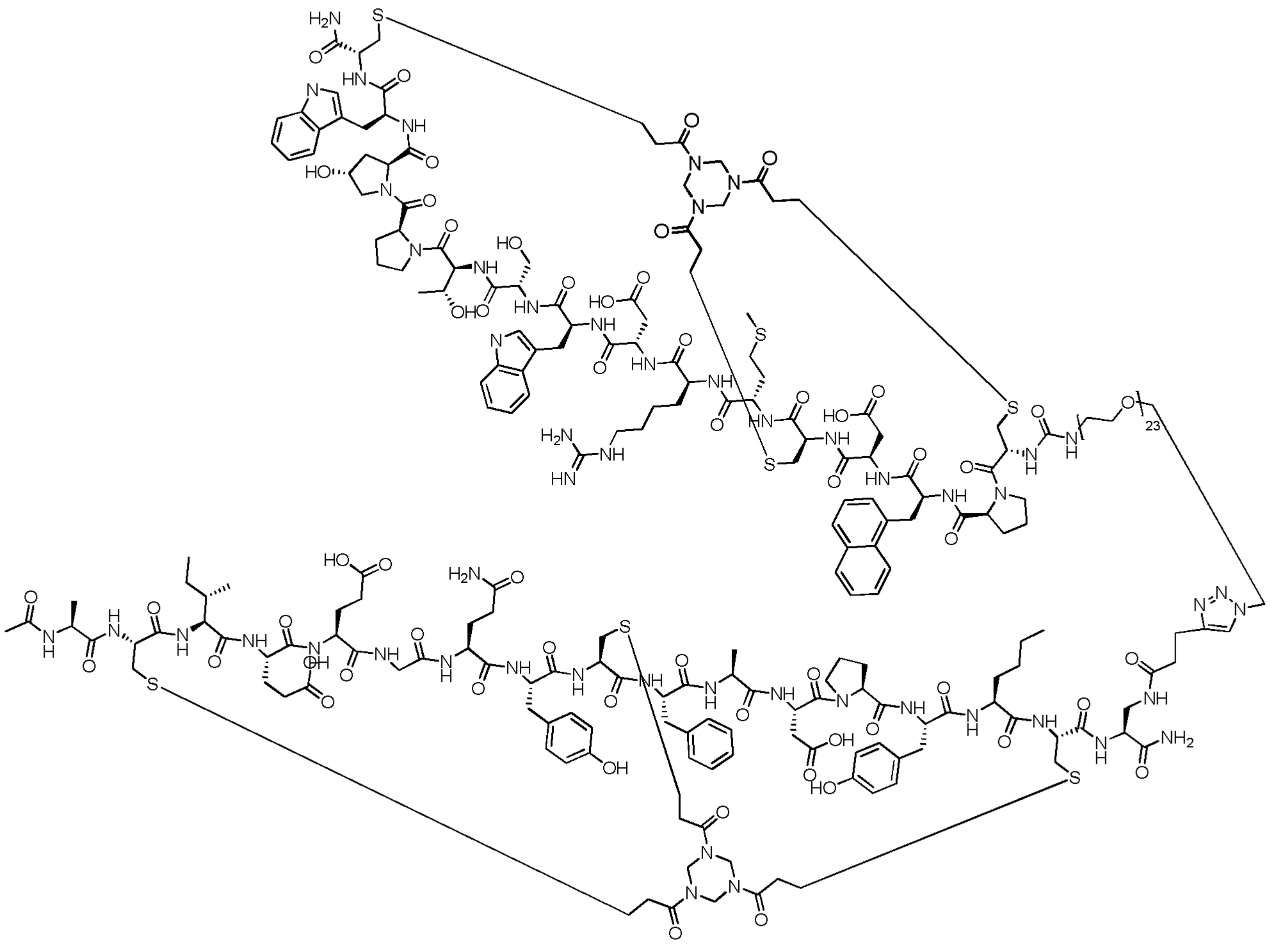
Procedure for preparation of BCY9399

[BCY00008116]-[COM00000134] + **BCY00007741** $\xrightarrow[\text{t-BuOH}/H_2O]{CuSO_4\ \text{VcNa THPTA}}$ **BCY00009399**

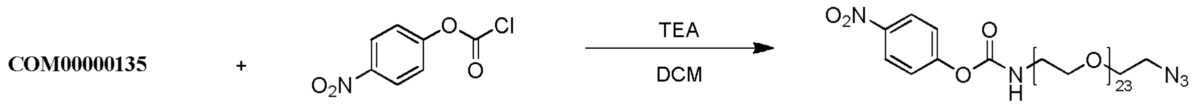
3

A mixture of Compound **3** (20.0 mg, 5.35 μmol, 1.0 eq), **BCY7741** (13.0 mg, 5.70 μmol, 1.01 eq), and THPTA (0.4 M, 13.4 μL, 1.0 eq) was dissolved in t-BuOH/H_2O (1:1, 2 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 13.4 μL, 1.0 eq) and VcNa (0.4 M, 26.8 μL, 2.0 eq) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 25-30 °C for 12 hr under N_2 atmosphere. LC-MS showed Compound **3** was consumed completely and one main peak with desired m/z [MW: 5006.64 observed m/z: 834.9([M/6+H]$^+$), 1002.3([M/5+H]$^+$), 1252.4([M/4+H]$^+$)] was detected. The reaction mixture was directly purified by prep-HPLC (**TFA condition)**. **BCY9399** (9.1 mg, 27.20% yield, 96.29% purity) was obtained as a white solid.

<u>**BCY9400**</u>



Procedure for preparation of Compound 2



1 **2**

To a solution of **COM135** (which may be prepared as described in BCY9648; 30.0 mg, 27.29 µmol), Compound 1 (8.3 mg, 40.94 µmol) in DCM (2 mL) was added TEA (4.14 mg, 40.94 µmol, 5.7 µL). Then the reaction mixture was stirred at 25-30 °C for 1 hr. LC-MS showed **COM135** was consumed completely and one main peak with desired m/z (calculated MW: 1264.40, observed *m/z*: 1281.4 ($[M+NH_4]^+$)) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent to give a residue. The residue was purified by prep-HPLC (neutral condition) to give compound **2** (18 mg) as a white solid.

Procedure for preparation of Compound 3

2 **3**

To a solution of Compound **2** (15.5 mg, 7.12 µmol) and **BCY8116** (9 mg, 7.12 µmol) in DMF (2 mL) was added DIEA (1.4 mg, 10.68 µmol, 1.9 µL). The mixture was stirred at 30 °C for 2 hrs. LC-MS showed Compound **2** was consumed completely and one main peak with desired m/z (MW: 3297.78, observed *m/z*: 1099.7($[M/3+H]^+$)) was detected. The reaction mixture was filtered and concentrated under reduced pressure to give a residue. The crude product was purified by reversed-phase HPLC (TFA condition). Compound **3** (19.5 mg, 5.91 µmol, 33.41% yield, 83.07% purity) was obtained as a white solid.

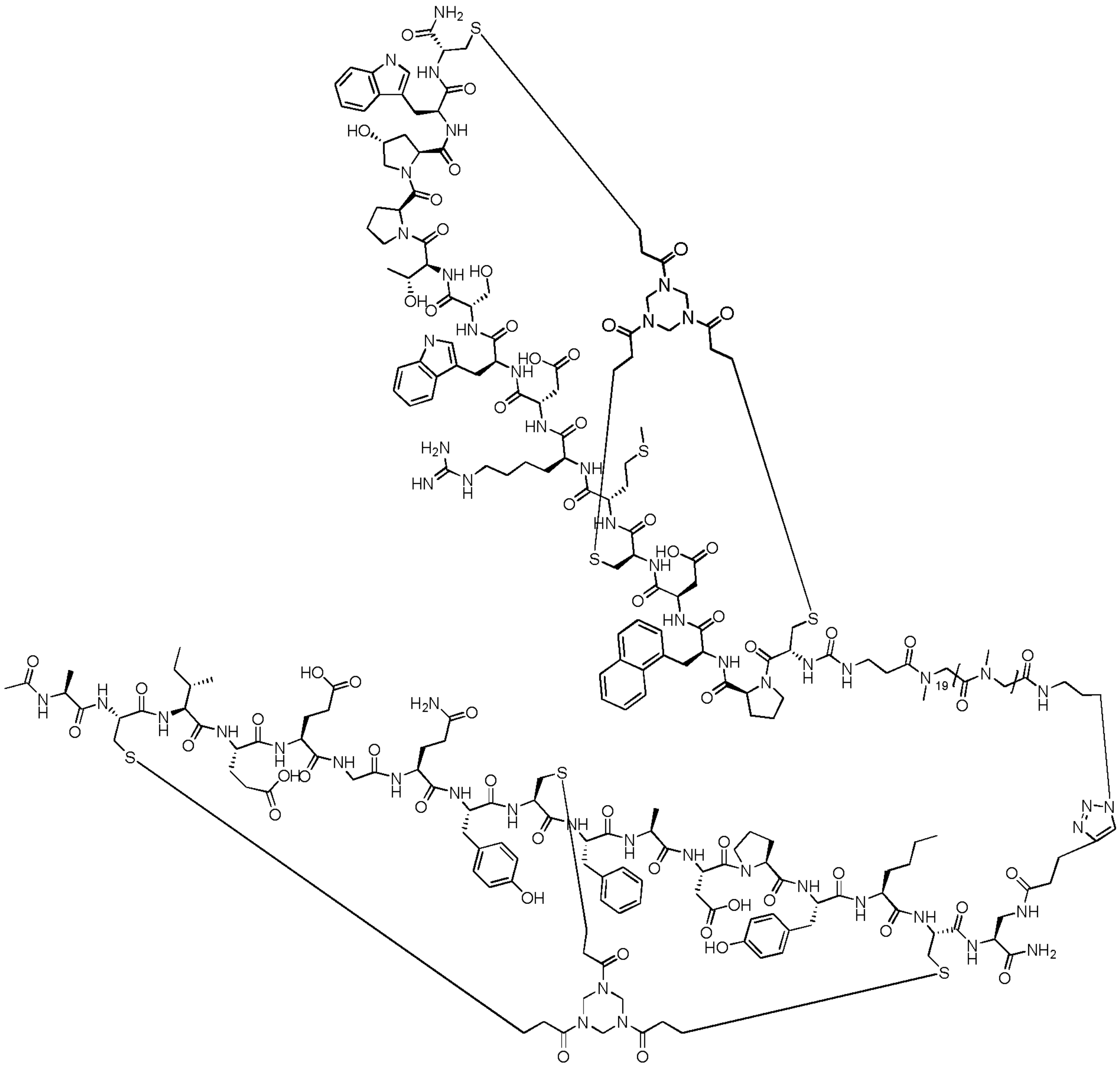
Procedure for preparation of BCY9400

$$\textbf{[BCY00008116]-[COM00000135]} + \textbf{BCY00007741} \xrightarrow[\text{t-BuOH/}H_2O]{CuSO_4\ \ \text{VcNa}\ \ \text{THPTA}} \textbf{BCY00009400}$$

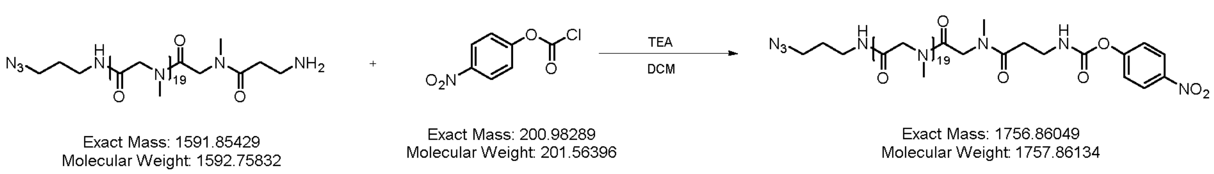
3

A mixture of Compound **3** (19.5 mg, 5.91 µmol), **BCY7741** (14 mg, 6.14 µmol, 1.01eq), and THPTA (0.4 M, 15 µL, 1 eq) was dissolved in t-BuOH/H_2O (1:1, 2 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 15 µL, 1 eq) and VcNa (0.4 M, 30 µL, 2 eq) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 25-30 °C for 12 hr under N_2 atmosphere. LC-MS showed Compound **3** was consumed completely and one main peak with desired m/z [MW: 5579.31 observed m/z: 930.5($[M/6+H]^+$), 1116.6($[M/5+H]^+$)] was detected. The reaction mixture was directly purified by prep-HPLC (TFA condition)**.** **BCY9400** (13.9 mg, 2.33 µmol, 27.20% yield, 93.56% purity) was obtained as a white solid.

<u>**BCY9401**</u>

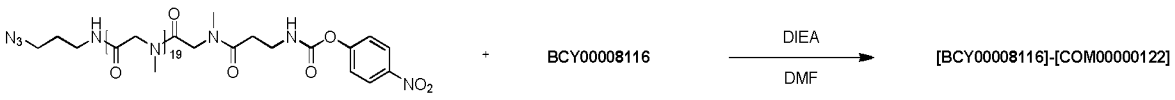


Procedure for preparation of compound 3



To a solution of Compound **1** (50.0 mg, 31.39 µmol, 1 *eq*), Compound **2** (6.6 mg, 32.96 µmol, 1.05 *eq*) in DCM (2 mL) was added TEA (4.8 mg, 47.09 µmol, 6.6 µL, 1.5 *eq*). The mixture was stirred at 25-30 °C for 2 hr. LC-MS showed Compound **1** was consumed completely and one main peak with desired m/z (MW:1757.86 observed *m/z*: 879.10([M/2+H]$^{+}$)) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent to give a residue. The residue was purified by prep-HPLC (TFA condition). Compound **3** (0.02 g, 6.56 µmol, 20.91% yield, 57.7% purity) was obtained as a white solid.

Procedure for preparation of compound 4



3 4

To a solution of Compound **3** (20 mg, 11.38 μmol, 1 *eq*), **BCY8116** (25 mg, 11.51 μmol, 1.01 *eq*) in DMF (4 mL) was added DIEA (2.2 mg, 17.07 μmol, 2.97 μL, 1.5 *eq*). The mixture was stirred at 25-30 °C for 12 hr. LC-MS showed Compound **3** was consumed completely and one main peak with desired m/z (MW: 3791.23, observed *m/z*: 1263.2([M/3+H]$^+$)) was detected. The reaction was directly purified by prep-HPLC (neutral condition). Compound **4** (10 mg, 2.43 μmol, 21.33% yield, 92% purity) was obtained as colorless oil.

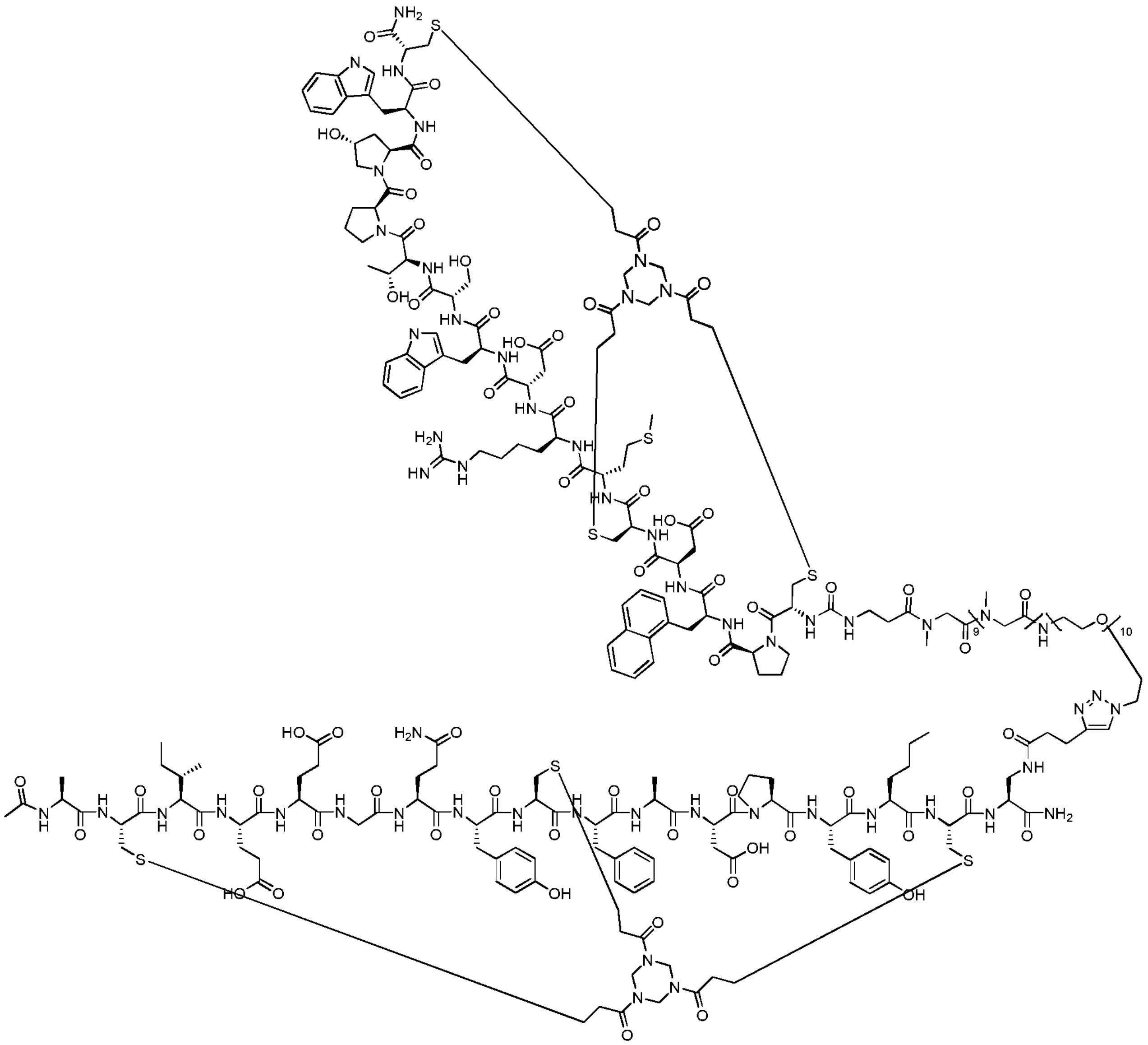
Procedure for preparation of BCY9401

[BCY00008116]-[COM00000122] + **BCY00007741** —(CuSO4,VcNa,THPTA; t-BuOH/H_2O)→ **BCY00009401**

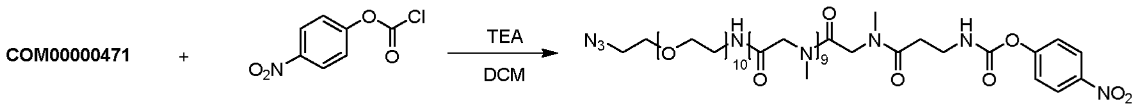
4

A mixture of Compound **4** (10 mg, 2.43 μmol, 0.9 *eq*), **BCY7741** (6.32 mg, 2.77 μmol, 1.0 *eq*) and THPTA (0.4 M, 6.7 μL, 1.0 *eq*) was dissolved in t-BuOH/H_2O (1:1, 2 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 6.7 μL, 1.0 *eq*) and VcNa (0.4 M, 13.4 μL, 2.0 *eq*) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 25-30 °C for 12 hr under N_2 atmosphere. LC-MS showed Compound **4** was consumed completely and one main peak with desired m/z [MW: MW: 6072.77, observed *m/z*: 1012.00([M/6+H]$^+$)] was detected. The reaction mixture was directly purified by prep-HPLC (TFA condition). **BCY9401** (8.4 mg, 1.56 μmol, 59.31% yield, 95.52% purity) was obtained as a white solid.

<u>**BCY9403**</u>



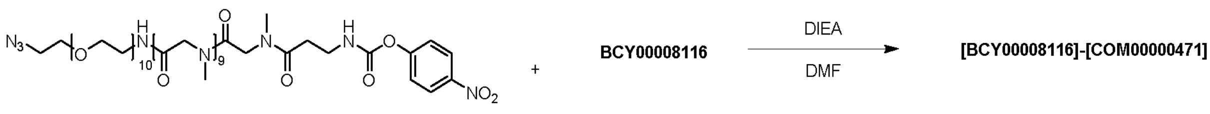
Procedure for preparation of Compound 2



1 2

To a solution of **COM471** (100.0 mg, 76.42 µmol, 1.0 eq), 4-nitrophenylchloroformate (16.2 mg, 80.25 µmol, 1.05 eq) in DCM (10 mL) was added TEA (11.6 mg, 114.64 µmol, 16.0 µL, 1.5 eq). The mixture was stirred at 25-30 °C for 2 hr. LC-MS showed **COM471** was consumed completely and one main peak with desired m/z (MW: 1473.58, observed *m/z*: 736.83 ($[M/2+H]^+$)) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent to give a residue. The residue was purified by prep-HPLC (TFA condition). Compound 2 (62.8 mg, 42.67 µmol, 55.84% yield, 48.37% purity) was obtained as a white oil.

Procedure for preparation of Compound 3



2 3

To a solution of Compound **2** (44 mg, 29.46 μmol, 1.0 eq), **BCY8116** (63 mg, 29.18 μmol, 1.0 eq) in DMF (2 mL) was added DIEA (5.66 mg, 43.77 μmol, 7.62 μL, 1.5 eq). The mixture was stirred at 40 °C for 12 hr. LC-MS showed Compound **2** was consumed completely and one main peak with desired m/z (MW: 3506.95, observed *m/z*: 1168.58 ([M/3+H]$^+$))was detected. The residue was purified by prep-HPLC (TFA condition). Compound **3** (20 mg, 5.42 μmol, 18.57% yield, 95.04% purity) was obtained as a white solid.

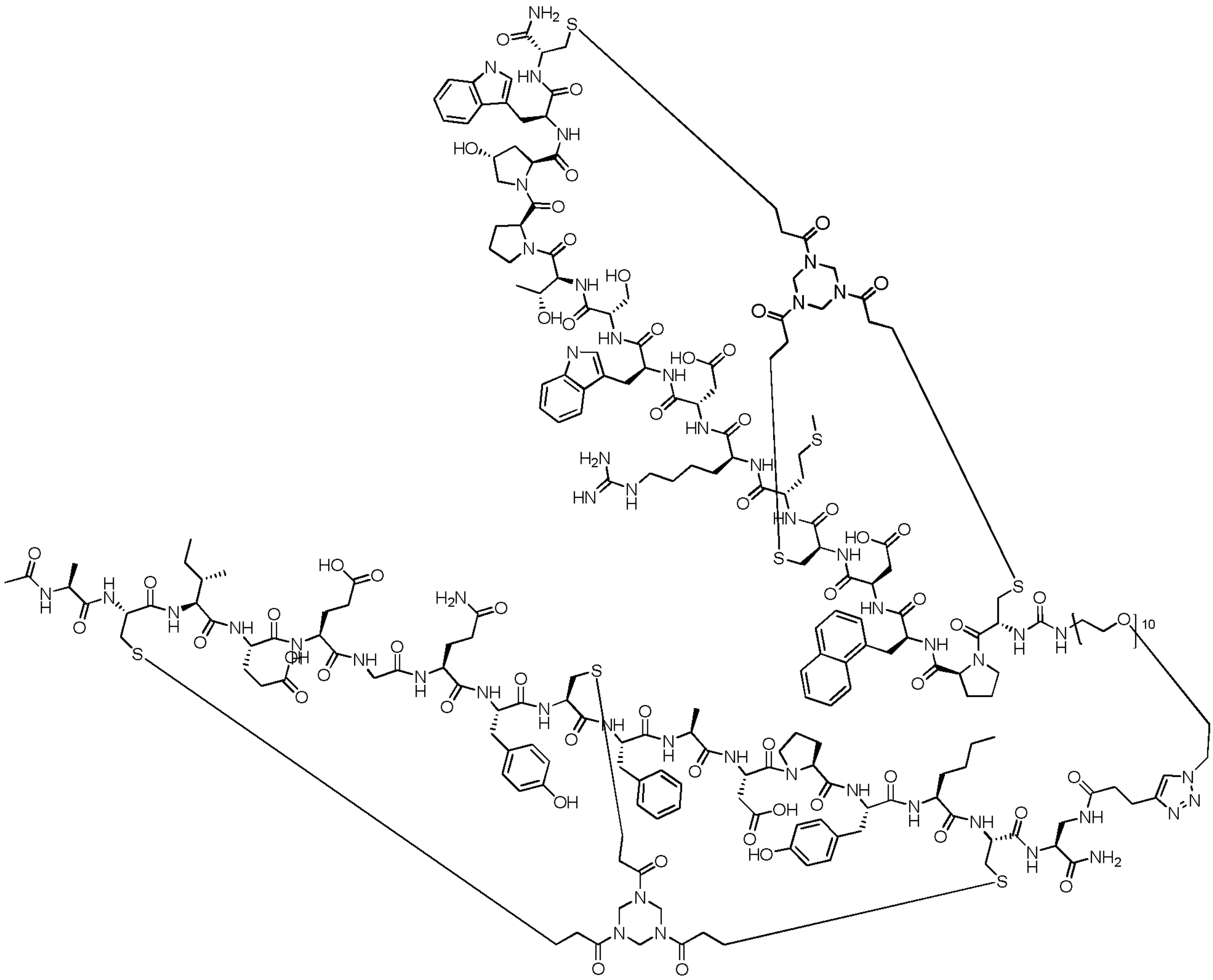
Procedure for preparation of BCY9403

[BCY00008116]-[COM00000471] + **BCY00007741** —($CuSO_4$ VcNa THPTA; t-BuOH/H_2O)→ **BCY00009403**

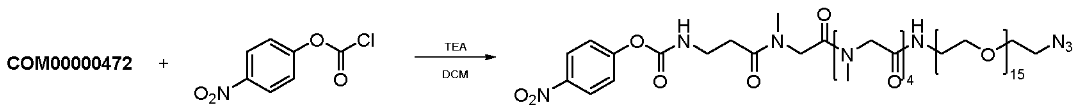
3

A mixture of Compound **3** (10.0 mg, 2.71 μmol, 1.0 eq), **BCY7741** (6.83 mg, 2.99 μmol, 1.1 *eq*), and THPTA (0.4 M, 7 μL, 1.0 eq) was dissolved in t-BuOH/H_2O (1:1, 2 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 7 μL, 1.0 eq) and VcNa (0.4 M, 14 μL, 2.0 eq) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 25-30 °C for 12 hr under N_2 atmosphere. LC-MS showed Compound **3** was consumed completely and one main peak with desired m/z [MW: 5788.49, observed *m/z*: 1157.00 ([M/5+H]$^+$) and 964.60 ([M/6+H]$^+$)] was detected. The reaction mixture was directly purified by prep-HPLC (TFA condition). **BCY9403** (2.1 mg, 0.34 μmol, 11.93% yield, 93.80% purity) was obtained as a white solid.

BCY9405



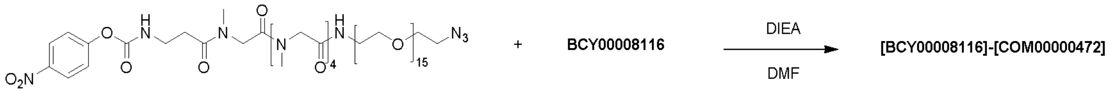
Procedure for preparation of Compound 2



1 2

To a solution of **COM472** (44.7 mg, 38.1 µmol), Compound **1** (9.2 mg, 45.72 µmol) in DCM (4 mL) was added TEA (5.8 mg, 57.14 µmol, 8 µL). The mixture was stirred at 25 °C for 2 hr. LC-MS showed **COM472** was consumed completely and one main peak with desired m/z (MW: 1338.45, observed *m/z*: 686.23([M/2+NH4$^+$])) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent to give a residue. The residue was purified by prep-HPLC (neutral condition). Compound **2** (20 mg, 14.94 µmol, 39.2% yield) was obtained as colorless oil.

Procedure for preparation of Compound 3



2 3

To a solution of Compound **2** (20 mg, 14.94 μmol) and **BCY8116** (38.96 mg, 17.93 μmol) in DMF (4 mL) was added DIEA (1.9 mg, 14.94 μmol, 2.6 μL). The mixture was stirred at 30 °C for 2 hrs. LC-MS showed Compound **2** was consumed completely and one main peak with desired m/z (MW: 3371.82, observed *m/z*: 1123.94([M/3+H$^+$])) was detected. The reaction mixture was filtered and concentrated under reduced pressure to give a residue. The crude product was purified by reversed-phase HPLC (TFA condition). Compound **3** (10 mg, 99.07% yield, 19.66 purity) was obtained as a white solid.

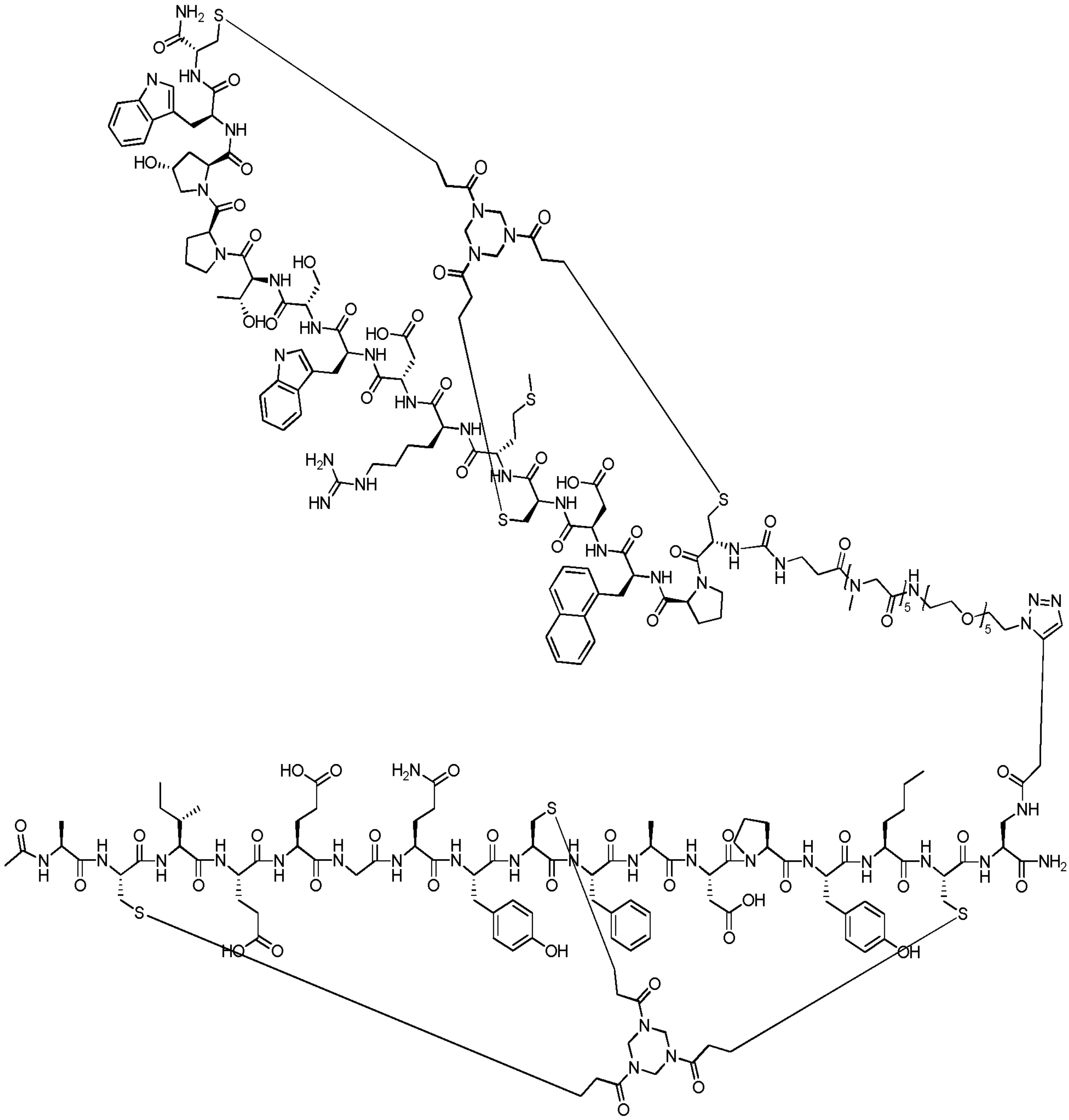
Procedure for preparation of BCY9405

[BCY00008116]-[COM00000472] + **BCY00007741** $\xrightarrow[\text{t-BuOH}/H_2O]{CuSO_4\ \text{VcNa THPTA}}$ **BCY00009405**

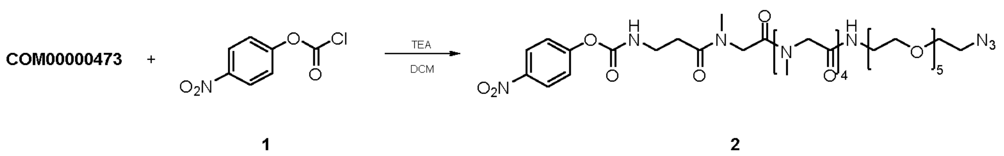
3

A mixture of Compound **3** (10.0 mg, 2.97 μmol, 1.0 eq), **BCY7741** (7.4 mg, 3.26 μmol, 1.1 *eq)*, and THPTA (1.3 mg, 2.97 μmol, 1.0 eq) was dissolved in t-BuOH/H_2O (1:1, 2 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 7.5 μL, 1.0 eq) and VcNa (0.4 M, 151 μL, 2.0 eq) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 25-30 °C for 12 hr under N_2 atmosphere. LC-MS showed Compound **3** was consumed completely and one main peak with desired m/z [MW: 5653.36, observed *m/z*: 1130.47 ([M/5+H]$^+$)] was detected. The reaction mixture was directly purified by prep-HPLC (TFA condition). **BCY9405** (7.8 mg, 46.08% yield, 97.8% purity) was obtained as a white solid.

<u>BCY9406</u>

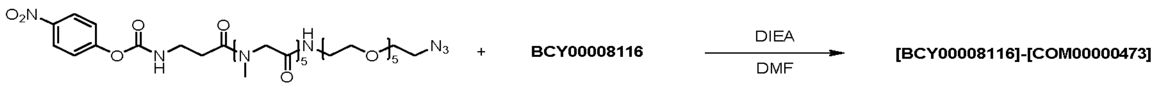


Procedure for preparation of Compound 2



To a solution of **COM473** (130.0 mg, 177.40 µmol, 1.0 eq), (4-nitrophenyl) carbonochloridate (36.4 mg, 180.59 µmol, 1.02 eq) in DCM (3 mL) was added TEA (27.0 mg, 266.09 µmol, 37 µL, 1.5 eq). The mixture was stirred at 35 °C for 2 hr. LC-MS showed **COM473** was consumed completely and one main peak with desired m/z (MW: 897.93, observed *m/z*: 897.65($[M+H]^+$), 914.60($[M+NH4]^+$)) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent to give a residue. The residue was purified by prep-HPLC (TFA condition). Compound **2** (90 mg, 95.87 µmol, 54.04% yield, 95.65% purity) was obtained as colorless oil.

Procedure for preparation of Compound 3



2 3

To a solution of Compound **2** (10 mg, 11.14 µmol, 1 eq), **BCY8116** (25 mg, 11.51 µmol, 1.03 eq) in DMF (2 mL) was added DIEA (2.16 mg, 16.71 µmol, 2.91 µL, 1.5 eq). The mixture was stirred at 25-30 °C for 12 hr. LC-MS showed one main peak with desired m/z (MW:2931.30, observed *m/z*: 977.00($[M/3+H]^+$)) was detected. The reaction mixture was filtered and concentrated under reduced pressure to give a residue. The crude product was purified by reversed-phase HPLC (FTA condition). Compound **3** (15 mg, 5.12 µmol, 45.79% yield, 99.66% purity) was obtained as a white solid.

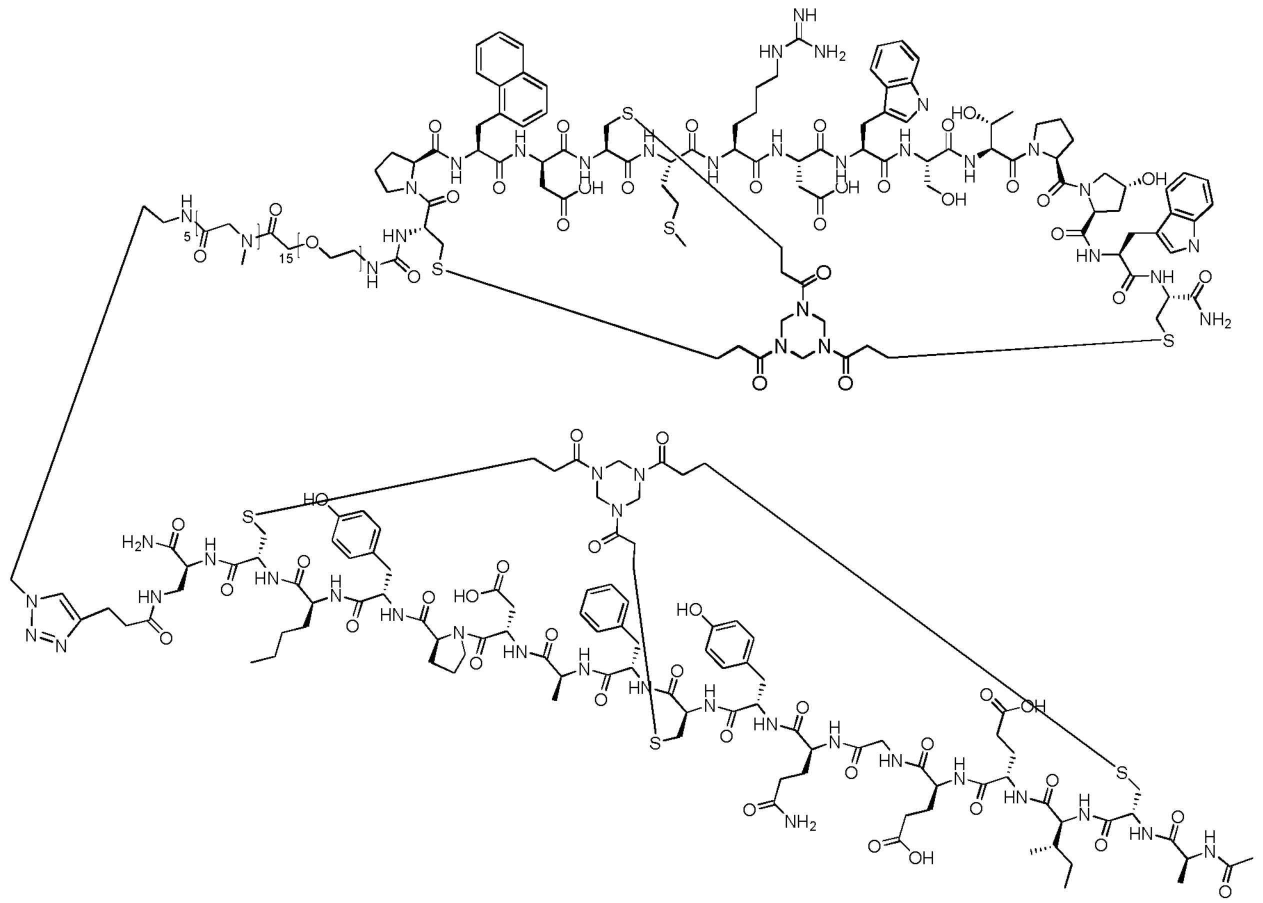
Procedure for preparation of BCY9406

[BCY00008116]-[COM00000473] + **BCY00007741** $\xrightarrow[\text{t-BuOH/}H_2O]{CuSO_4\ \text{VcNa THPTA}}$ **BCY00009406**

3

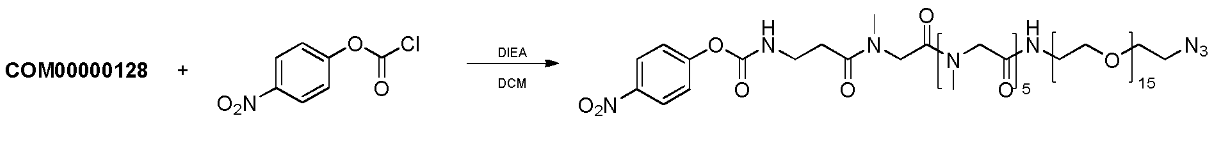
A mixture of Compound **3** (15 mg, 5.12 µmol, 1.0 eq), **BCY7741** (12 mg, 5.26 µmol, 1.03 eq), and THPTA (0.4 M, 12.8 µL, 1.0 eq) was dissolved in t-BuOH/H_2O (1:1, 2 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 12.8 µL, 1.0 eq) and VcNa (0.4 M, 25.6 µL, 2.0 eq) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 25-30 °C for 12 hr under N_2 atmosphere. LC-MS showed Compound **3** was consumed completely and one main peak with desired m/z [MW: 5212.84 observed *m/z*: 1042.74 ($[M/4+H]^+$)] was detected. The reaction mixture was directly purified by prep-HPLC (TFA condition). **BCY9406** (14.4 mg, 2.57 µmol, 50.21% yield, 93.01% purity) was obtained as a white solid.

<u>**BCY9407**</u>



BCY00009407

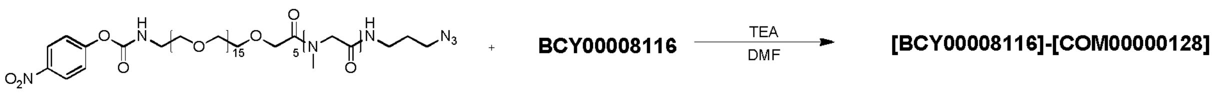
Procedure for preparation of Compound 2



1 2

To a solution of **COM128** (60 mg, 50.53 μmol, 1.0 eq), compound **1** (13 mg, 64.50 μmol, 1.28 eq), DIEA (9.80 mg, 75.80 μmol, 13.20 μL, 1.5 eq) in DCM (5 mL) was degassed and purged with N_2 for 3 times, and then the mixture was stirred at 25-30 °C for 1 hr under N_2 atmosphere. LC-MS showed **COM128** was consumed completely and one main peak with desired m/z (calculated MW: 1352.48, observed *m/z*: 676.7 ([M/2+H]$^+$)) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent to give a residue. The residue was purified by prep-HPLC (TFA condition). Compound **2** (12 mg, 8.87 μmol, 17.56% yield) was obtained as colorless oil.

Procedure for preparation of **[BCY8116]-[COM128]**



2

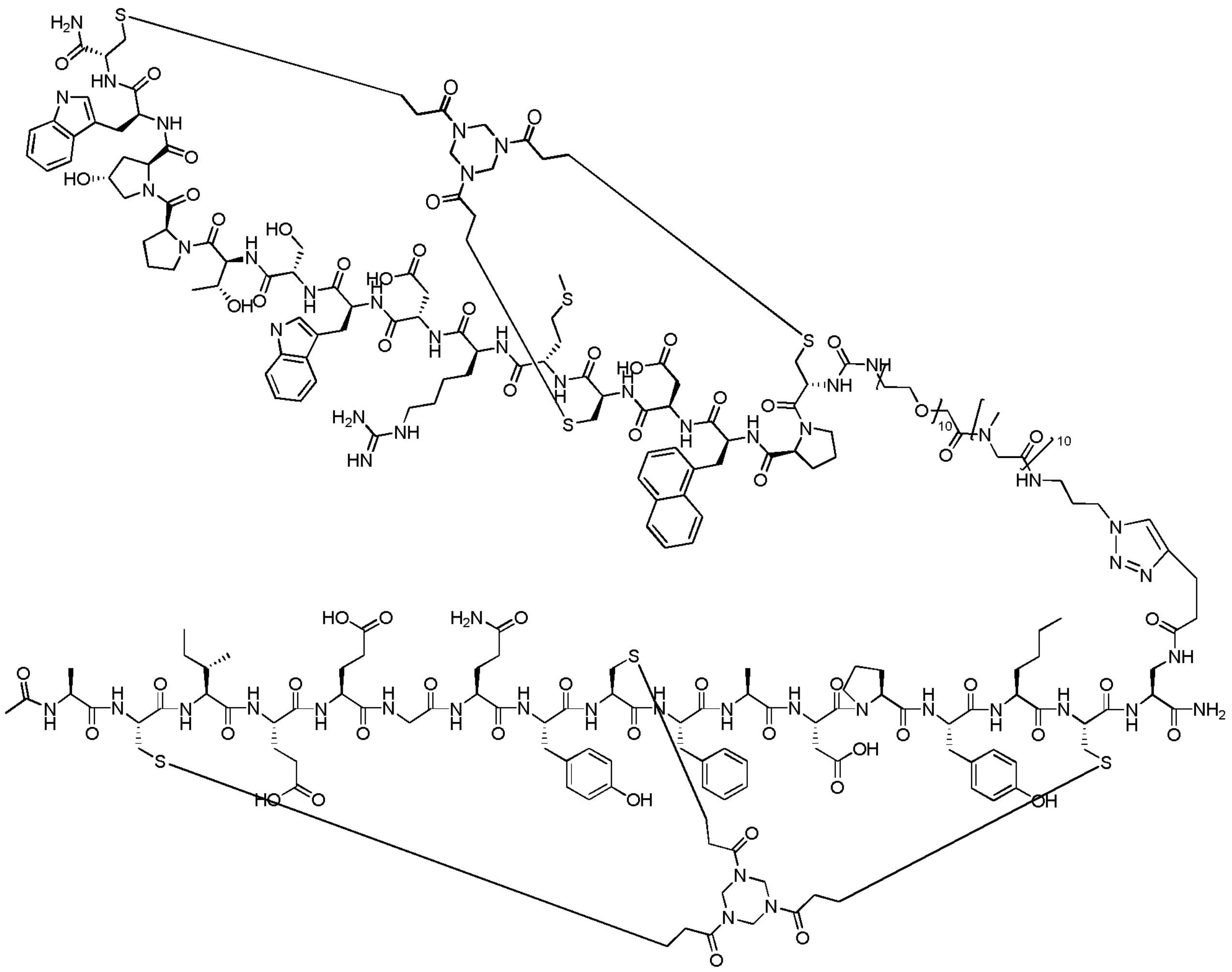
To a solution of compound **2** (7 mg, 5.18 µmol, 1.0 eq) and **BCY8116** (11 mg, 5.06 µmol, 1.0 eq), DIEA(2.01 mg, 15.53 µmol, 2.70 µL, 3.0 eq) in DMF (3 mL) was degassed and purged with N_2 for 3 times, and then the mixture was stirred at 25-30 °C for 1 hr under N_2 atmosphere. LC-MS showed one main peak with desired m/z (calculated MW: 3385.85, observed *m/z*: 1129.3 ($[M/3+H]^+$)) was detected. The reaction mixture was filtered and concentrated under reduced pressure to give a residue. The crude product was purified by reversed-phase HPLC (TFA condition). **[BCY8116]-[COM128]** (15.6 mg, 4.46 µmol, 86.13% yield, 96.75% purity) was obtained as a white solid

Procedure for preparation of BCY9407

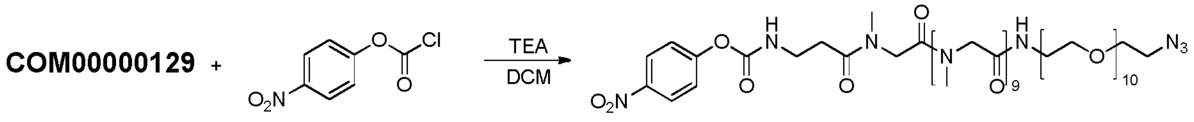
[BCY00008116]-[COM00000128] + **BCY00007741** —($CuSO_4$ VcNa THPTA / t-BuOH/H_2O)→ **BCY00009407**

A mixture of **[BCY8116]-[COM128]** (15.6 mg, 4.61 µmol, 1.0 eq), **BCY7741** (11 mg, 4.82 µmol, 1.05 eq), and THPTA (0.8 M, 5.8 µL, 1.0 eq) was dissolved in t-BuOH/H_2O (1:1, 2 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 11.6 µL, 1.0 eq) and VcNa (0.4 M, 23.2 µL, 2.0 eq) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 25-30 °C for 12 hr under N_2 atmosphere. LC-MS showed one peak with desired m/z (calculated MW: 5667.39, observed *m/z*: 945.6 ($[M/6+H]^+$) and 1134.2 ($[M/5+H]^+$)) was detected. The reaction mixture was directly purified by prep-HPLC (TFA condition). **BCY9407** (1.3 mg, 0.23 µmol, 4.33% yield, 86.90% purity) was obtained as a white solid.

<u>BCY9408</u>



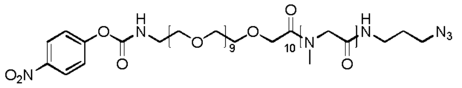
Procedure for preparation of Compound 2



1 2

To a solution of **COM129** (45.0 mg, 34.39 μmol, 1.0 eq), compound **1** (15.0 mg, 74.42 μmol, 2.1 eq) in DCM (5 mL) was added TEA (5.5 mg, 53.88 μmol, 7.5 μL, 1.5 eq), and then the mixture was stirred at 25-30 °C for 1 hr under N_2 atmosphere. LC-MS showed **COM129** was consumed completely and one main peak with desired m/z (MW: 1473.58, observed *m/z*: 737.3 ($[M/2+H]^+$)) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent to give a residue. The residue was purified by prep-HPLC (TFA condition). Compound **2** (9 mg, 6.11 μmol, 17.01% yield, 95.76% purity) was obtained as a white solid.

Procedure for preparation of Compound 3



+ **BCY00008116** —TEA / DMF→ **[BCY00008116]-[COM00000129]**

2 **3**

To a solution of compound **2** (9.0 mg, 6.11 μmol, 1.0 eq) and **BCY8116** (13.3 mg, 6.11 μmol, 1.0 eq) in DMF (3 mL) was added DIEA (2.4 mg, 18.32 μmol, 3.2 μL, 3.0 eq). All solvents were degassed and purged with N_2 for 3 times, and then the mixture was stirred at 25-30 °C for 1 hr under N_2 atmosphere. LC-MS showed compound **2** was consumed completely and one main peak with desired m/z (MW: 3506.95, observed *m/z*: 877.4($[M/4+H]^+$) and *m/z*: 1169.6($[M/3+H]^+$)) was detected. The reaction mixture was filtered and concentrated under reduced pressure to give a residue. The crude product was purified by reversed-phase HPLC (TFA condition). Compound **3** (7.2 mg, 2.05 μmol, 31.93% yield, 95% purity) was obtained as a white solid.

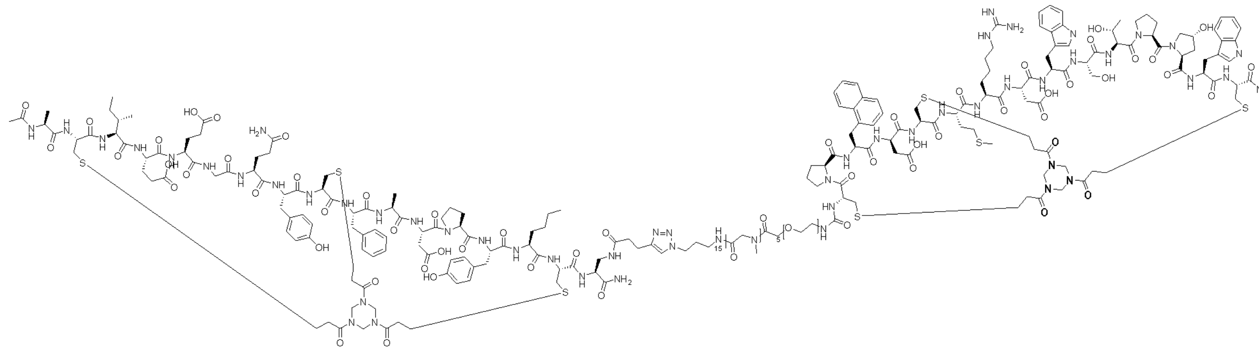
Procedure for preparation of BCY9408

[BCY00008116]-[COM00000129] + **BCY00007741** —$CuSO_4$ VcNa THPTA / t-BuOH/H_2O→ **BCY00009408**

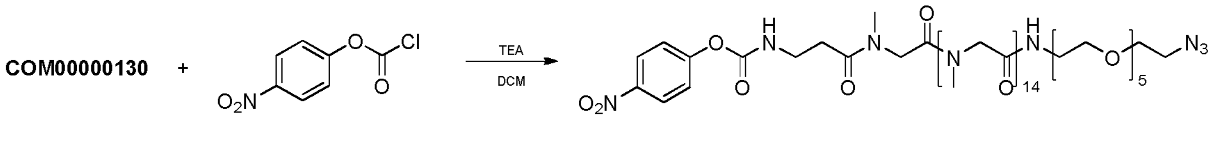
3

A mixture of Compound **3** (7.2 mg, 2.05 μmol, 1.0 eq), **BCY7741** (5.0 mg, 2.19 μmol, 1.03 eq), and THPTA (0.4 M, 5.1 μL, 1.0 eq) was dissolved in t-BuOH/H_2O (1:1, 2 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 5.1 μL, 1.0 eq) and VcNa (0.4 M, 10.2 μL, 2.0 eq) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 25-30 °C for 12 hr under N_2 atmosphere. LC-MS showed Compound **3** was consumed completely and one main peak with desired m/z [MW: 5788.49 observed *m/z*: 968.9($[M/6+H]^+$) and 1158.0($[M/5+H]^+$)] was detected. The reaction mixture was directly purified by prep-HPLC (TFA condition). **BCY9408** (3.1 mg, 4.97e-1 μmol, 24.23% yield, 92.87% purity) was obtained as a white solid.

<u>**BCY9409**</u>



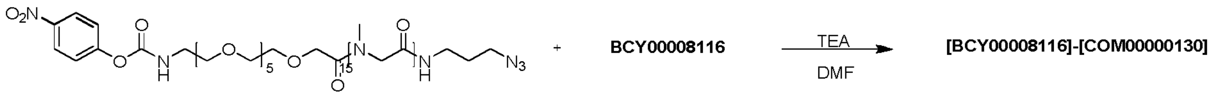
Procedure for preparation of Compound 2



1 **2**

To a solution of compound **1** (30 mg, 20.78 µmol), **COM130** (6.28 mg, 31.17 µmol) in DCM (3 mL) was added TEA (3.15 mg, 31.17 µmol, 4.34 µL, 1.5 *eq*). The mixture was stirred at 25-30°C for 1 hr. LC-MS showed compound **1** was consumed completely and one main peak with desired m/z (MW: 1608.70 observed *m/z*: 804.8 ([M/2+H]$^+$) was detected. The reaction mixture was concentrated under reduced pressure and then lyophilized to give Compound **2** (10.2 mg, crude) as a white solid.

Procedure for preparation of Compound 3



2 **3**

To a solution of compound **2** (10.2 mg, 6.34 µmol) and **BCY8116** (13.50 mg, 6.22 µmol) in DMF (2 mL) was added DIEA (0.8 mg, 6.22 µmol, 1.1 µL, 1.0 eq).The mixture was stirred at 30 °C for 2 hr. LC-MS detected desired m/z (MW: 3642.08, observed *m/z*: 1214.4([M/3+H]$^+$). The reaction mixture was filtered and concentrated under reduced pressure to give a residue. The crude product was purified by reversed-phase HPLC (TFA condition). Compound **3** (15.0 mg, 4.12 µmol, 62.94% yield, 95% purity) was obtained as a white solid.

Procedure for preparation of BCY9409

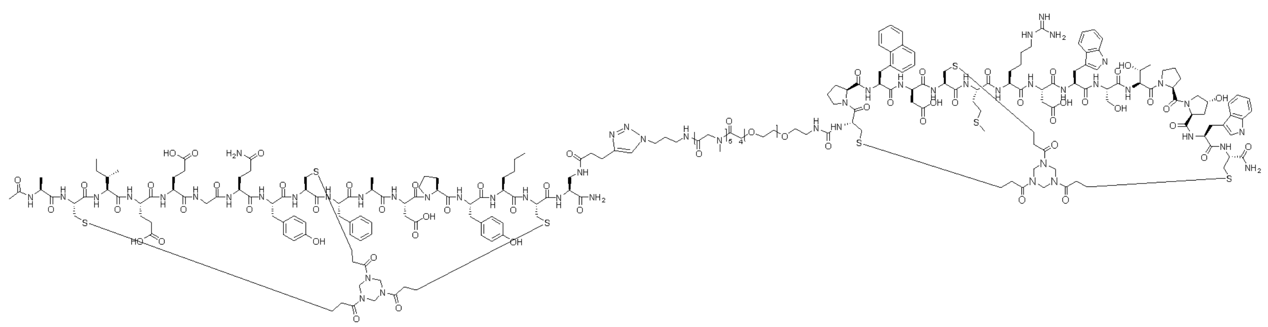
[BCY00008116]-[COM00000130] + **BCY00007741** —($CuSO_4$ VcNa THPTA / t-BuOH/H_2O)→ **BCY00009409**

3

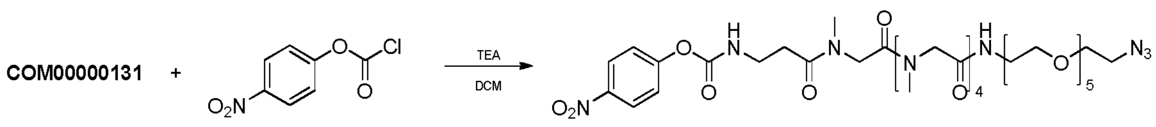
A mixture of Compound **3** (15 mg, 4.12 µmol, 1.0 eq), **BCY7741** (10 mg, 4.38 µmol, 1.03 eq), and THPTA (0.4 M, 10.3 µL, 1.0 eq) was dissolved in t-BuOH/H_2O (1:1, 2 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 10.3 µL, 1.0 eq) and

VcNa (0.4 M, 20.6 μL, 2.0 eq) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 25-30 °C for 12 hr under N_2 atmosphere. LC-MS showed Compound **3** was consumed completely and one main peak with desired m/z [MW: 5923.61, observed *m/z*: 988.2([M/6+H]$^+$)] was detected. The reaction mixture was directly purified by prep-HPLC (TFA condition). **BCY9409** (3.1 mg, 0.52 μmol, 12.62% yield, 90.89% purity) was obtained as a white solid.

BCY9410



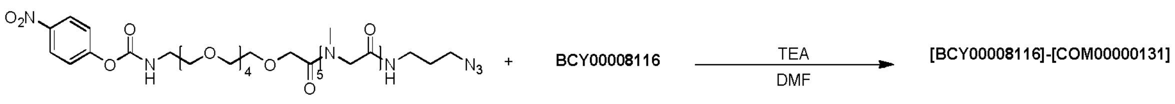
Procedure for preparation of Compound 2



1 2

To a solution of **COM131** (167.0 mg, 227.89 μmol, 1.0 eq), compound **1** (55.0 mg, 272.87 μmol, 1.2 eq) in DCM (5 mL) was added TEA (36.4 mg, 359.23 μmol, 50.0 μL, 1.6 eq). The mixture was stirred at 25-30 °C for 1 hr. LC-MS showed one main peak with desired m/z (MW: 897.93 observed 920.3([M+Na$^+$]) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent to give a residue. The residue was purified by prep-HPLC (TFA condition). Compound **2** (35 mg, 33.74 μmol, 14.81% yield, 86.56% purity) was obtained as colorless oil.

Procedure for preparation of Compound 3



2 3

To a solution of compound **2** (20 mg, 22.27 μmol, 1.0 eq) and **BCY8116** (48 mg, 22.09 μmol, 1.0 eq) in DMF (2 mL) was added DIEA (8.64 mg, 66.82 μmol, 11.64 μL, 3.0 eq). The mixture was stirred at 30 °C for 2 hr. LC-MS showed compound 3 was consumed completely and one main peak with desired m/z (MW: 2931.32, observed *m/z*: 977.7([M+H]+))was detected. The residue was purified by prep-HPLC (TFA condition). Compound **3** (40 mg, 13.08 μmol, 58.7% yield, 95.82% purity) was obtained as a white solid.

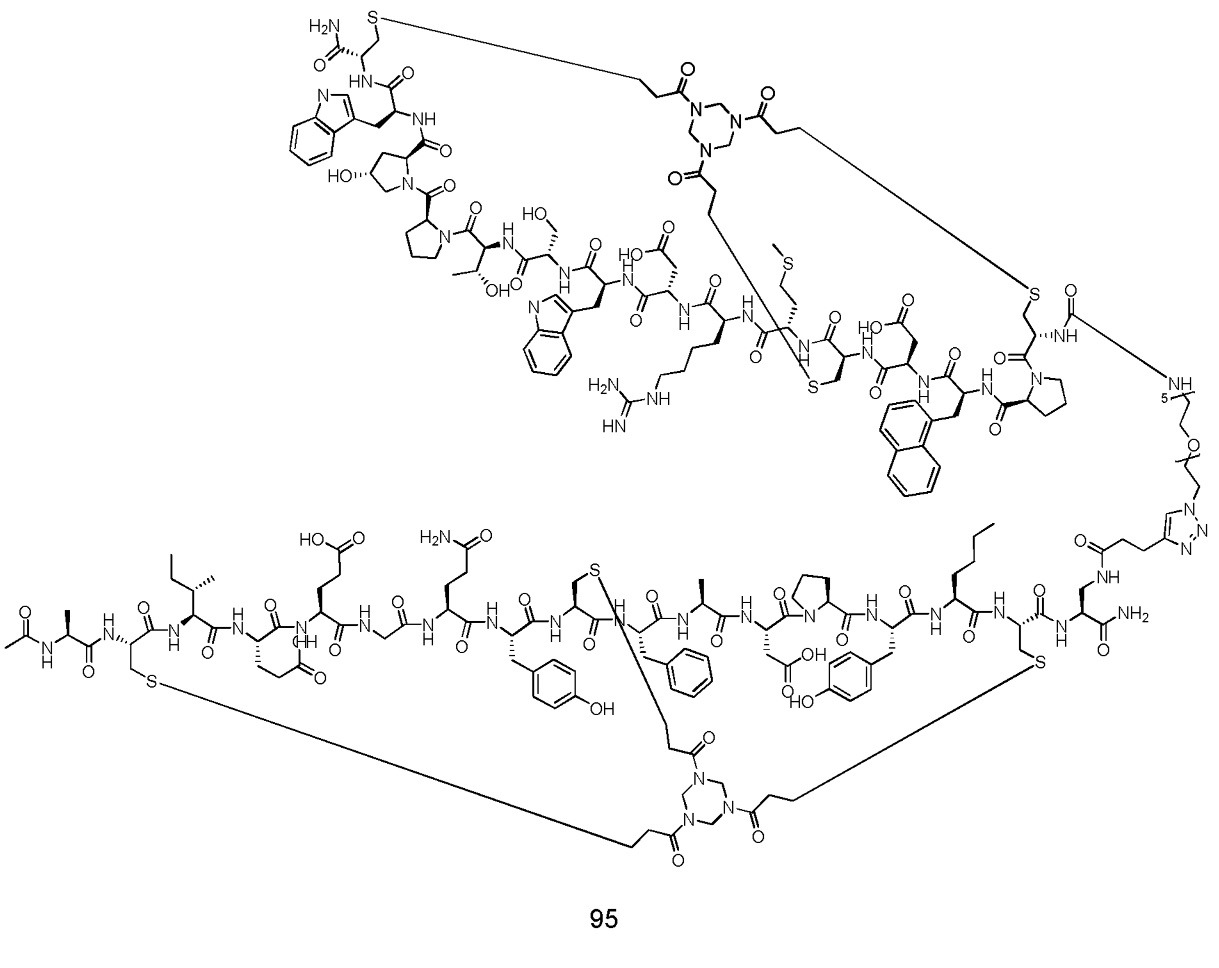
Procedure for preparation of BCY9410

[BCY00008116]-[COM00000131] + **BCY00007741** $\xrightarrow[\text{t-BuOH/H}_2\text{O}]{\text{CuSO}_4\ \text{VcNa}\ \text{THPTA}}$ **BCY00009410**

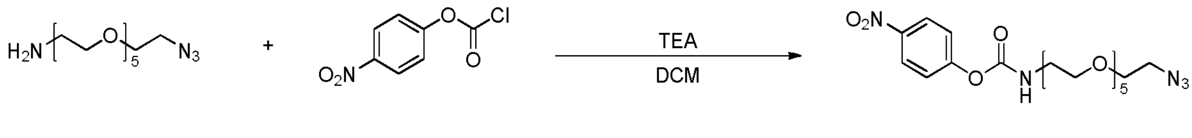
3

A mixture of Compound **3** (40 mg, 13.08 μmol, 1.0 eq), **BCY7741** (35 mg, 15.34 μmol, 1.17 eq), and THPTA (0.4 M, 34 μL, 1.0 eq) was dissolved in t-BuOH/H_2O (1:1, 2 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 34 μL, 1.0 eq) and VcNa (0.4 M, 68 μL, 2.0 eq) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O) and the solution turned to light yellow. The reaction mixture was stirred at 25-30 °C for 12 hr under N_2 atmosphere. LC-MS showed Compound **3** was consumed completely and one main peak with desired m/z [MW: 5212.85, observed *m/z*: 1043.2 ([M/5+H]+)] was detected. The reaction mixture was directly purified by prep-HPLC (TFA condition). **BCY9410** (38.6 mg, 6.78 μmol, 49.71% yield, 91.6% purity) was obtained as a white solid.

<u>**BCY9411**</u>



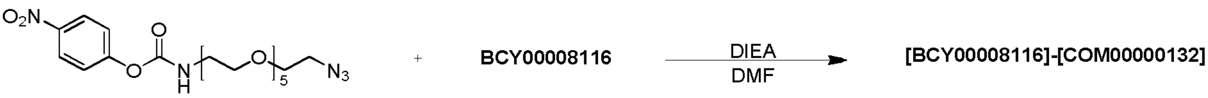
Procedure for preparation of Compound 2



COM00000132 **1** **2**

To a solution of **COM132** (5 mg, 16.32 μmol, 1 *eq*), Compound **1** (4 mg, 19.85 μmol, 1.22 *eq*) in DCM (5 mL) was added TEA (2.8 mg, 24.48 μmol, 3.4 μL, 1.5 *eq*). The mixture was stirred at 25 °C for 1 hr. LC-MS showed one peak with desired m/z (calculated MW: 471.46, observed *m/z*: 489.2([M+NH4]$^+$)) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent, and then lyophilized to give compound **2** (8 mg, crude) as a white solid.

Procedure for preparation of Compound 3



2 **3**

To a solution of Compound **2** (3.3 mg, 6.9 μmol, 1.5 *eq*) and **BCY8116** (10.0 mg, 4.6 μmol, 1.0 *eq*) in DMF (5 mL) was added DIEA (0.7 mg, 6.90 μmol, 1 μL, 1.5 *eq*). The mixture was stirred at 30 °C for 2 hrs. LC-MS showed Compound **2** was consumed completely and one main peak with desired m/z (calculated MW: 2504.83, observed *m/z*: 1252.3([M/2+H]$^+$)) was detected. The reaction mixture was filtered and concentrated under reduced pressure to give a residue. The crude product was purified by reversed-phase HPLC (TFA condition). Compound **3** (4.2 mg, 1.51 μmol, 32.78% yield, 90% purity) was obtained as a white solid.

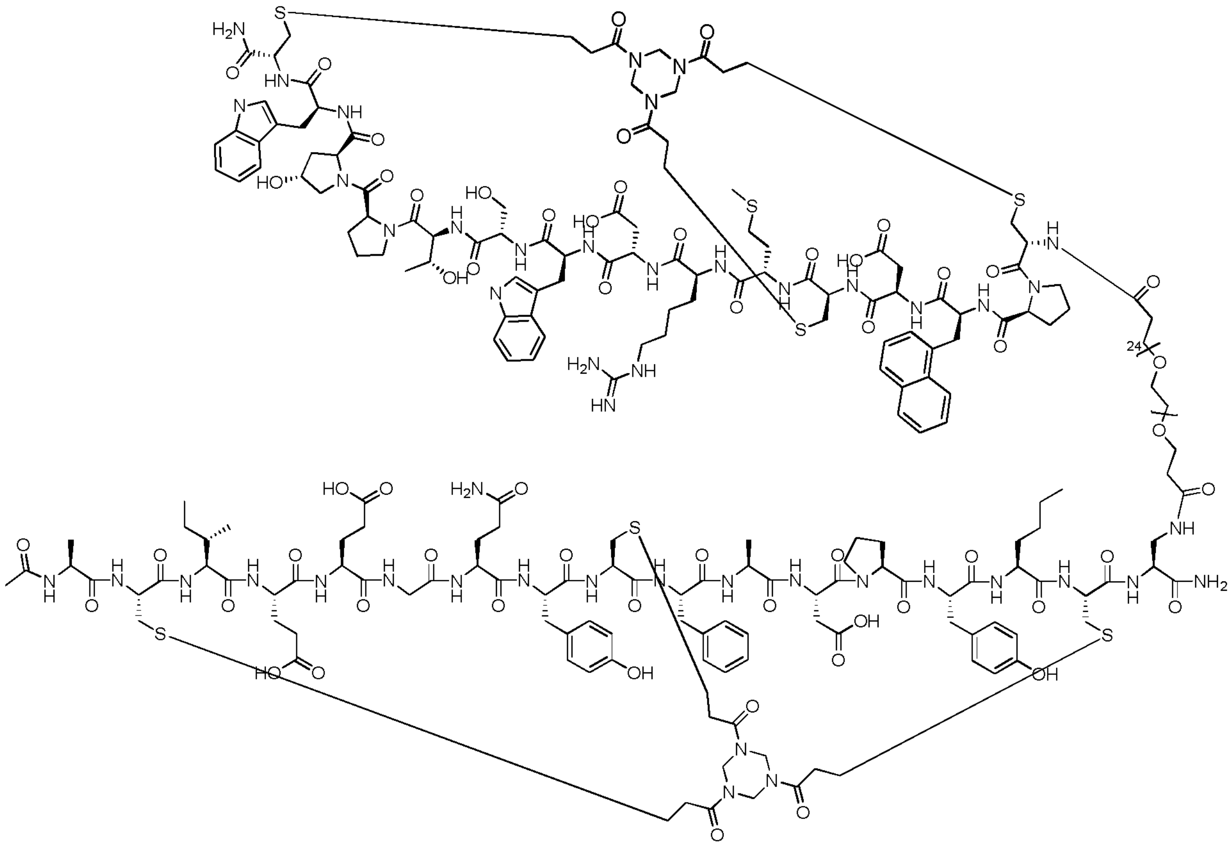
Procedure for preparation of BCY9411

[BCY00008116]-[COM00000132] + **BCY00007741** —($CuSO_4$ VcNa THPTA; t-BuOH/H_2O)→ **BCY00009411**

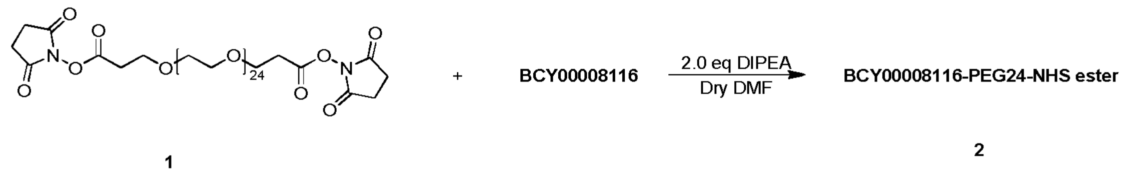
3

A mixture of Compound **3** (4.2 mg, 1.68 μmol, 1.0 *eq*), **BCY7741** (4.0 mg, 1.75 μmol, 1.05 *eq*), and THPTA (0.04 M, 84 μL, 2.0 *eq*) was dissolved in t-BuOH/H_2O (1:1, 2 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.04 M, 84 μL, 2.0 *eq*) and VcNa (0.04 M, 168 μL, 4.0 *eq*) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 25-30 °C for 12 hr under N_2 atmosphere. LC-MS showed Compound **3** was consumed completely and one main peak with desired m/z [MW: 4786.37 observed *m/z*: 1596.2([M/3+H]$^+$), 1196.9([M/4+H]$^+$)] was detected. The reaction mixture was directly purified by prep-HPLC (TFA condition). **BCY9411** (4.1 mg, 0.86 μmol, 50.20% yield, 98.26% purity) was obtained as a white solid.

<u>**BCY9759**</u>



Procedure for preparation of Compound 2



To a solution of compound **1** (5.0 mg, 3.54 μmol, 1.0 eq), **BCY8116** (7.7 mg, 3.54 μmol, 1.0 eq) in DMF (3 mL) was added DIEA (0.9 mg, 7.07 μmol, 1.2 μL, 2.0 eq). The mixture was stirred at 0 °C for 20 min. LC-MS detected mass corresponding to compound 2 with NHS group falling off (calculated MW: 3470.95, hydrolyzed MW: 3373.81, observed *m/z*: 1125.0($[M/3+H]^+$). The reaction mixture was filtered and concentrated under reduced pressure and lyophilized to give compound **2** (15 mg, crude) was obtained as a white solid.

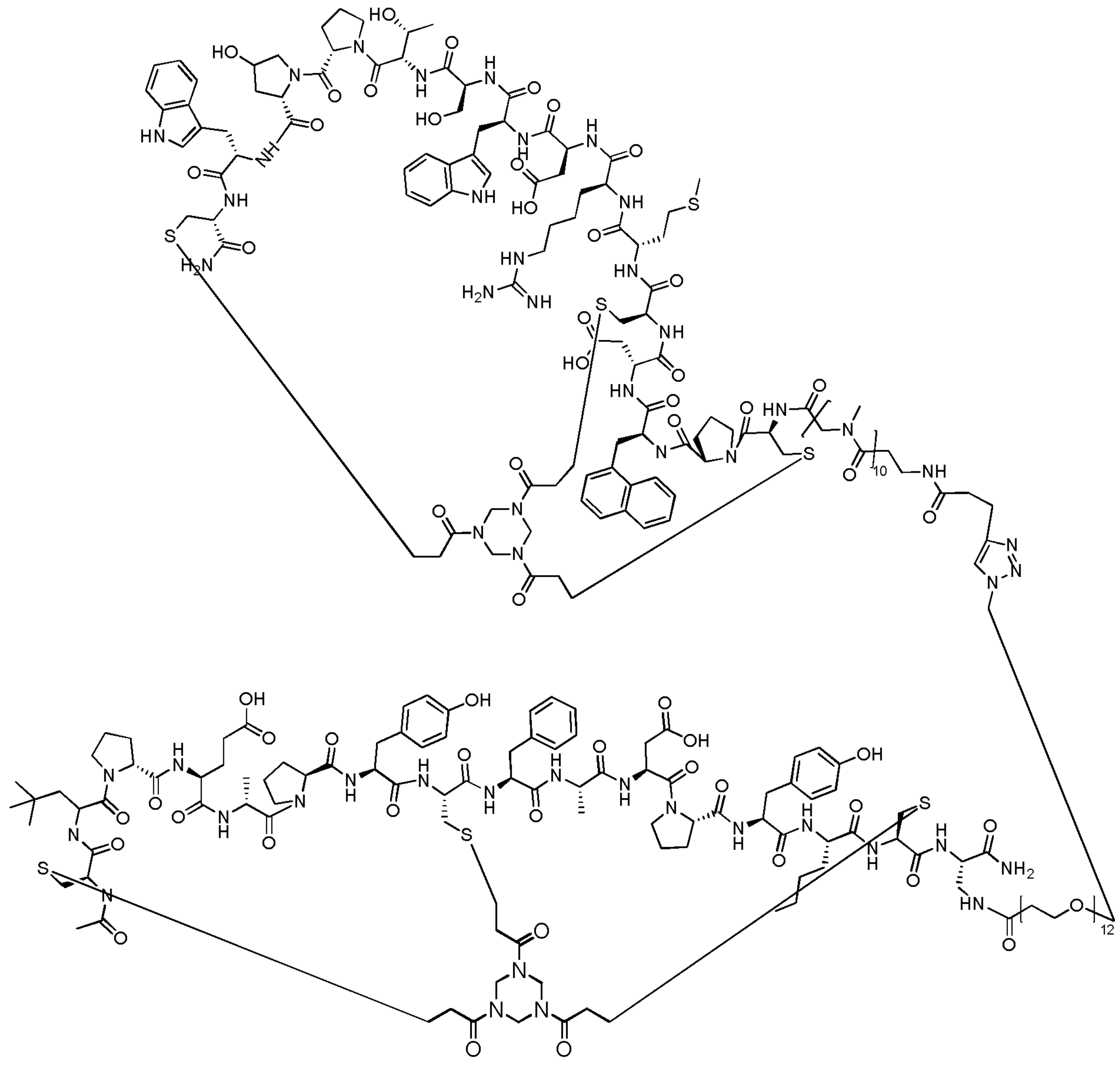
Procedure for preparation of BCY9759

BCY00008116-PEG24-NHS ester + **BCY00007732** —(2.0 eq DIPEA / Dry DMF)→ **BCY00009759**

2

To a solution of compound **2** (20 mg, 5.76 μmol, 1.0 eq) and **BCY7732** (12.7 mg, 5.76 μmol, 1.0 eq) in DMF (3 mL) was added DIEA (1.5 mg, 11.52 μmol, 2.0 μL, 2.0 eq). The mixture was stirred at 25~30 °C for 2 hrs. LC-MS showed compound **2** was consumed completely and one main peak with desired m/z (MW: 5557.3, observed *m/z*: 927.0 ($[M/6+H]^+$) and 1112.2($[M/5+H]^+$)) was detected. The reaction mixture was filtered and concentrated under reduced pressure to give a residue. The crude product was purified by reversed-phase HPLC (TFA condition). **BCY9759** (2.3 mg, 6.92% yield, 96.29% purity) was obtained as a white solid.

<u>BCY10000</u>



BCY00010000

Procedure for preparation of BCY9172-PEG12-N$_3$

BCY00009172 + **NHS-PEG12-N$_3$** $\xrightarrow[\text{DMF}]{\text{DIEA}}$ **BCY00009172-PEG12-N$_3$**

1 **2**

BCY9172 (520 mg, 248.16 µmol, 1.0 eq) and compound **1** (370 mg, 499.47 µmol, 2.01 eq) were dissolved in DMF (5 mL), then the mixture was added with DIEA (48.11 mg, 372.24 µmol, 64.84 µL, 1.5 eq) and stirred at 30°C for 12 hr. LC-MS showed **BCY9172** was consumed completely and one main peak with desired m/z (calculated MW: 2721.12, observed *m/z*: 1360.9 ([M/2+H]$^+$)) was detected. The reaction mixture was purified by prep-HPLC (TFA condition) and compound **2** (284 mg, 101.10 µmol, 40.74% yield, 96.87% purity) was obtained as a white solid.

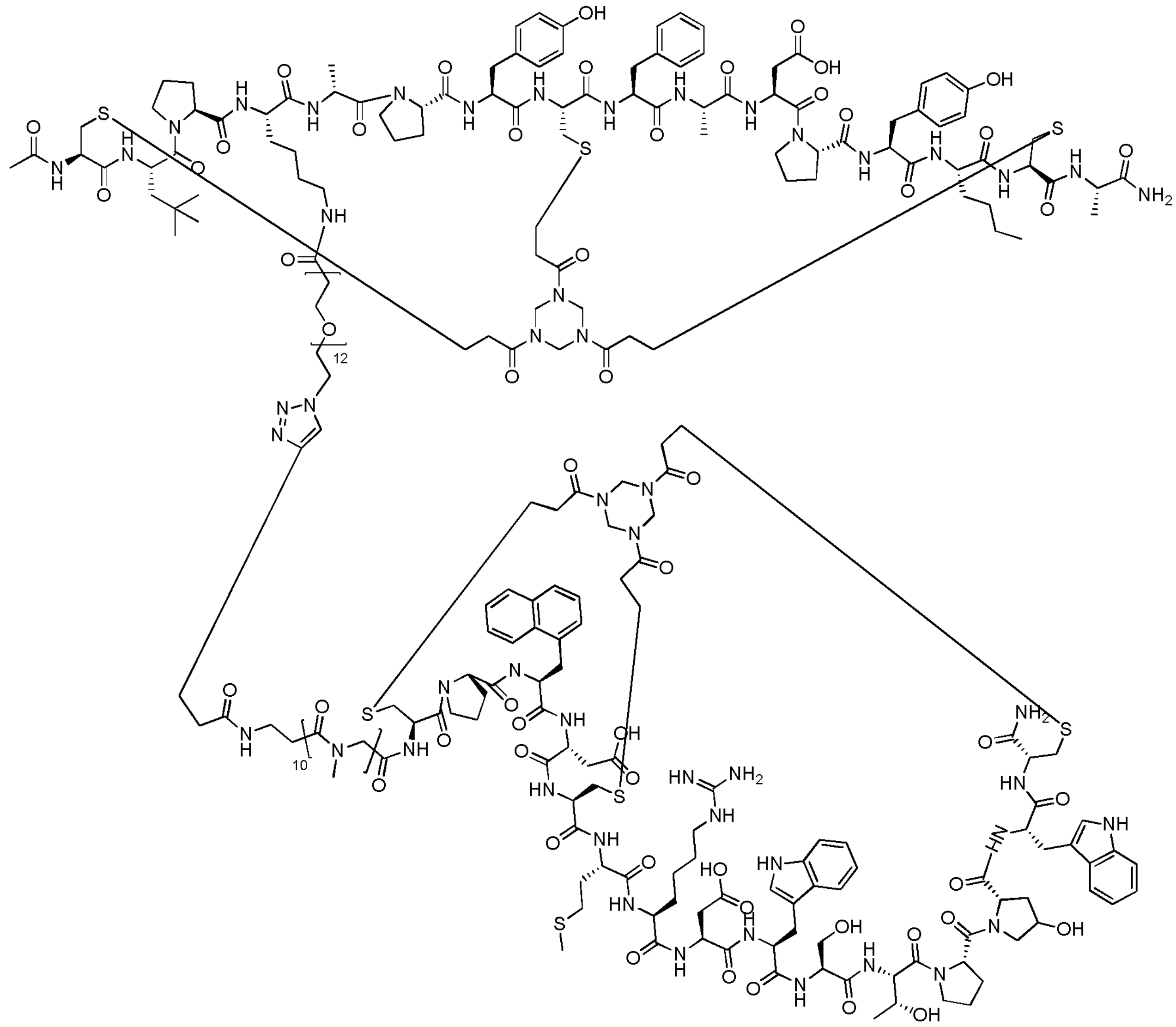
Procedure for preparation of BCY10000

BCY00009172-PEG12-N_3 + **BCY00008846** —($CuSO_4$, VcNa, THPTA; t-BuOH/H_2O)→ **BCY00010000**

2

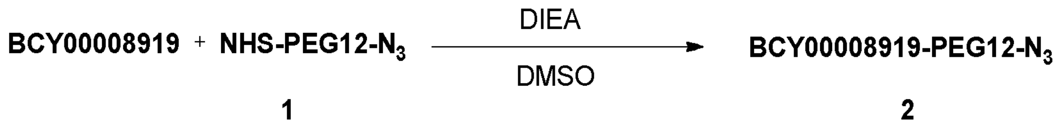
This reaction was performed in two independent containers in parallel. For one container, Compound **2** (142 mg, 52.18 μmol, 1.0 eq) and **BCY8846** (157 mg, 51.74 μmol, 1.0 eq) were first dissolved in 10 mL of t-BuOH/H_2O (1:1), and then $CuSO_4$ (0.4 M, 130.5 μL, 1.0 *eq*), VcNa (0.4 M, 261.0 μL, 2.0 eq) and THPTA (0.4 M, 130.5 μL, 1.0 eq) were added. Finally 1 M NH_4HCO_3 was added to adjust pH to 8. All solvents here were degassed and purged with N_2 for 3 times. The reaction mixture was stirred at 30 °C for 12 hr under N_2 atmosphere. LC-MS showed compound **2** was consumed completely and one main peak with desired m/z (calculated MW: 5755.54, observed *m/z*: 959.60 ($[M/6+H]^+$) and 1151.55 ($[M/5+H]^+$)) was detected. The reaction mixture was purified by prep-HPLC (TFA condition) and **BCY10000** (314.9 mg, 51.99 μmol, 49.82% yield, 95.03% purity) was obtained as a white solid.

BCY10567



BCY00010567

Procedure for preparation of BCY8919-PEG12-N₃



BCY8919 (60.0 mg, 28.85 µmol, 1.0 eq) and compound **1** (22.2 mg, 30.01 µmol, 1.04 eq) were dissolved in DMSO (1 mL). The solution was added with DIPEA (5.6 mg, 43.28 µmol, 7.6 µl, 1.5 eq), and then the mixture was stirred at 25-30°C for 2 hr. LC-MS showed **BCY8919** was consumed completely and one main peak with desired m/z (calculated MW: 2705.16, observed *m/z*: 1353.15([M/2+H]$^+$)) was detected. The reaction mixture was purified by prep-HPLC (TFA condition) and compound **2** (BCY8919-PEG12-N_3, 18.5 mg, 6.77 µmol, 23.47% yield, 99.04% purity) was obtained as a white solid.

Procedure for preparation of BCY10567

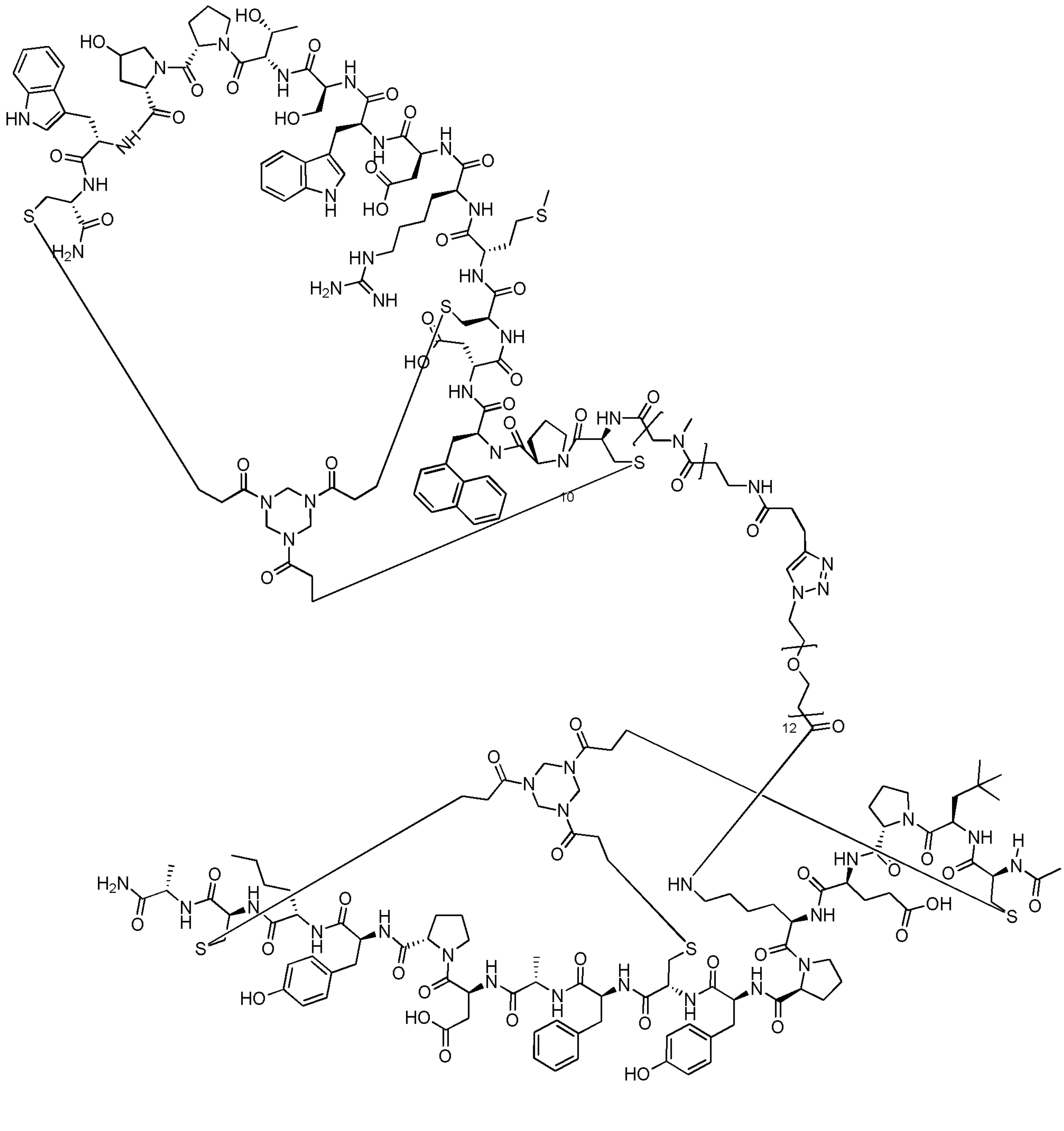
BCY00008919-PEG12-N_3 + **BCY00008846** —($CuSO_4$, VcNa, THPTA; t-BuOH/H_2O)→ **BCY00010567**

2

Note: This reaction has been performed twice, and the first one is described below.

Compound **2** (9.0 mg, 3.33 μmol, 1.0 eq) and **BCY8846** (10.1 mg, 3.33 μmol, 1.0 eq) were first dissolved in 2 mL of t-BuOH/H_2O (1:1), and then $CuSO_4$ (0.4 M, 8.3 μL, 1.0 eq), VcN (1.3 mg, 6.56 μmol, 2.0 eq) and THPTA (1.4 mg, 3.22 μmol, 1.0 eq) were added. Finally 0.4 M NH_4HCO_3 was added to adjust pH to 8. All solvents here were degassed and purged with N_2 for 3 times. The reaction mixture was stirred at 30°C for 16 hr under N_2 atmosphere. LC-MS showed compound **2** was consumed completely and one main peak with desired m/z (calculated MW: 5739.58, observed *m/z*: 956.75([M/6+H]$^+$)). The reaction mixture was purified by prep-HPLC (TFA condition) and **BCY10567** (6.85 mg, 1.18 μmol, 35.48% yield, 98.91% purity) was obtained as a white solid.

<u>BCY10569</u>



BCY00010569

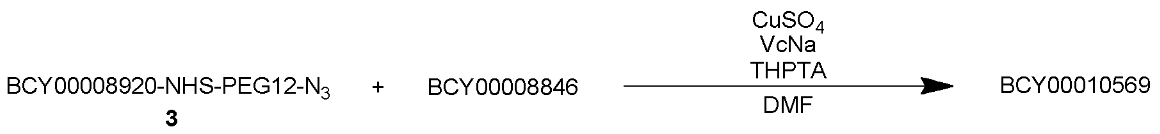
Procedure for preparation of Compound 3

BCY00008920 + NHS-PEG12-N_3 —(DIEA / DMF)→ BCY00008920-PEG12-N_3

NHS-PEG12-N_3 = **2**; BCY00008920-PEG12-N_3 = **3**

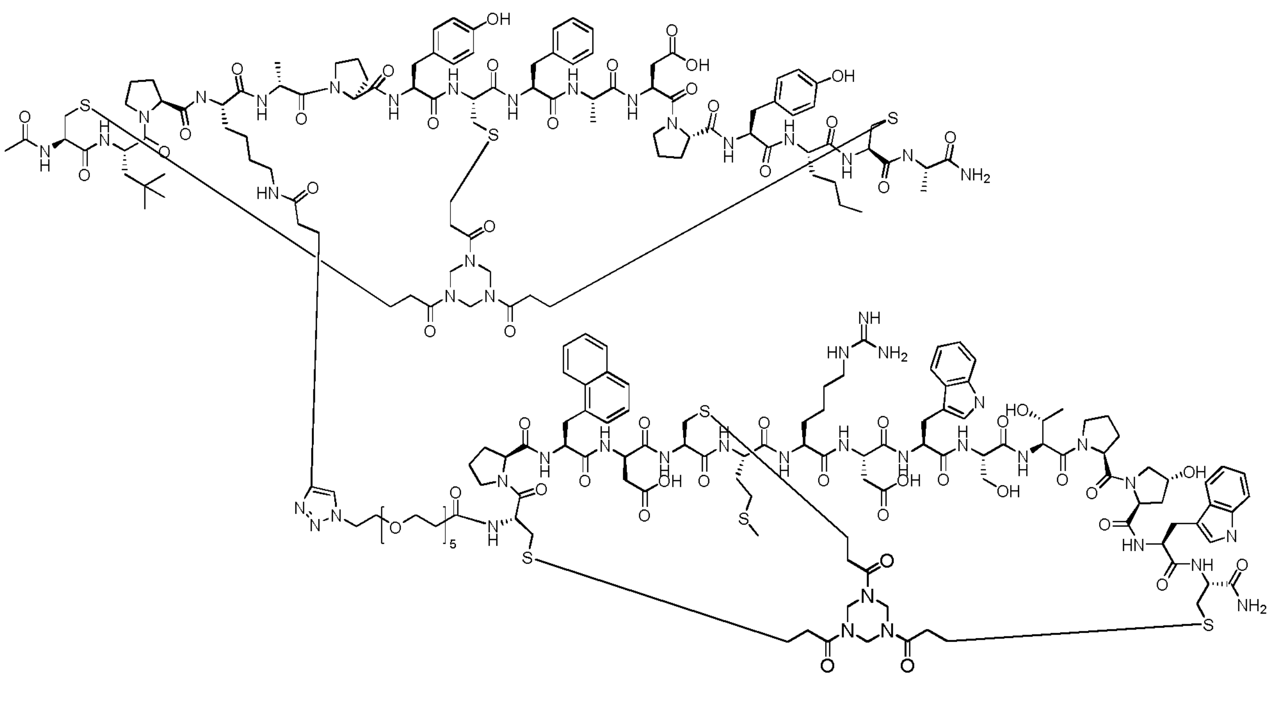
A mixture of compound **BCY8920** (40.0 mg, 18.71 µmol, 1.0 eq.), compound **2** (16.0 mg, 21.6 µmol, 1.15 eq.) and DIEA (5.0 µL, 28.0 µmol, 1.5 eq.) was dissolved in DMF. The reaction mixture was stirred at 40 °C for 1 hr, till LC-MS showed one main peak with desired m/z (calculated MW:2763.2, observed *m/z*: 912.17([(M-28)/2+H]$^+$) was detected. The reaction mixture was then concentrated under reduced pressure to remove solvent and produced a residue, following by purification by prep-HPLC (TFA condition). Compound **3** (23.4 mg, 8.47 μmol, 45.25% yield, 99.0% purity) was obtained as a white solid.

Procedure for preparation of BCY10569



A mixture of compound **3** (5.0 mg, 1.81 μmol, 1.0 eq.), **BCY8846** (5.8 mg, 1.9 μmol, 1.05 eq.), and THPTA (1.0 mg, 2.3 μmol, 1.3 eq.) was dissolved in t-BuOH/H_2O (1:1, 1 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 5.0 μL, 1.0 eq.) and VcNa (0.4 M, 5.0 μL, 1.0 eq.) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 40 °C for 2 hr under N_2 atmosphere. LC-MS showed compound **3** was consumed completely and one main peak with desired m/z (calculated MW: 5797.62, observed *m/z*: 1160.7 ([M/5+H]$^+$) was detected. The reaction mixture was filtered and concentrated under reduced pressure to give a residue. The crude product was purified by prep-HPLC (TFA condition), and **BCY10569** (5.7 mg, 1.18 μmol, 52.25% yield, 96.16% purity) was obtained as a white solid.

<u>BCY10571</u>



BCY00010571

Procedure for preparation of BCY8116-PEG5-N$_3$

$$\textbf{BCY00008116} + \textbf{NHS-PEG5-N}_3 \xrightarrow[\text{DMSO}]{\text{DIEA}} \textbf{BCY00008116-PEG5-N}_3$$

1 **2**

BCY8116 (60 mg, 27.62 µmol, 1.0 eq) and compound **1** (12.0 mg, 27.75 µmol, 1.0 eq) were first dissolved in DMSO (1 mL), then the mixture was added with DIEA (5.4 mg, 41.43 µmol, 7.22 µL, 1.5 eq). The mixture was stirred at 30 °C for 12 hr. LC-MS showed one main peak with desired m/z (MW: 2489.82. observed m/z: 1245.1700 ($[M/2+H]^+$)) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent to give a residue. The residue was purified by prep-HPLC (TFA condition). Compound **2** (48 mg, 19.28 µmol, 69.80% yield, 100% purity) was obtained as a white solid.

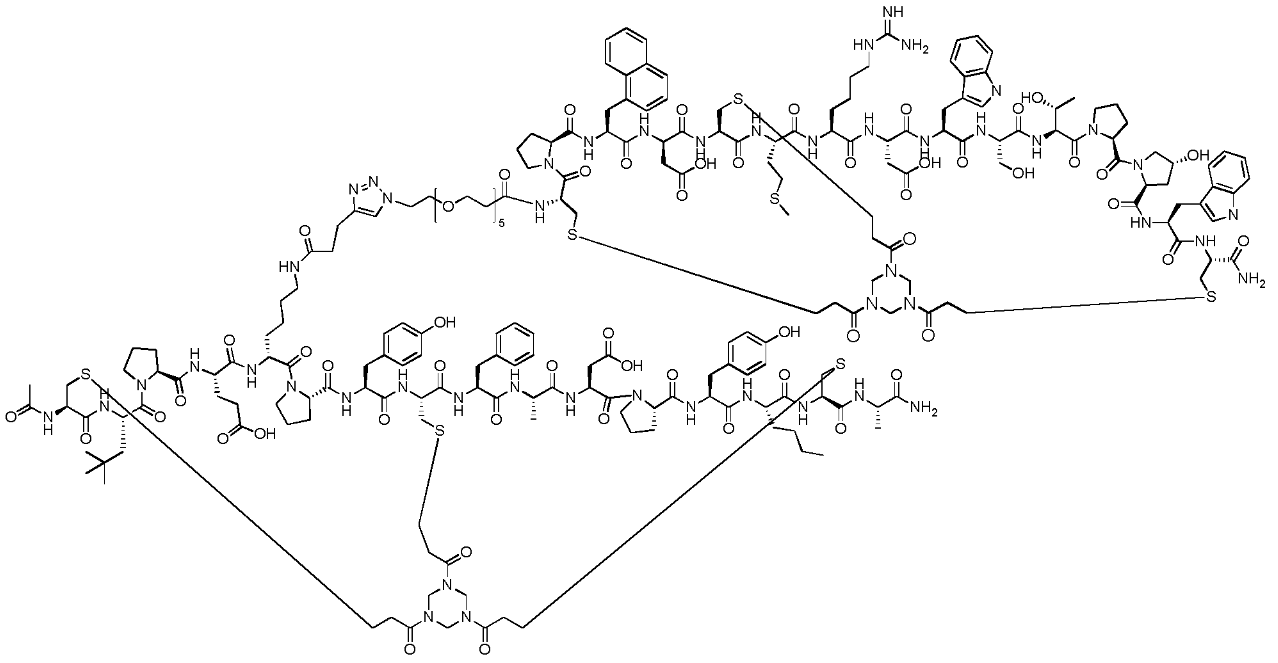
Procedure for preparation of BCY10571

$$\textbf{BCY00008116-PEG5-N}_3 + \textbf{BCY00008927} \xrightarrow[\text{t-BuOH/H}_2\text{O}]{\text{CuSO}_4,\ \text{VcNa},\ \text{THPTA}} \textbf{BCY00010571}$$

2

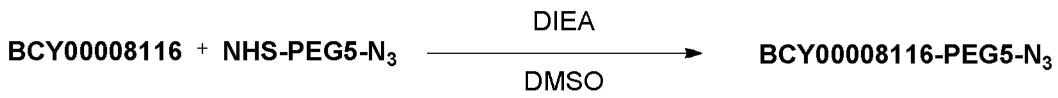
This reaction was performed in two independent containers in parallel. For one container, Compound **2** (10 mg, 4.02 µmol, 1.0 eq) and **BCY8927** (9 mg, 4.17 µmol, 1.04 eq) were first dissolved in 2 mL of t-BuOH/H_2O (1:1), and then $CuSO_4$ (0.4 M, 10.0 µL, 1.0 eq), VcNa (0.4 M, 20.1 µL, 2.0 eq) and THPTA (0.4 M, 10.0 µL, 1 eq) were added. Finally 0.4 M NH_4HCO_3 was added to adjust pH to 8. All solvents here were degassed and purged with N_2 for 3 times. The reaction mixture was stirred at 30 °C for 4 hr under N_2 atmosphere. LC-MS showed compound **3** was consumed completely and one main peak with desired m/z (MW: 4649.36, observed *m/z*: 1162.57 ($[M/4+H]^+$), 1549.69 ($[M/3+H]^+$)) was detected. The residue was purified by prep-HPLC (TFA condition). **BCY10571** (13 mg, 2.79 µmol, 34.88% yield, 96.48% purity) was obtained as a white solid.

<u>**BCY10572**</u>



BCY00010572

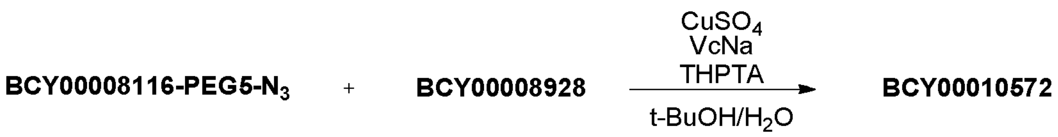
Procedure for preparation of BCY8116-PEG5-N₃



1 **2**

BCY8116 (60 mg, 27.62 μmol, 1.0 eq) and compound **1** (12.0 mg, 27.75 μmol, 1.0 eq) were first dissolved in DMSO (1 mL), then the mixture was added with DIEA (5.4 mg, 41.43 μmol, 7.22 μL, 1.5 eq). The mixture was stirred at 30 °C for 12 hr. LC-MS showed one main peak with desired m/z (MW: 2489.82. observed m/z: 1245.1700 ($[M/2+H]^+$)) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent to give a residue. The residue was purified by prep-HPLC (TFA condition). Compound **2** (48 mg, 19.28 μmol, 69.80% yield, 100% purity) was obtained as a white solid.

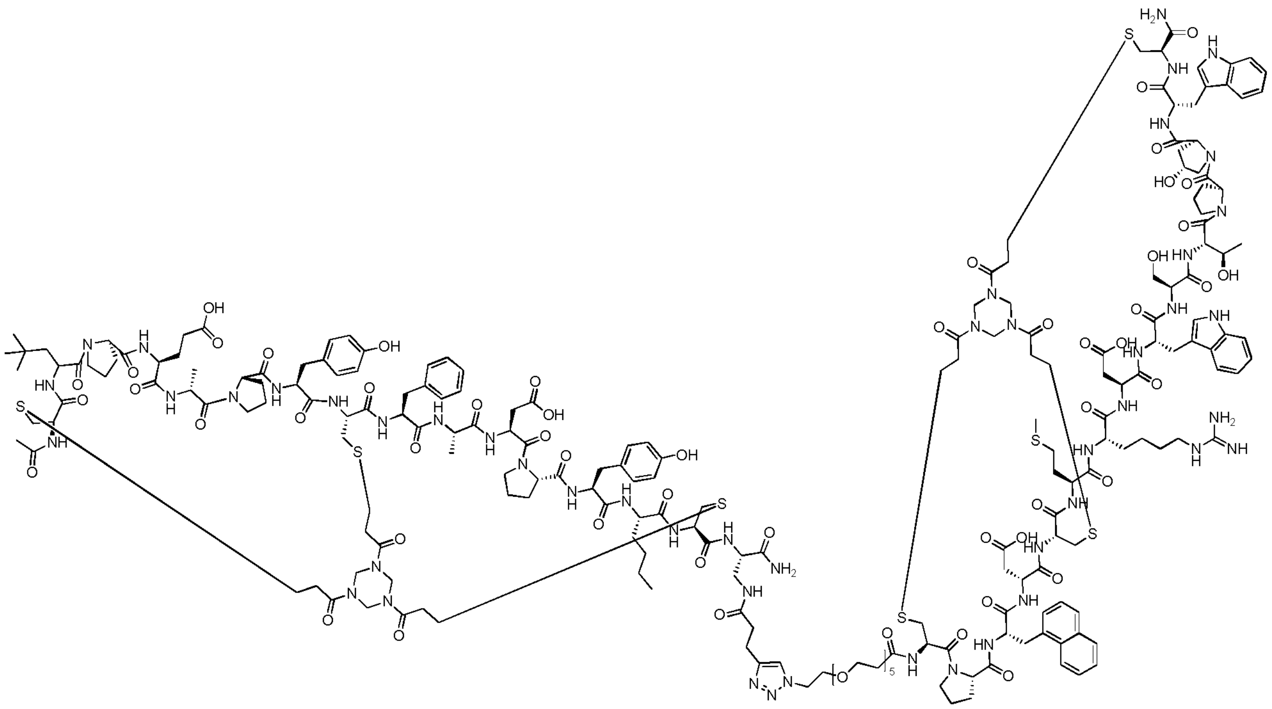
Procedure for preparation of BCY10572



2

This reaction was performed in two independent containers in parallel. For one container, Compound **2** (10 mg, 4.02 μmol, 1.0 eq) and **BCY8928** (9 mg, 4.06 μmol, 1.01 eq) were first dissolved in 2 mL of t-BuOH/H_2O (1:1), and then $CuSO_4$ (0.4 M, 10.1 μL, 1 eq), VcNa (0.4 M, 20.2 μL, 2.0 eq) and THPTA (0.4 M, 10.1 μL, 1.0 eq) was added. Finally 0.4 M NH_4HCO_3 was added to adjust pH to 8. All solvents here were degassed and purged with N_2 for 3 times. The reaction mixture was stirred at 30 °C for 4 hr under N_2 atmosphere. LC-MS showed compound 1 was consumed completely and one main peak with desired m/z (MW: 4707.40. observed m/z: 1568.29 ($[M/3+H]^+$) and 1176.83 ($[M/4+H]^+$)) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent to give a residue. The residue was purified by prep-HPLC (TFA condition). **BCY10572** (21 mg, 4.46 µmol, 55.7% yield, 97.51% purity) was obtained as a white solid.

__BCY10573__



BCY00010573

Procedure for preparation of Compound 2

BCY00008116 + **NHS-PEG5-N_3** —DIEA / DMSO→ **BCY00008116-PEG5-N_3**

1 **2**

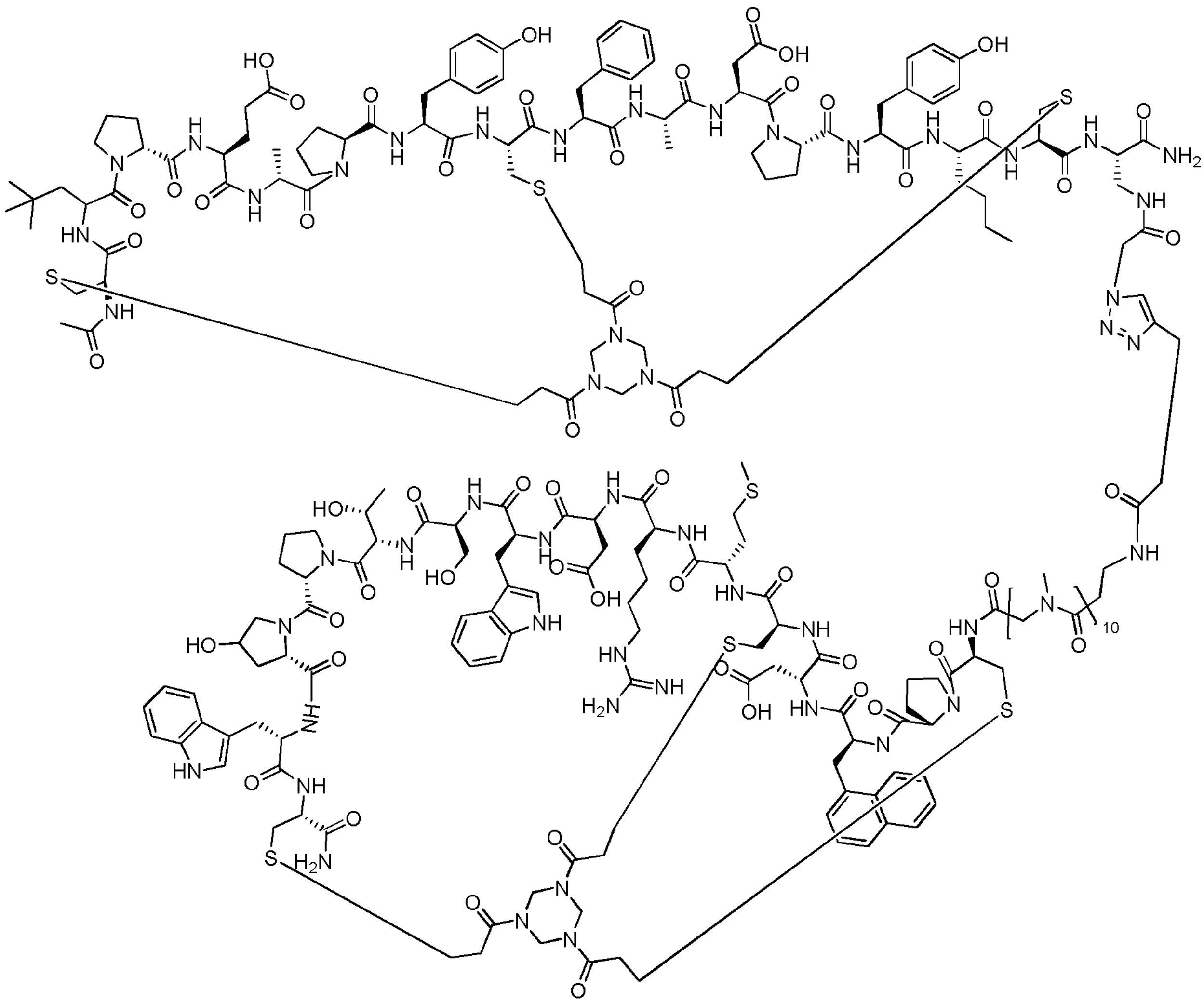
To a solution of **BCY8116** (35 mg, 16.11 µmol, 1 *eq*), Compound **1** (7.00 mg, 16.19 µmol, 1 *eq*) in DMSO (1 mL) was added DIEA (3.12 mg, 24.17 µmol, 4.21 µL, 1.5 *eq*). The mixture was stirred at 25-30 °C for 2 hr. LC-MS showed majority of **BCY8116** was consumed and one main peak with desired m/z (calculated MW: 2489.82, observed *m/z*: 1245.37 ($[M/2+H]^+$) and 830.25($[M/3+H]^+$)) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent to give a residue. The residue was purified by prep-HPLC (TFA condition). Compound **2** (26.8 mg, 10.76 µmol, 66.81% yield, 100% purity) was obtained as a white solid.

Procedure for preparation of BCY10573

BCY00008116-PEG5-N_3 (**2**) + **BCY00011014** $\xrightarrow[\text{t-BuOH/}H_2O]{CuSO_4\ \text{VcNa}\ \text{THPTA}}$ **BCY00010573**

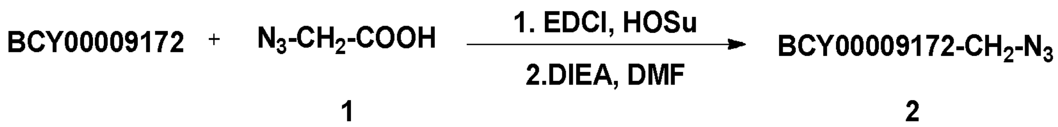
A mixture of Compound **2** (15 mg, 6.02 µmol, 1.0 *eq*), **BCY11014** (13.50 mg, 6.21 µmol, 1.03 *eq*), and THPTA (0.4 M, 15.1 µL, 1.0 *eq*) was dissolved in t-BuOH/H_2O (1:1, 2 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 15.1 µL, 1.0 *eq*) and VcNa (0.4 M, 30.2 µL, 2.0 *eq*) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 25-30 °C for 12 hr under N_2 atmosphere. LC-MS showed Compound **2** was consumed completely and one main peak with desired m/z [MW: 4665.32, observed *m/z*: 1167.50 ([M/4+H^+])] was detected. The reaction mixture was directly purified by prep-HPLC (**TFA condition)**. **BCY10573** (11.5 mg, 2.42 µmol, 40.14% yield, 98.11% purity) was obtained as a white solid.

<u>**BCY10578**</u>



BCY00010578

Procedure for preparation of Compound 2



Compound **1** (5.0 mg, 49.5 μmol, 1.0 eq) was first activated by mixing with EDCI (8.5 mg, 54.8 μmol, 1.1 eq) and HOSu (5.7 mg, 49.5 μmol, 1.0 eq)**.** The mixture was stirred at 25-30 °C for 30 min. TLC indicated compound **1** was consumed completely and **one new spot** formed. Then compound **BCY9172** (80.0 mg, 38.18 μmol, 0.8 eq.) and DIEA (6.3 mg, 8.5 μL, 49.5 μmol, 1.0 eq.) were added to this mixture, and stirred at 40 °C for 1 hr, till LC-MS showed one main peak with desired m/z (calculated MW:2178.46, observed *m/z*: 1089.44 ($[M/2+H]^+$) was detected. The reaction mixture was then concentrated under reduced pressure to remove solvent and produced a residue, following by purification by prep-HPLC

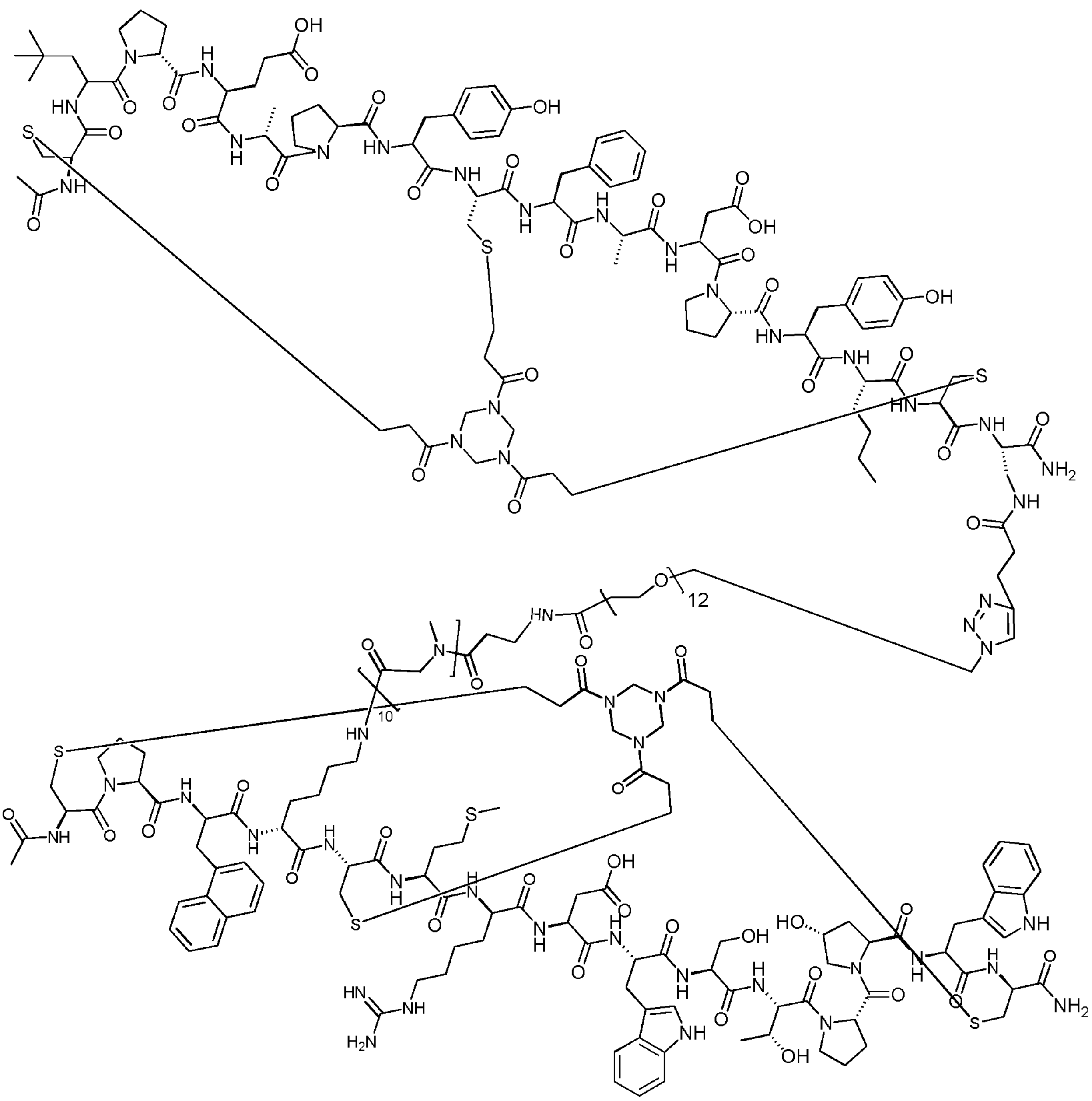
(TFA condition). Compound **2** (15 mg, 6.88 μmol, 18.66% yield, 73.3% purity) was obtained as a white solid.

Procedure for preparation of BCY10578

BCY00009172-CH2-N3 (**2**) + BCY00008846 —($CuSO_4$, VcNa, THPTA / DMF)→ BCY00010578

A mixture of compound **2** (9.8 mg, 4.5 μmol, 1.0 eq.), **BCY8846** (14.0 mg, 4.6 μmol, 1.0 eq.), and THPTA (2.0 mg, 4.6 μmol 1.0 eq.) was dissolved in t-BuOH/H_2O (1:1, 1 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 12 μL, 1.0 eq.) and VcNa (0.4 M, 24 μL, 2.0 eq.) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 40 °C for 2 hr under N_2 atmosphere. LC-MS showed compound **2** was consumed completely and one main peak with desired m/z (calculated MW: 5212.88, observed *m/z*: 1304.2 ($[M/4+H]^+$)) was detected. The reaction mixture was filtered and concentrated under reduced pressure to give a residue. The crude product was purified by prep-HPLC (TFA condition), and **BCY10578** (13.78 mg, 2.64 μmol, 58.66% yield, 96.23% purity) was obtained as a white solid.

<u>**BCY10917**</u>



Procedure for preparation of BCY8831-PEG12-N$_3$

BCY00008831 + **NHS-PEG12-N$_3$** $\xrightarrow[\text{DMF}]{\text{DIEA}}$ **BCY00008831-PEG12-N$_3$**

1 **2**

BCY8831 (40.0 mg, 13.29 µmol, 1.0 eq) and compound **1** (10.5 mg, 14.17 µmol, 1.07 eq) were dissolved in DMF (1 mL). The solution was added with DIPEA (2.6 mg, 20.09 µmol, 3.5 µl, 1.5 eq), and then the mixture was stirred at 30°C for 16 hr. LC-MS showed **BCY8831** was consumed completely and one main peak with desired m/z (calculated MW: 3635.16 observed *m/z*: 1212.0([M/3+H]$^+$)) was detected. The reaction mixture was purified by prep-HPLC (TFA condition) and compound **2** (22.0 mg, 5.83 µmol, 43.85% yield, 96.39% purity) was obtained as a white solid.

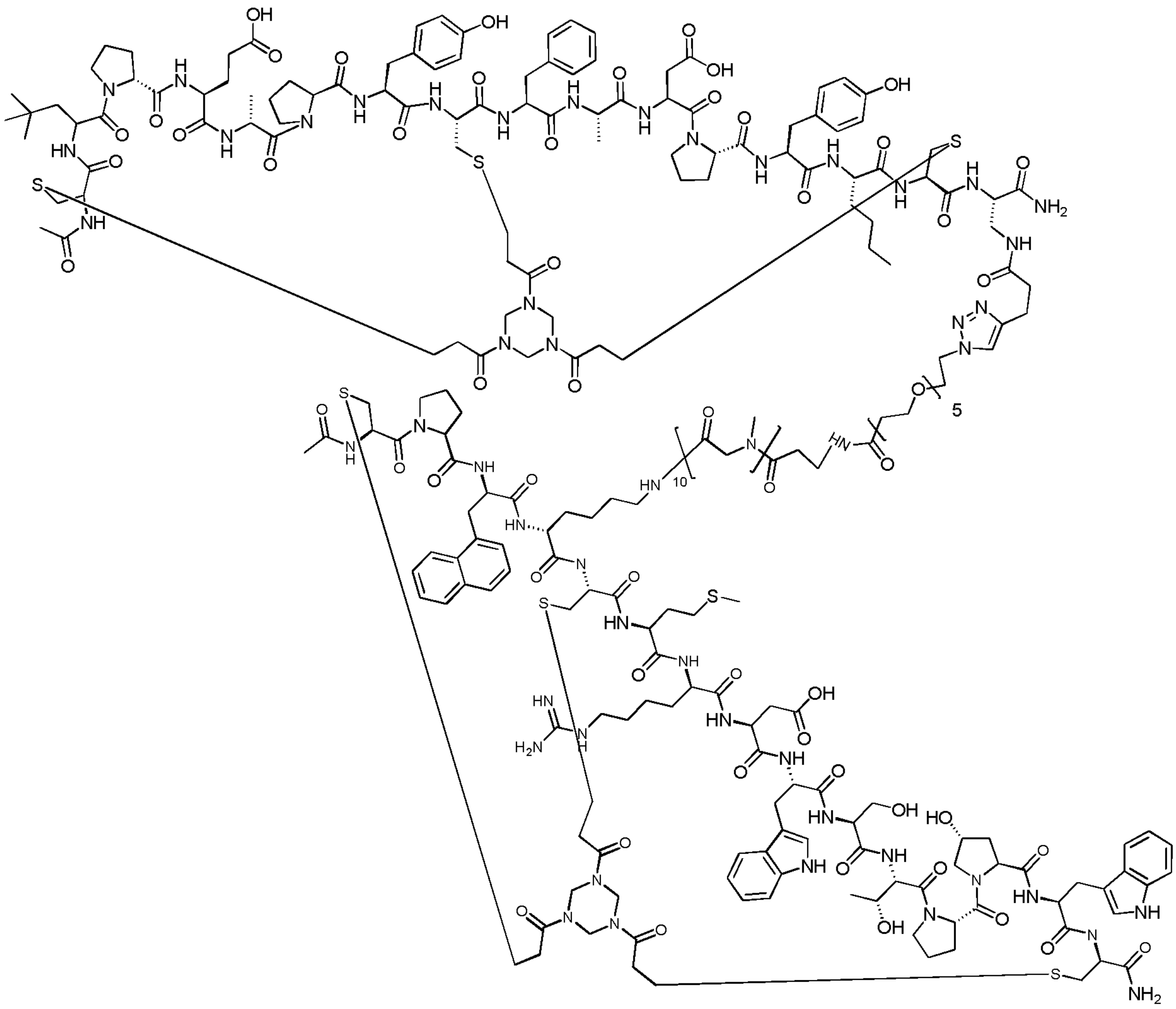
Procedure for preparation of BCY10917

$$\text{BCY00008831-PEG12-N}_3 \; (\mathbf{2}) + \text{BCY00011014} \xrightarrow[\text{t-BuOH/H}_2\text{O}]{\text{CuSO}_4,\ \text{VcNa},\ \text{THPTA}} \text{BCY00010917}$$

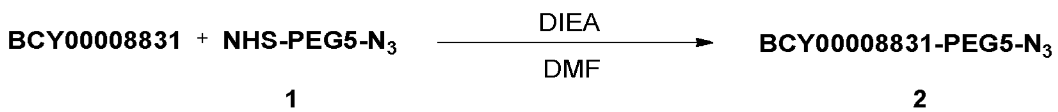
Note: Two batches were made, and the first one was written for final report.

Compound **2** (10.0 mg, 2.75 μmol, 1.0 eq) and **BCY11014** (5.98 mg, 2.75 μmol, 1.0 eq) , were first dissolved in 2 mL of t-BuOH/H_2O (1:1), and then $CuSO_4$ (0.4 M, 13.7 μL, 2.0 eq), VcNa（1.1 mg, 5.55 μmol, 2.0 eq） and THPTA (1.2 mg, 2.76 μmol, 1.0 eq) was added. Finally 1 M NH_4HCO_3 was added to adjust pH to 8. All solvents here were degassed and purged with N_2 for 3 times. The reaction mixture was stirred at 30°C for 16 hr under N_2 atmosphere. LC-MS showed compound 2 was consumed completely and one main peak with desired m/z (calculated MW: 5810.66 observed *m/z*: 1163.0($[M/5+H]^+$)). The reaction mixture was purified by prep-HPLC (TFA condition) and **BCY10917** (6.4 mg, 1.07 μmol, 39.03% yield, 97.49% purity) was obtained as a white solid.

<u>**BCY11020**</u>



Procedure for preparation of BCY8831-PEG5-N₃



BCY8831 (25.0 mg, 8.31 μmol, 1.0 eq) and compound **1** (3.9 mg, 9.02 μmol, 1.09 eq), were dissolved in DMF (1 mL). The solution was added with DIPEA (1.6 mg, 12.46 μmol, 2.2 μl, 1.5 eq), and then the mixture was stirred at 35°C for 2 hr. LC-MS showed **BCY8831** was consumed completely and one main peak with desired m/z (calculated MW: 3326.79 observed *m/z*: 1109.66($[M/3+H]^+$)) was detected. The reaction mixture was purified by prep-HPLC (TFA condition) and compound 2 (7.3 mg, 2.09 μmol, 25.20% yield, 95.41% purity) was obtained as a white solid.

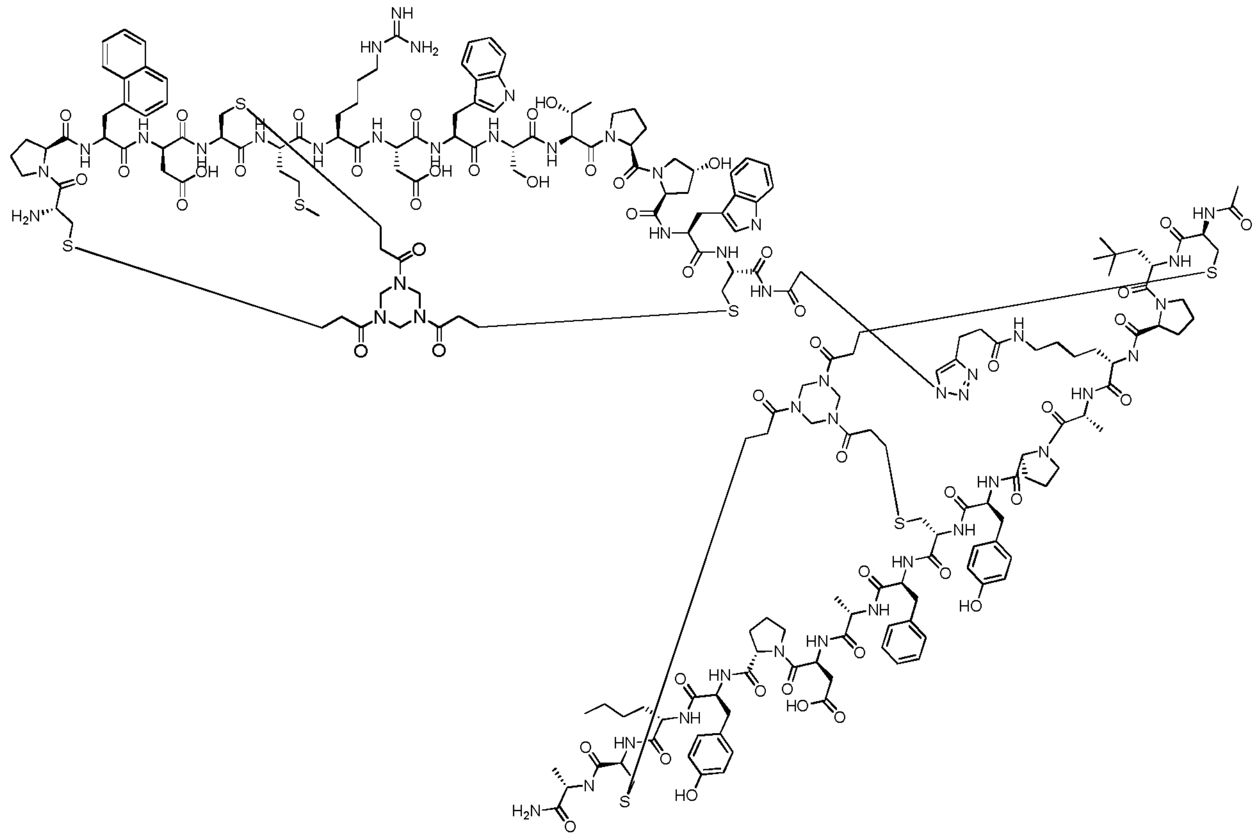
Procedure for preparation of BCY11020

BCY00008831-PEG5-N_3 + **BCY00011014** $\xrightarrow[\text{t-BuOH/}H_2O]{CuSO_4\text{, VcNa, THPTA}}$ **BCY00011020**

2

Compound **2** (7.3 mg, 2.19 µmol, 1.0 eq) and **BCY11014** (4.8 mg, 2.19 µmol, 1.0 eq) , were first dissolved in 2 mL of t-BuOH/H_2O (1:1), and then $CuSO_4$ (0.4 M, 5.5 µL, 1.0 eq), VcNa (1.0 mg, 5.05 µmol, 2.3 eq) and THPTA (1.0 mg, 2.30 µmol, 1.0 eq) was added. Finally 1 M NH_4HCO_3 was added to adjust pH to 8. All solvents here were degassed and purged with N_2 for 3 times. The reaction mixture was stirred at 30°C for 12 hr under N_2 atmosphere. LC-MS showed compound **2** was consumed completely and one main peak with desired m/z (calculated MW: 5502.29, observed *m/z*: 1101.74([M/5+H]$^+$)) was detected. The reaction mixture was purified by prep-HPLC (TFA condition) and **BCY11020** (3.3 mg, 0.577 µmol, 26.30% yield, 96.24% purity) was obtained as a white solid.

<u>**BCY11373**</u>



BCY00011373

Procedure for preparation of Compound 2

BCY00008116 + N_3-CH_2-COOH (**1**) —(1. EDCI, HOSu; 2.DIEA, DMF)→ BCY00008116-CH_2-N_3 (**2**)

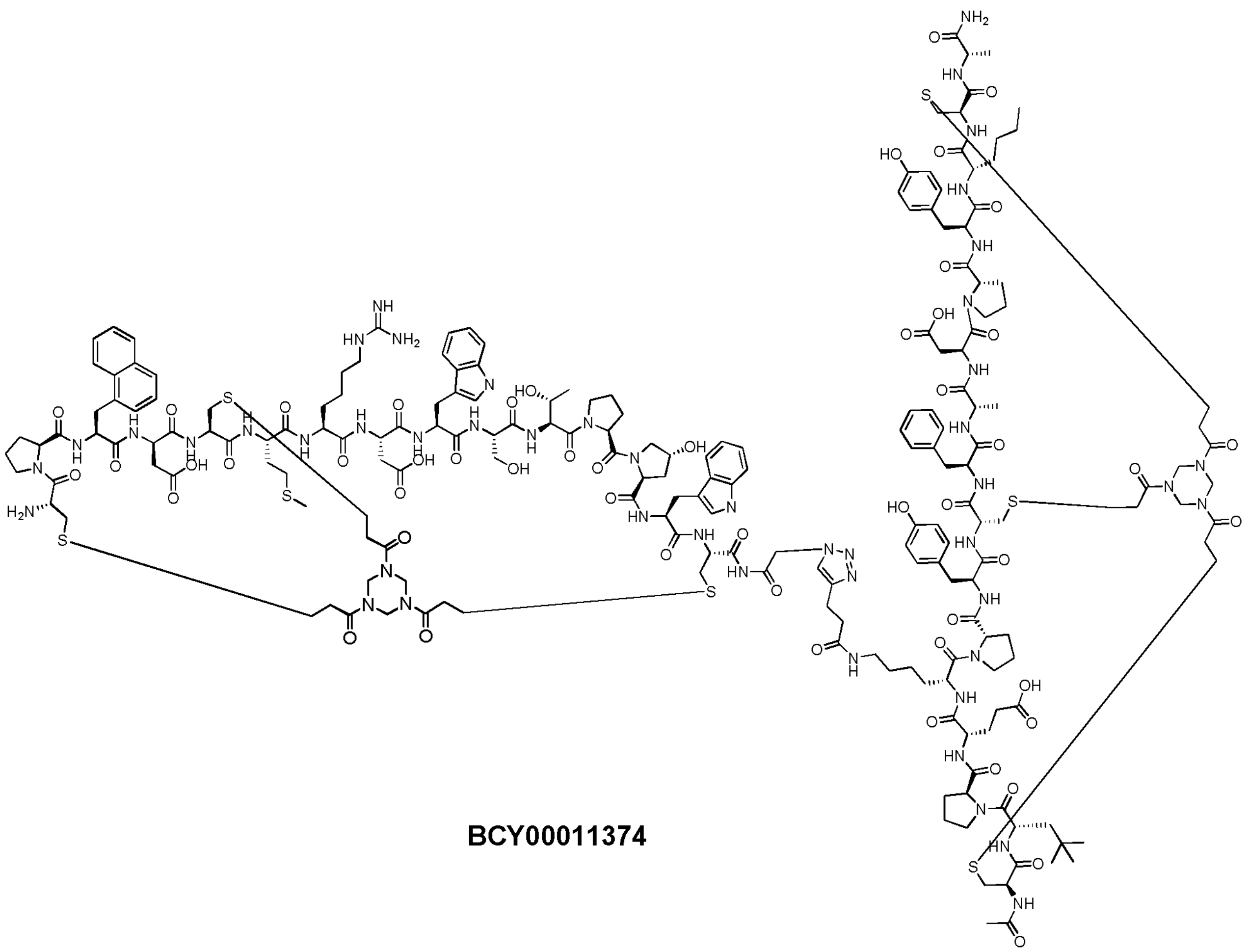
To a solution of compound **1** (5.0 mg, 49.5 µmol, 1.0 eq) in DMF (1 mL) was added EDCI (8.5 mg, 54.8 µmol, 1.1 eq) and HOSu (5.7 mg, 49.5 µmol, 1.0 eq)**.** The mixture was stirred at 25-30 °C for 30 min. TLC indicated compound **1** was consumed completely and **one new spot** formed. Then 0.3 mL of this mixture was added with **BCY8116** (30.0 mg, 13.81 µmol, 0.28 eq.) and DIEA (2.4 µL, 13.81 µmol, 0.28 eq.), and stirred at 25-30 °C for 2 hr. LC-MS showed **BCY8116** was consumed completely and one main peak with desired m/z (calculated MW:2255.53, observed *m/z*: 1128.34($[M/2+H]^+$) was detected. The reaction mixture was then concentrated under reduced pressure to remove solvent and produced a residue, following by purification by prep-HPLC (TFA condition). Compound **2** (21 mg, 8.9 µmol, 64.43% yield, 95.56% purity) was obtained as a white solid.

Procedure for preparation of BCY11373

BCY00008116-CH_2-N_3 (**2**) + BCY00008927 —($CuSO_4$, VcNa, THPTA; t-BuOH/H_2O)→ BCY00011373

A mixture of compound **2** (5 mg, 2.22 µmol, 1.0 eq.), **BCY8928** (4.79 mg, 2.22 µmol, 1.0 eq.), and THPTA (1.0 mg, 2.30 µmol, 1.0 eq.) was dissolved in t-BuOH/H_2O (1:1, 1 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 5.6 µL, 1.0 eq.) and VcNa (0.4 M, 5.6 µL, 1.0 eq.) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 40 °C for 2 hr under N_2 atmosphere. LC-MS showed compound **2** was consumed completely and one main peak with desired m/z (calculated MW: 4415.07, observed *m/z*: 1471.5($[M/3+H]^+$ and 1103.8($[M/4+H]^+$) was detected. The reaction mixture was filtered and concentrated under reduced pressure to give a residue. The crude product was purified by prep-HPLC (TFA condition), and **BCY11373** (4.9 mg, 1.03 µmol, 46.26% yield, 92.4% purity) was obtained as a white solid.

<u>**BCY11374**</u>



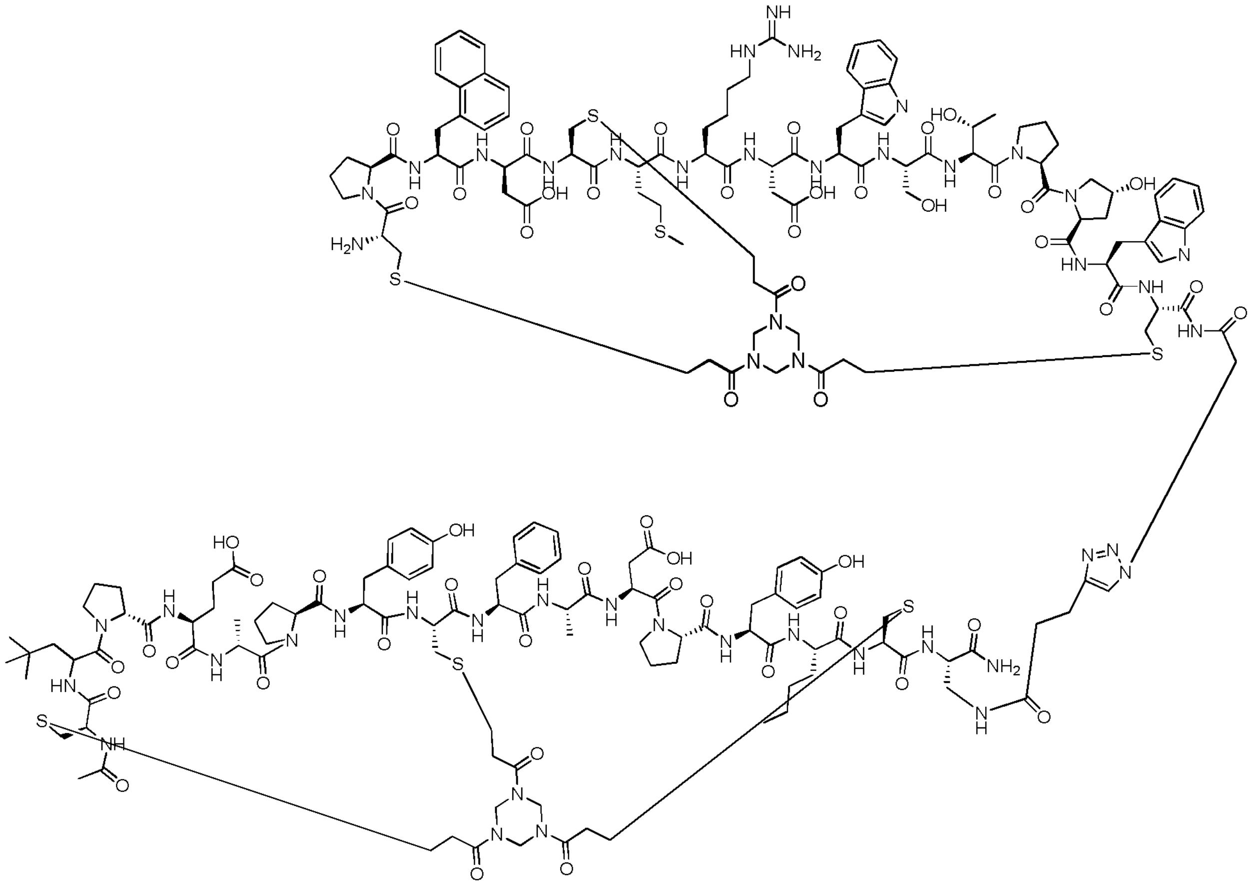
BCY00011374

Procedure for preparation of BCY11374

BCY00008116-CH2-N_3 (2) + **BCY00008928** → ($CuSO_4$, VcNa, THPTA; t-BuOH/H_2O) → **BCY00011374**

A mixture of compound **2** (which may be prepared as described in the procedure for preparing BCY11373; 5 mg, 2.22 μmol, 1.0 eq.), **BCY8928** (4.9 mg, 2.22 μmol, 1.0 eq.), and THPTA (1.0 mg, 2.30 μmol, 1.0 eq.) was dissolved in t-BuOH/H_2O (1:1, 1 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 5.6 μL, 1.0 eq.) and VcNa (0.4 M, 5.6 μL, 1.0 eq.) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 40 °C for 2 hr under N_2 atmosphere. LC-MS showed compound **2** was consumed completely and one main peak with desired m/z (calculated MW: 4473.11, observed *m/z*: 1491.5($[M/3+H]^+$ and 1118.5($[M/4+H]^+$) was detected. The reaction mixture was filtered and concentrated under reduced pressure to give a residue. The crude product was purified by prep-HPLC (TFA condition), and **BCY11374** (4.1 mg, 1.27 μmol, 38.04% yield, 92.0% purity) was obtained as a white solid.

BCY11375



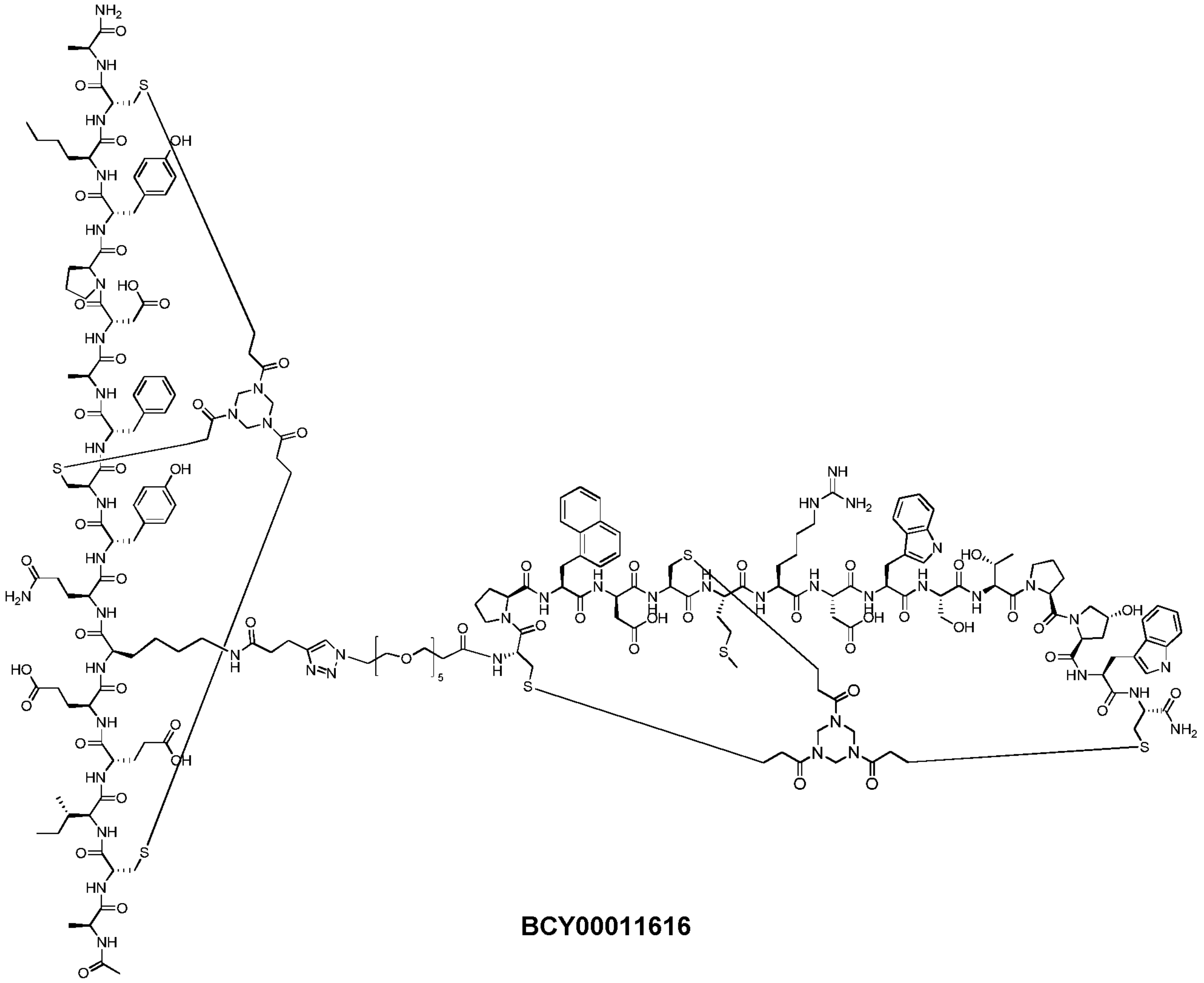
BCY00011375

Procedure for preparation of BCY11375

BCY00008116-CH_2-N_3 (2) + **BCY00011014** —($CuSO_4$, VcNa, THPTA / DMF)→ **BCY00011375**

A mixture of compound 2 (which may be prepared as described in BCY11373; 5 mg, 2.22 μmol, 1.0 eq.), **BCY11014** (4.8 mg, 2.22 μmol, 1.0 eq.), and THPTA (0.5 mg, 2.30μmol, 1.0 eq.) was dissolved in t-BuOH/H_2O (1:1, 1 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 5.6 μL, 1.0 eq.) and VcNa (0.4 M, 5.6 μL, 1.0 eq.) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 40 °C for 2 hr under N_2 atmosphere. LC-MS detected some desired m/z (calculated MW: 4431.03, observed *m/z*: 1107.59($[M/4+H]^+$ and 1477.90($[M/3+H]^+$). The reaction mixture was filtered and concentrated under reduced pressure to give a residue. The crude product was purified by prep-HPLC (TFA condition), and **BCY11375** (6 mg, 1.31 μmol, 59.13% yield, 96.8% purity) was obtained as a white solid.

<u>**BCY11616**</u>



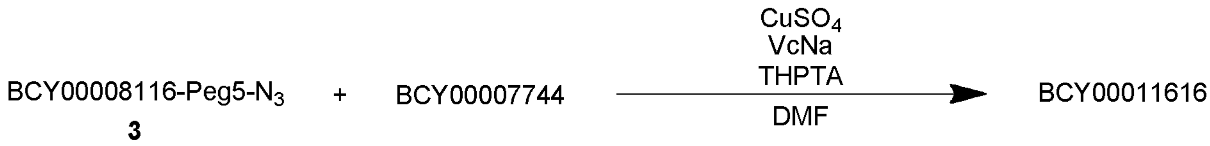
BCY00011616

Procedure for preparation of Compound 3

BCY00008116	+	NHS-Peg5-N_3	$\xrightarrow[\text{DMF}]{\text{DIEA}}$	BCY00008116-Peg5-N_3
1		**2**		**3**

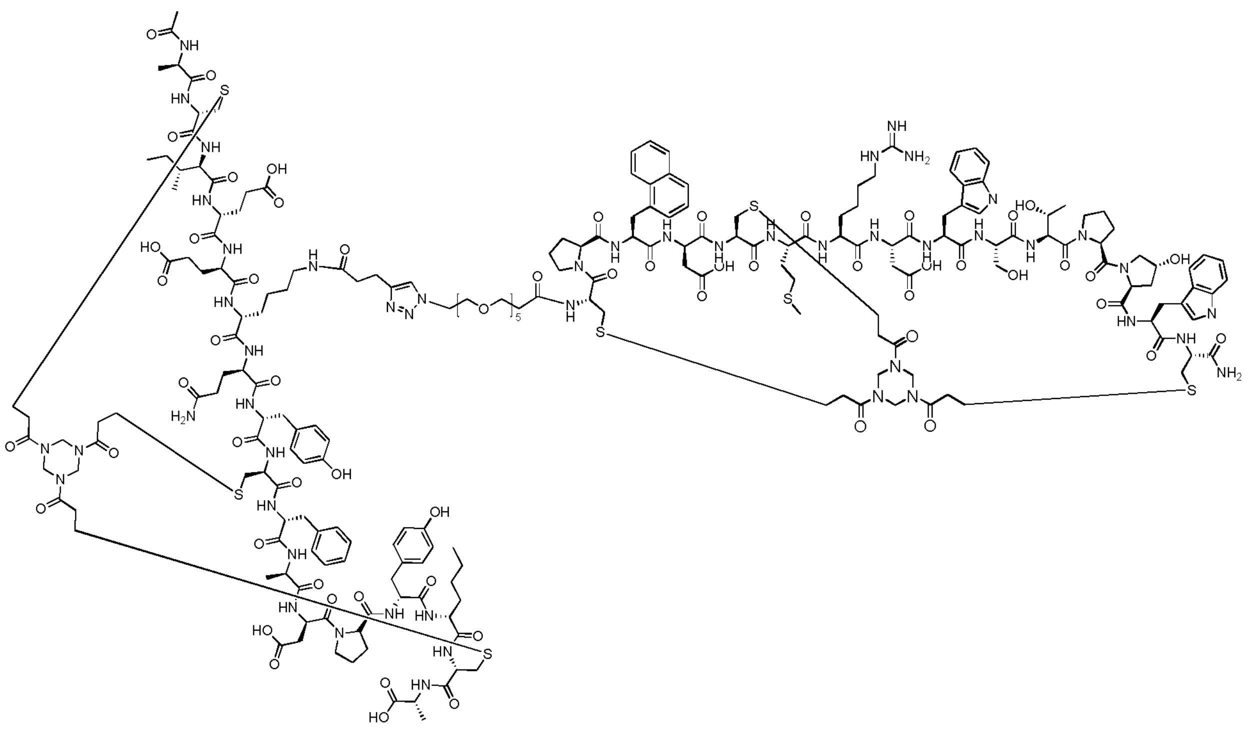
A mixture of compound **BCY8116** (30.0 mg, 13.81 μmol, 1.0 eq.), compound **2** (6.0 mg, 13.88 μmol, 1.0 eq.) and DIEA (2.4 μL, 13.82 μmol, 1.0 eq.) was dissolved in DMF. The reaction mixture was stirred at 40 °C for 1 hr, till LC-MS showed compound **1** was consumed completely and one main peak with desired m/z (calculated MW:2489.82, observed *m/z*: 1245.4 ($[M/2+H]^+$) was detected. The reaction mixture was then concentrated under reduced pressure to remove solvent and produced a residue, following by purification by prep-HPLC (TFA condition). Compound **3** (27 mg, 10.29 μmol, 74.52% yield, 94.9% purity) was obtained as a white solid.

Procedure for preparation of BCY11616



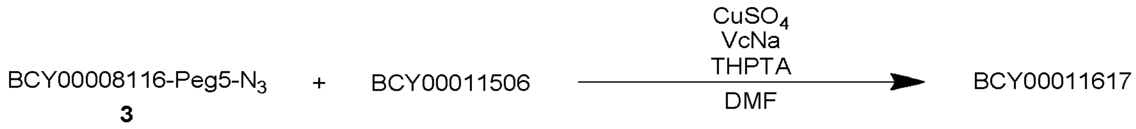
A mixture of compound **3** (5 mg, 2.01 µmol, 1.0 eq.), **BCY7744** (5.2 mg, 2.21 µmol, 1.1 eq.), and THPTA (1.0 mg, 2.30 µmol, 1.0 eq.) was dissolved in t-BuOH/H_2O (1:1, 1 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 5.0 µL, 1.0 eq.) and VcNa (0.4 M, 5.0 µL, 1.0 eq.) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 40 °C for 2 hr under N_2 atmosphere. LC-MS showed compound **3** was consumed completely and one main peak with desired m/z (calculated MW: 4827.46, observed *m/z*: 1207.12 ($[M/4+H]^+$) was detected. The reaction mixture was filtered and concentrated under reduced pressure to give a residue. The crude product was purified by prep-HPLC (TFA condition), and **BCY11616** (4.7 mg, 1.0 µmol, 48.48% yield, 94.7% purity) was obtained as a white solid.

<u>**BCY11617**</u>



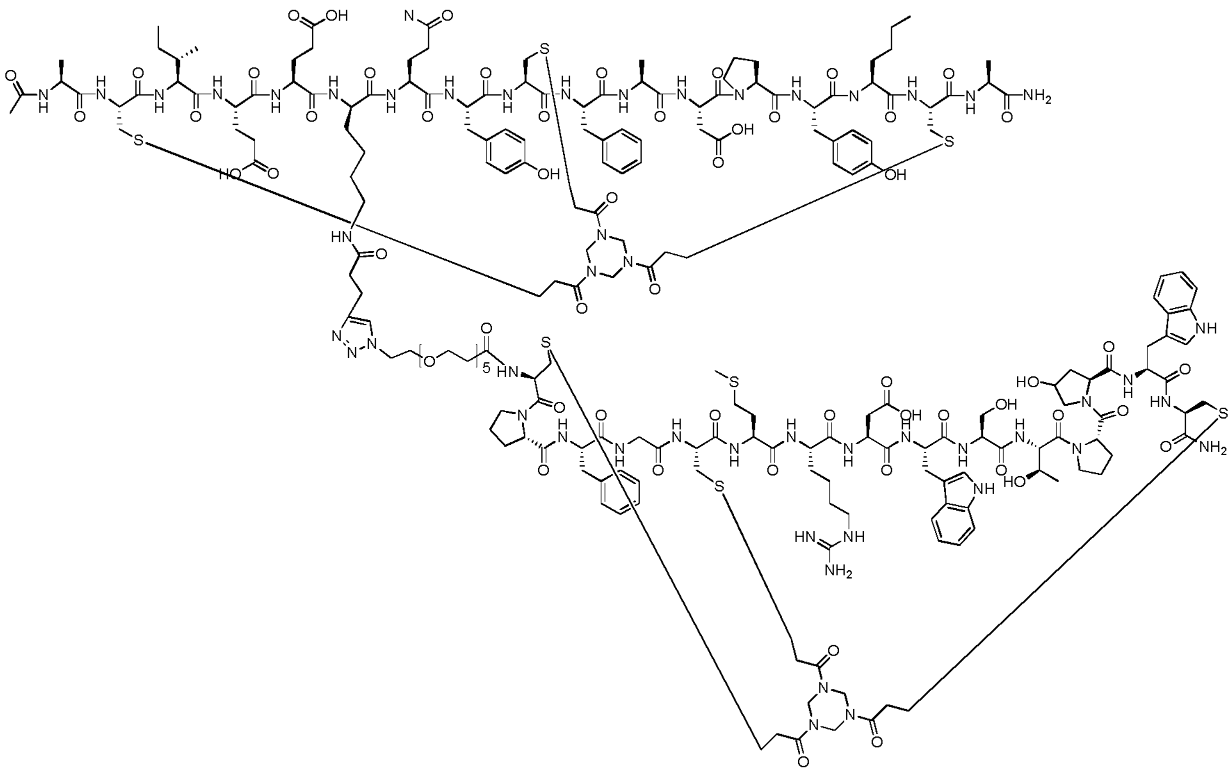
BCY00011617

Procedure for preparation of BCY11617



A mixture of compound **3** (which may be prepared as described in the procedure for preparing BCY11616; 5 mg, 2.01 μmol, 1.0 eq.), **BCY11506** (5.2 mg, 2.21 μmol, 1.1 eq.), and THPTA (1.0 mg, 2.30 μmol, 1.1 eq.) was dissolved in t-BuOH/H_2O (1:1, 1 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 5.0 μL, 1.0 eq.) and VcNa (0.4 M, 5.0 μL, 1.0 eq.) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 40 °C for 2 hr under N_2 atmosphere. LC-MS showed compound **3** was consumed completely and one main peak with desired m/z (calculated MW: 4828.45, observed *m/z*: 1206.97 ($[M/4+H]^+$) and 965.91 ($[M/5+H]^+$)) was detected. The reaction mixture was filtered and concentrated under reduced pressure to give a residue. The crude product was purified by prep-HPLC (TFA condition), and **BCY11617** (3.2 mg, 0.63 μmol, 31.37% yield, 95.05% purity) was obtained as a white solid.

BCY11857



BCY00011857

Procedure for preparation of BCY11414-PEG5-N₃

$$\textbf{BCY00011414} + \textbf{NHS-PEG5-}\mathbf{N_3} \xrightarrow[\text{MeCN}/H_2O]{NaHCO_3} \textbf{BCY00011414-PEG5-}\mathbf{N_3}$$

1 **2**

BCY11414 (60.0 mg, 29.06 µmol, 1.0 eq) and compound **1** (13.0 mg, 30.06 µmol, 1.03 eq) were dissolved in 2 mL of MeCN/H_2O (1:1). Adjust pH to 8 with $NaHCO_3$ (0.4 M), and then the mixture was stirred at 25-30°C for 2 hr. LC-MS showed one main peak with desired m/z (calculated MW: 2381.72, observed *m/z*: 1191.07($[M/2+H]^+$)) was detected. The reaction mixture was purified by prep-HPLC (TFA condition) and compound **2** (38.0 mg, 15.9 µmol, 54.71% yield, 97.35% purity) was obtained as a white solid.

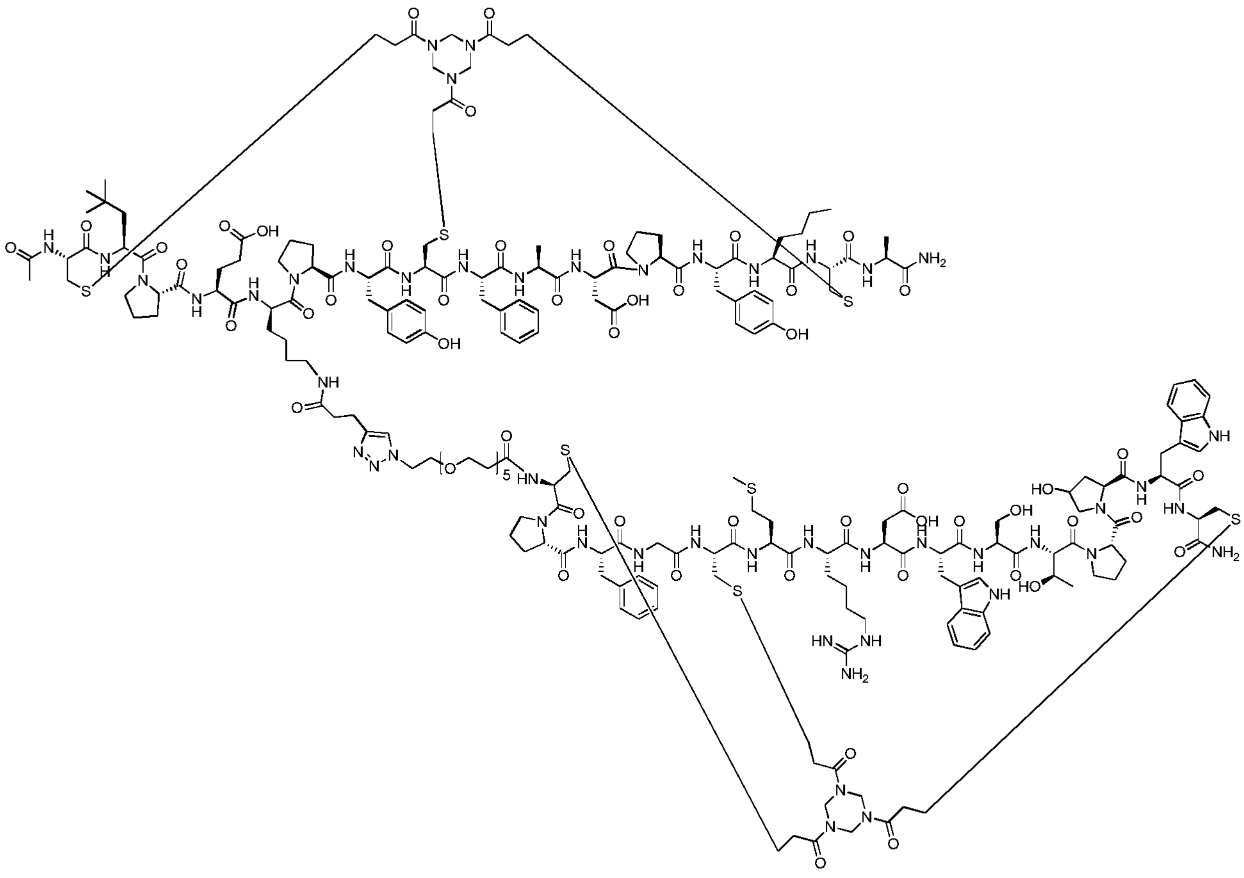
Procedure for preparation of BCY11857

$$\textbf{BCY00011414-PEG5-}\mathbf{N_3} + \textbf{BCY00007744} \xrightarrow[\text{t-BuOH}/H_2O]{CuSO_4,\ \text{VcNa},\ \text{THPTA}} \textbf{BCY00011857}$$

2

Compound **2** (10.0 mg, 4.20 µmol, 1.0 eq) and **BCY7744** (11.5 mg, 4.92 µmol, 1.2 eq) were first dissolved in 2 mL of t-BuOH/H_2O (1:1), and then $CuSO_4$ (0.4 M, 11.0 µL, 1.0 eq), VcNa (2.0 mg, 10 µmol, 2.4 eq) and THPTA (2.0 mg, 4.6 µmol, 1.1 eq) were added. Finally 0.2 M NH_4HCO_3 was added to adjust pH to 8. All solvents here were degassed and purged with N_2 for 3 times. The reaction mixture was stirred at 30°C for 16 hr under N_2 atmosphere. LC-MS showed compound **2** was consumed completely and one main peak with desired m/z (calculated MW: 4719.37, observed *m/z*: 1180.24($[M/4+H]^+$)). The reaction mixture was purified by prep-HPLC (TFA condition) and **BCY11857** (10.3 mg, 2.18 µmol, 51.90% yield, 96.02% purity) was obtained as a white solid.

BCY11858



BCY00011858

Procedure for preparation of BCY11414-PEG5-N$_3$

BCY00011414 + **NHS-PEG5-N$_3$** $\xrightarrow[MeCN/H_2O]{NaHCO_3}$ **BCY00011414-PEG5-N$_3$**

1 **2**

BCY11414 (60.0 mg, 29.06 µmol, 1.0 eq) and compound **1** (13.0 mg, 30.06 µmol, 1.03 eq), were dissolved in 2 mL of MeCN/H_2O (1:1). Adjust pH to 8 with $NaHCO_3$(0.4 M), and then the mixture was stirred at 25-30°C for 2 hr. LC-MS showed one main peak with desired m/z (calculated MW: 2381.72, observed *m/z*: 1191.07($[M/2+H]^+$)) was detected. The reaction mixture was purified by prep-HPLC (TFA condition) and compound **2** (38.0 mg, 15.9 µmol, 54.71% yield, 97.35% purity) was obtained as a white solid.

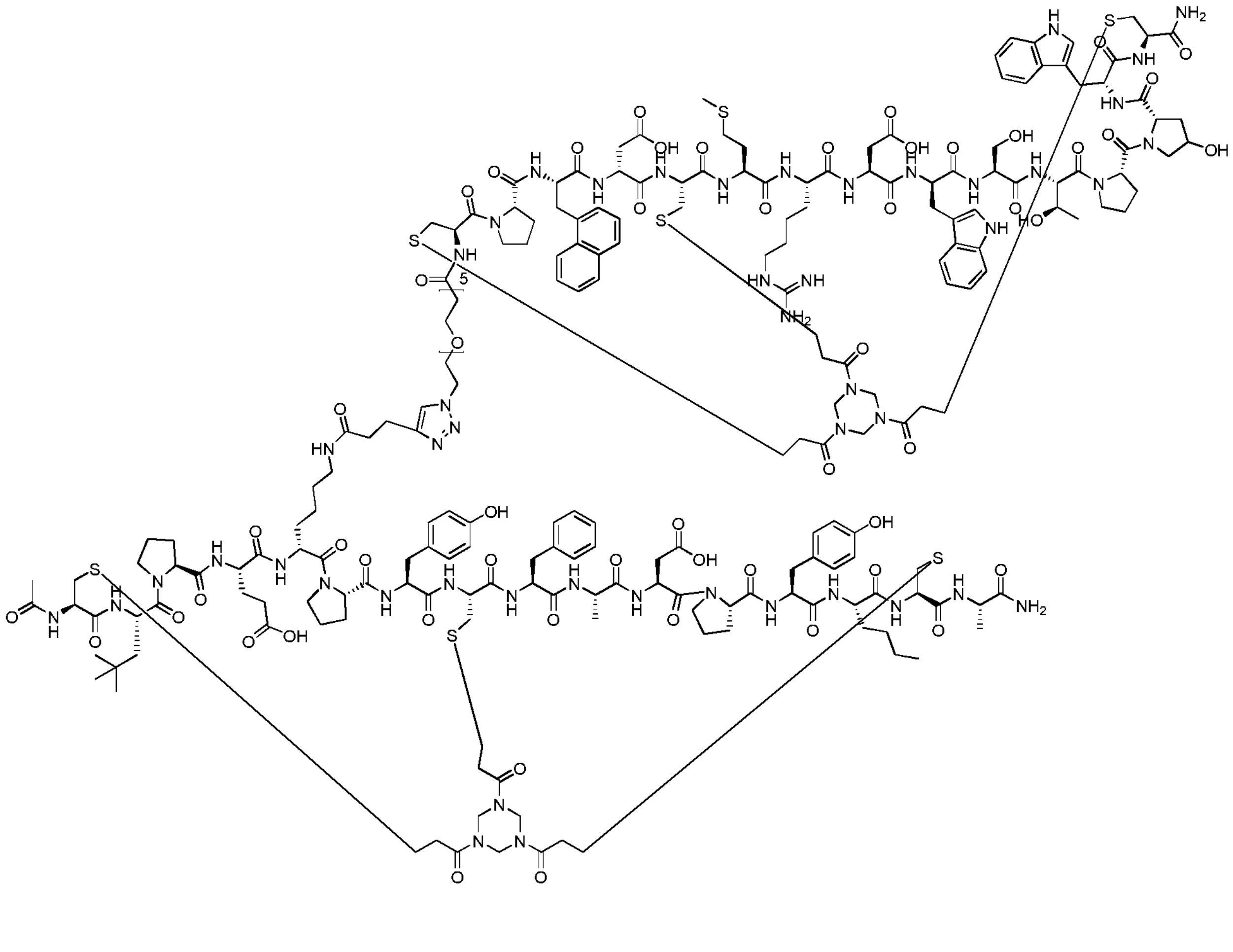
Procedure for preparation of BCY11858

BCY00011414-PEG5-N$_3$ + **BCY00008928** $\xrightarrow[t\text{-}BuOH/H_2O]{CuSO_4,\ VcNa,\ THPTA}$ **BCY00011858**

2

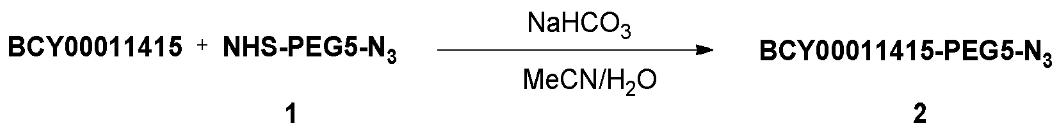
Compound **2** (20.0 mg, 8.40 µmol, 1.0 eq) and **BCY8928** (22.0 mg, 9.92 µmol, 1.1 eq) were first dissolved in 2 mL of t-BuOH/H_2O (1:1), and then $CuSO_4$ (0.4 M, 21.0 µL, 1.0 eq), VcNa (4.0 mg, 20.19 μmol, 2.4 eq) and THPTA (4.0 mg, 9.20 μmol, 1.1 eq) were added. Finally 0.4 M NH_4HCO_3 was added to adjust pH to 8. All solvents here were degassed and purged with N_2 for 3 times. The reaction mixture was stirred at 30°C for 16 hr under N_2 atmosphere. LC-MS showed compound 2 was consumed completely and one main peak with desired m/z (calculated MW: 4599.30, observed *m/z*: 920.38($[M/5+H]^+$), 1150.79 ($[M/4+H]^+$),1533.35($[M/3+H]^+$)). The reaction mixture was purified by prep-HPLC (TFA condition) and **BCY11858** (16.9 mg, 3.67 μmol, 43.43% yield, 99.25% purity) was obtained as a white solid.

BCY11859



BCY00011859

Procedure for preparation of BCY11415-PEG5-N₃



BCY11415 (30.0 mg, 13.81 µmol, 1.0 eq) and compound **1** (6.0 mg, 30.06 µmol, 1.0 eq), were dissolved in 2 mL of $MeCN/H_2O$ (1:1). Adjust pH to 8 with $NaHCO_3$ (0.4 M), and then the mixture was stirred at 25-30°C for 2 hr. LC-MS showed one main peak with desired m/z (calculated MW: 2489.82, observed *m/z*: 1245.18($[M/2+H]^+$)) was detected. The reaction mixture was purified by prep-HPLC (TFA condition) and compound 2 (24.0 mg, 9.63 µmol, 69.7% yield, 99.28% purity) was obtained as a white solid.

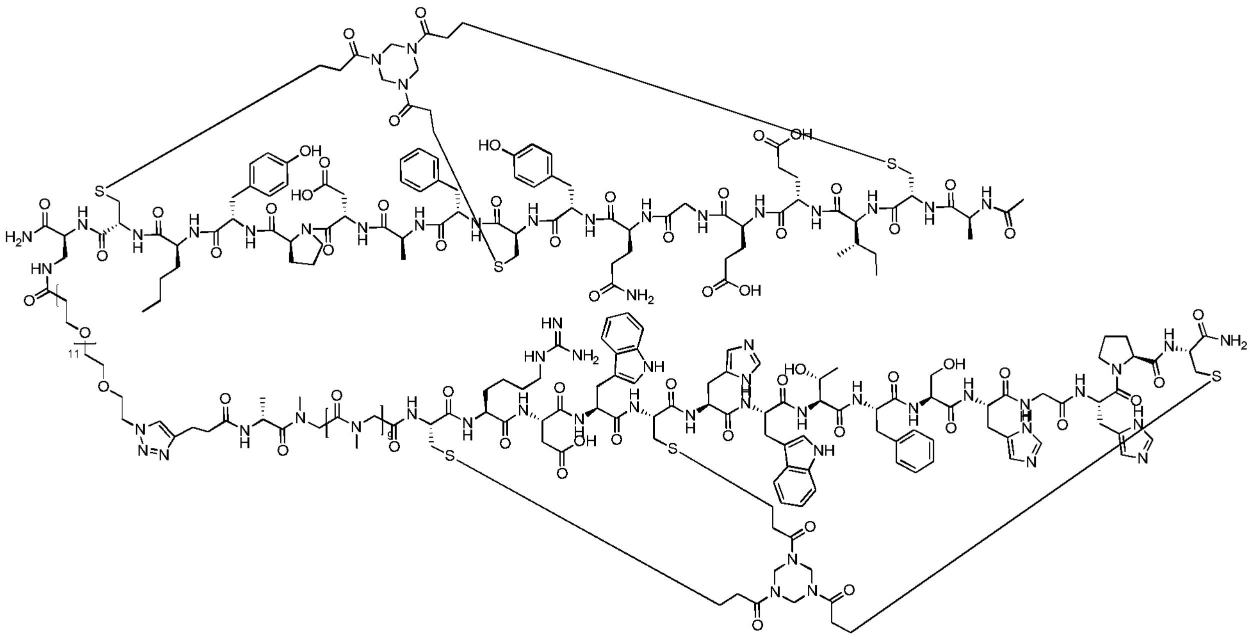
Procedure for preparation of BCY11859

BCY00011415-PEG5-N_3 (**2**) + **BCY00008928** —($CuSO_4$, VcNa, THPTA / t-$BuOH/H_2O$)→ **BCY00011859**

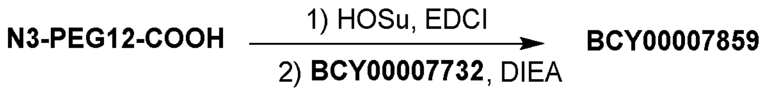
Compound 2 (20.0 mg, 8.03 µmol, 1.0 eq) and **BCY8928** (21.0 mg, 9.47 µmol, 1.1 eq) were first dissolved in 2 mL of t-$BuOH/H_2O$ (1:1), and then $CuSO_4$ (0.4 M, 21.0 µL, 1.0 eq), VcNa (4.0 mg, 2.5 eq) and THPTA (4.0 mg, 1.1 eq) was added. Finally 1 M NH_4HCO_3 was added to adjust pH to 8. All solvents here were degassed and purged with N_2 for 3 times. The reaction mixture was stirred at 30°C for 16 hr under N_2 atmosphere. LC-MS showed compound 2 was consumed completely and one main peak with desired m/z (calculated MW: 4707.40, observed *m/z*: 941.7($[M/5+H]^+$), 1176.9($[M/4+H]^+$), 1569.6($[M/3+H]^+$)). The reaction mixture was purified by prep-HPLC (TFA condition) and **BCY11859** (19.2 mg, 4.01 µmol, 49.87% yield, 98.22% purity) was obtained as a white solid.

Example 4: Synthesis of PD-L1/CD137 Binding Heterotandem Bicyclic Peptides

BCY8939

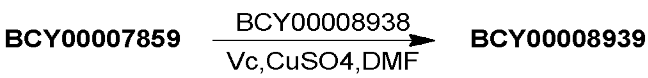


General procedure for preparation of BCY8939



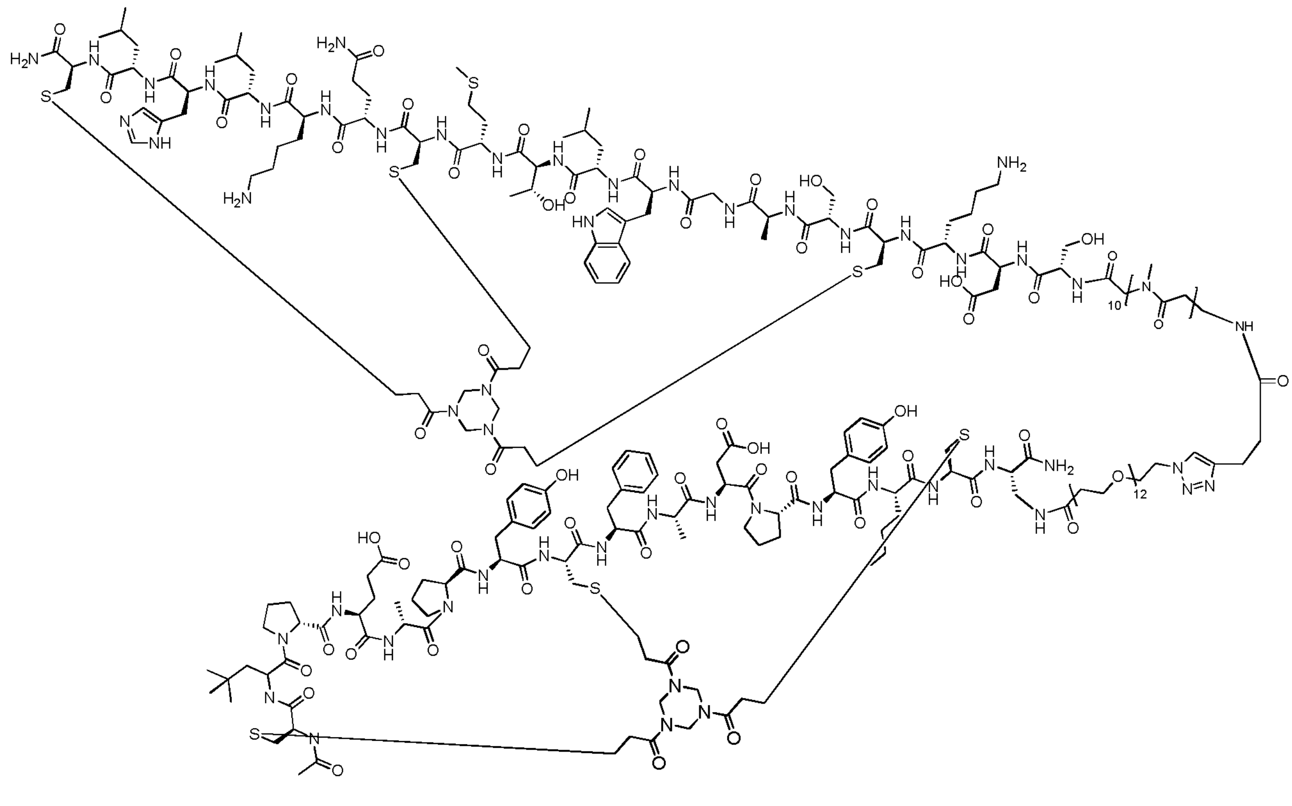
To a solution of **N3-PEG12-COOH** (250 mg, 388 µmol) and HOSu (67.0 mg, 583 µmol) in DMA (4.5 mL) and DCM (1.5 mL) was added EDCI (89.3 mg, 466 µmol) with stirring at 20 °C for 16 hr. LCMS showed the desired intermediate was formed completely. **BCY7732** (854.97 mg, 388.37 µmol, 1 *eq*) and DIEA (186 mg, 1.44 mmol, 250 µL) were added to the mixture with further stirring at 20 °C for additional 5 hr. LC-MS showed **BCY7732** was consumed completely and one main peak with desired mass was detected. The reaction mixture was purified by prep-HPLC (TFA condition) to give compound **BCY7859** (621 mg, 200.58 µmol, 51.65% yield, 95% purity, TFA) as a white solid. *Calculated* MW: 2817.16, *observed* m/z: 942.7 [M/3+H]$^+$

General procedure for preparation of BCY8939



To a solution of **BCY7859** (31.1 mg, 11.0 µmol) and **BCY8938** (30.0 mg, 10.0 µmol) in DMF (2 mL) was added (2R)-2-[(1S)-1,2-dihydroxyethyl]-3,4-dihydroxyl -2H-furan-5-one (1 M, 100 µL) and $CuSO_4$ (1 M, 30.0 µL) with stirring under nitrogen atmosphere for 2 hr at 20 °C. LC-MS showed **BCY7859** was consumed completely and one main peak with desired mass was detected. The reaction mixture was purified by prep-HPLC (TFA condition) to give compound **BCY8939** (16.1 mg, 2.72 µmol, 27.1% yield, 98.3% purity) as a white solid. *Calculated* MW: 5823.49, *observed* m/z: 1165.4 [M/5+H]$^+$, 971.0 [M/6+H]$^+$, 832.9[M/7+H]$^+$

BCY10580



BCY00010580

Procedure for preparation of BCY9172-PEG12-N$_3$

BCY00009172 + **NHS-PEG12-N$_3$** —(DIEA / DMSO)→ **BCY00009172-PEG12-N$_3$**

1 **2**

BCY9172 (100.0 mg, 47.72 μmol, 1 eq) and compound 1 (40.0 mg, 54.00 μmol, 1.13 eq) in DMSO (2 mL) was added DIEA (9.25 mg, 71.58 μmol, 12.47 μL, 1.5 eq). The mixture was stirred at 30 °C for 12 hr. LC-MS showed **BCY9172** was consumed completely and one main peak with desired m/z (MW: 2721.12, observed *m/z*: 1361.07([(M/2+H$^+$])) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent and produced a residue. The residue was then purified by prep-HPLC (neutral condition). Compound **2** (48 mg, 17.44 μmol, 45.68% yield, 98.87% purity) was obtained as a white solid.

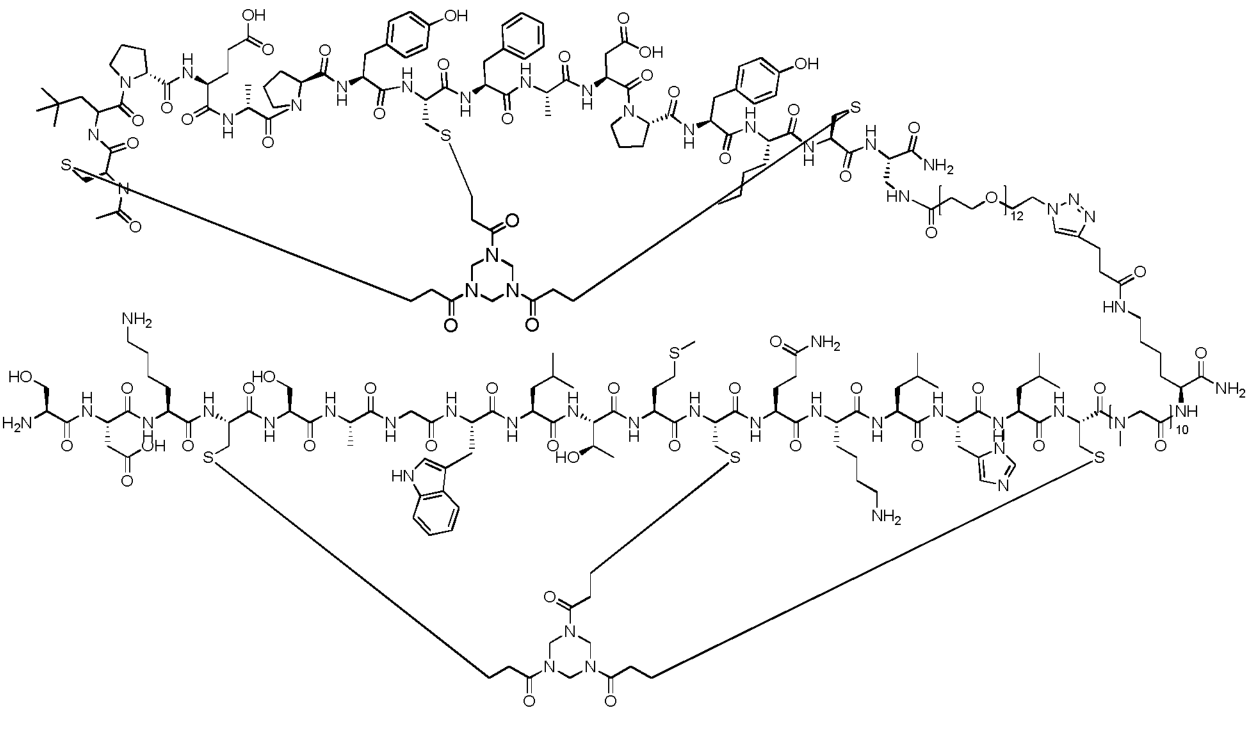
Procedure for preparation of BCY10580

BCY00009172-PEG12-N$_3$ + **BCY00010043** —($CuSO_4$, VcNa, THPTA / t-BuOH/H_2O)→ **BCY00010580**

2

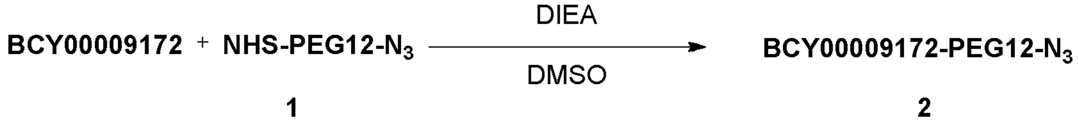
Compound **2** (20 mg, 7.35 µmol, 1.0 eq) and **BCY10043** (23.1 mg, 7.35 µmol, 1.0 eq) were first dissolved in 2 mL of t-BuOH/H_2O (1:1), and then $CuSO_4$ (0.4 M, 18.4 µL, 1.0 eq), VcNa(0.4 M, 36.8 µL, 2.0 eq) and THPTA (0.4 M, 18.4 µL, 1.0 eq) were added. Finally 1 M NH_4HCO_3 was added to adjust pH to 8. All solvents here were degassed and purged with N_2 for 3 times. The reaction mixture was stirred at 30 °C for 4 hr under N_2 atmosphere. LC-MS showed compound 3 was consumed completely and one main peak with desired m/z (MW: 5855.74 observed m/z: 976.40 ($[M/6+H]^+$) and 1171.67 ($[M/5+H]^+$)) was detected. The residue was purified by prep-HPLC (TFA condition). **BCY10580** (29 mg, 4.85 µmol, 65.95% yield, 97.879% purity) was obtained as a white solid.

BCY10581



BCY00010581

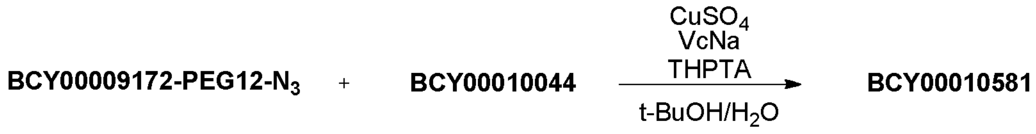
Procedure for preparation of BCY9172-PEG12-N₃



BCY9172 (100 mg, 47.72 µmol, 1 eq) and compound 1 (40.00 mg, 54.00 µmol, 1.13 eq) in DMSO (2 mL) was added DIEA (9.25 mg, 71.58 µmol, 12.47 µL, 1.5 eq). The mixture was stirred at 30 °C for 12 hr. LC-MS showed **BCY9172** was consumed completely and one main peak with desired m/z (MW: 2721.12, observed *m/z*: 1361.07($[(M/2+H^+]$)) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent and produced a residue. The residue was then purified by prep-HPLC (neutral condition).

Compound **2** (48 mg, 17.44 µmol, 45.68% yield, 98.87% purity) was obtained as a white solid.

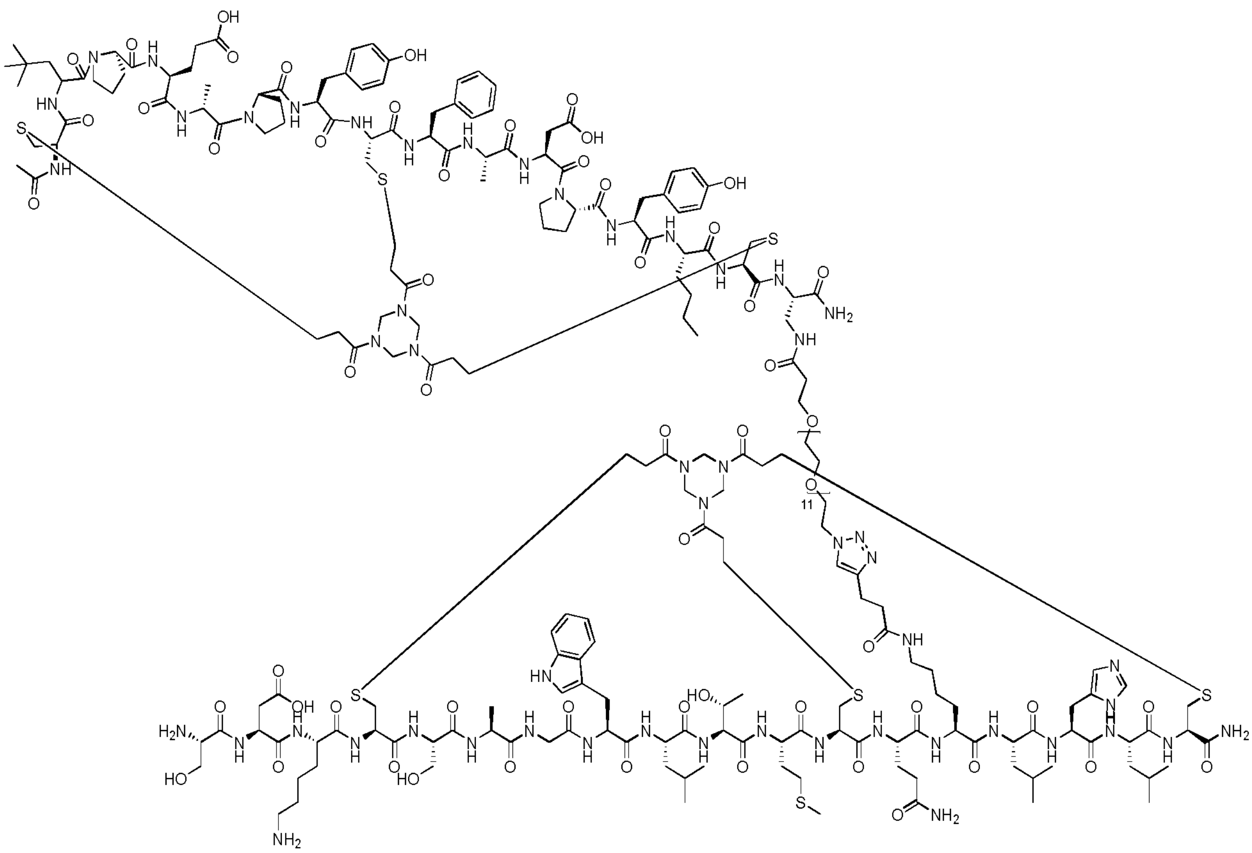
Procedure for preparation of BCY10581



2

Compound **2** (12 mg, 4.41 µmol, 1 eq) and **BCY10044** (14.08 mg, 4.41 µmol, 1eq) were first dissolved in 2 mL of t-BuOH/H_2O (1:1), and then $CuSO_4$ (0.4 M, 11.02 µL, 1 eq), VcNa (0.4 M, 22.05 µL, 2 eq) and THPTA (0.4 M, 10.04 µL, 1 eq) was added. Finally 1 M NH_4HCO_3 was added to adjust pH to 8. All solvents here were degassed and purged with N_2 for 3 times. The reaction mixture was stirred at 30 °C for 4 hr under N_2 atmosphere. LC-MS showed compound 3 was consumed completely and one main peak with desired m/z (MW: 5912.84, observed m/z: 985.90 ($[M/6+H]^+$) and 1183.28 ($[M/5+H]^+$)) was detected. The residue was purified by prep-HPLC (TFA condition). **BCY10581** (9.3 mg, 1.47 µmol, 33.36% yield, 93.541% purity) was obtained as a white solid.

<u>**BCY10582**</u>



Procedure for preparation of Compound 2

$$\textbf{BCY00009172} + \underset{\mathbf{1}}{\textbf{NHS-PEG12-N}_3} \xrightarrow[\text{DMSO}]{\text{DIEA}} \underset{\mathbf{2}}{\textbf{BCY00009172-PEG12-N}_3}$$

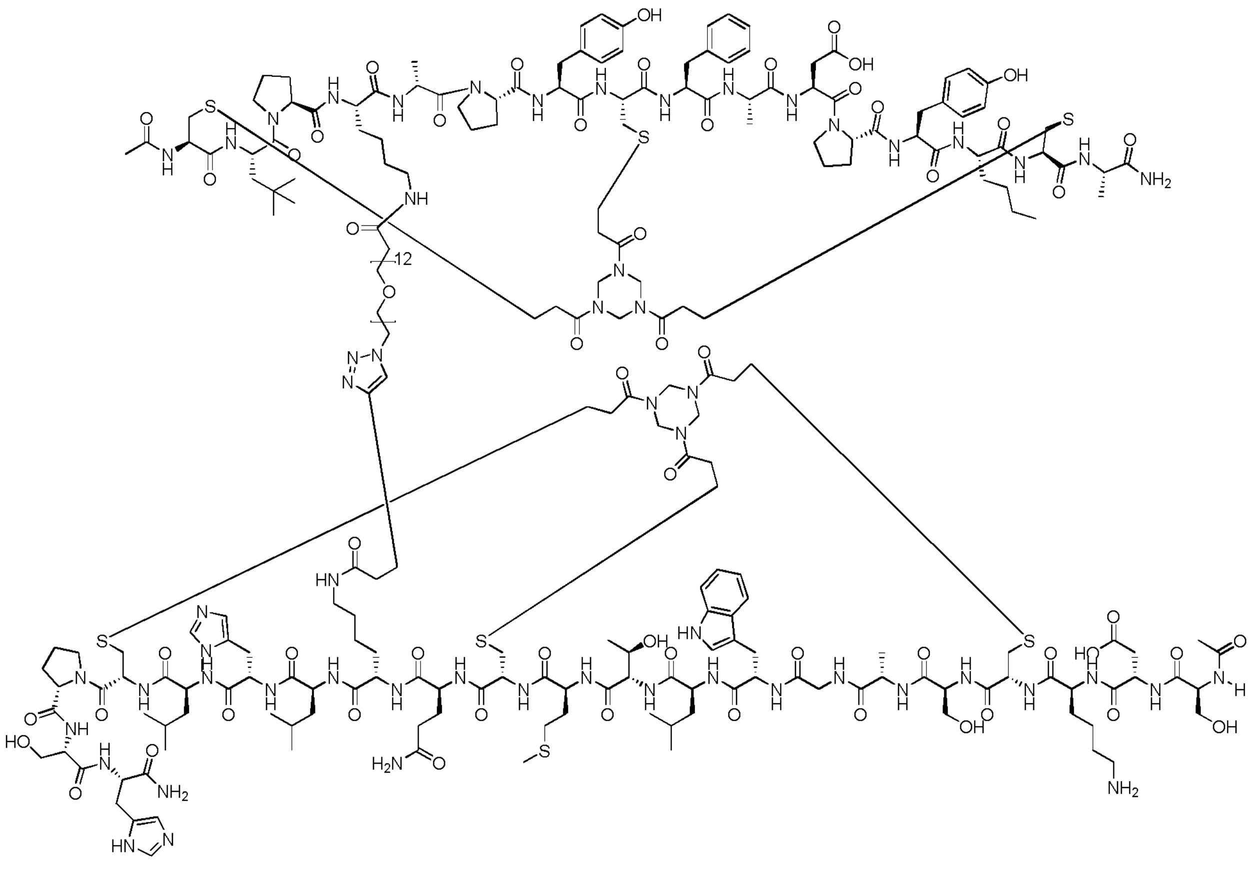
To a solution of **BCY9172** (100.0 mg, 47.7 µmol, 1.0 eq), Compound **1** (40.0 mg, 54.0 µmol, 1.13 eq) in DMSO (2 mL) was added DIEA (9.2 mg, 71.6 µmol, 12.5 µL, 1.5 eq). The mixture was stirred at 30°C for 12 hr. LC-MS showed **BCY9172** was consumed completely and one main peak with desired m/z (calculated MW: 2721.12, observed *m/z*: 1361.07($[M/2+H]^+$)) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent to give a residue. The residue was purified by prep-HPLC (TFA condition). Compound **2** (37 mg, 13.60 µmol, 28.49% yield) was obtained as a white solid.

Procedure for preparation of BCY10582

$$\underset{\mathbf{2}}{\textbf{BCY00009172-PEG12-N}_3} + \textbf{BCY00010045} \xrightarrow[\text{t-BuOH/}H_2O]{CuSO_4\ \text{VcNa}\ \text{THPTA}} \textbf{BCY00010582}$$

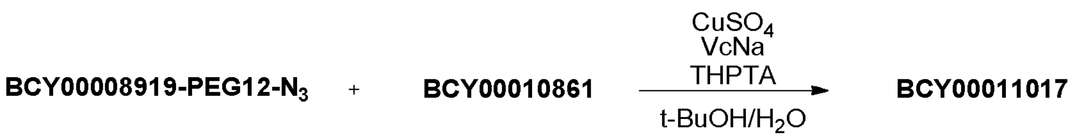
A mixture of Compound **2** (16.0 mg, 5.9 µmol, 1.0 *eq*), **BCY10045** (14.0 mg, 6.0 µmol, 1.01 *eq*), and THPTA (0.4 M, 14.7 µL, 1.0 *eq*) was dissolved in t-BuOH/H_2O (1:1, 2 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 14.7 µL, 1.0 *eq*) and VcNa (0.4 M, 29.4 µL, 2.0 *eq*) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 25-30 °C for 12 hr under N_2 atmosphere. LC-MS showed Compound **2** was consumed completely and one main peak with desired m/z [calculated MW: 5073.89, observed *m/z*: 1015.24 ($[M/5+H]^+$) and 1268.97($[M/4+H]^+$) was detected. The reaction mixture was directly purified by prep-HPLC (TFA condition). **BCY10582** (10 mg, 1.92 µmol, 32.58% yield, 97.21% purity) was obtained as a white solid.

<u>BCY11017</u>



BCY00011017

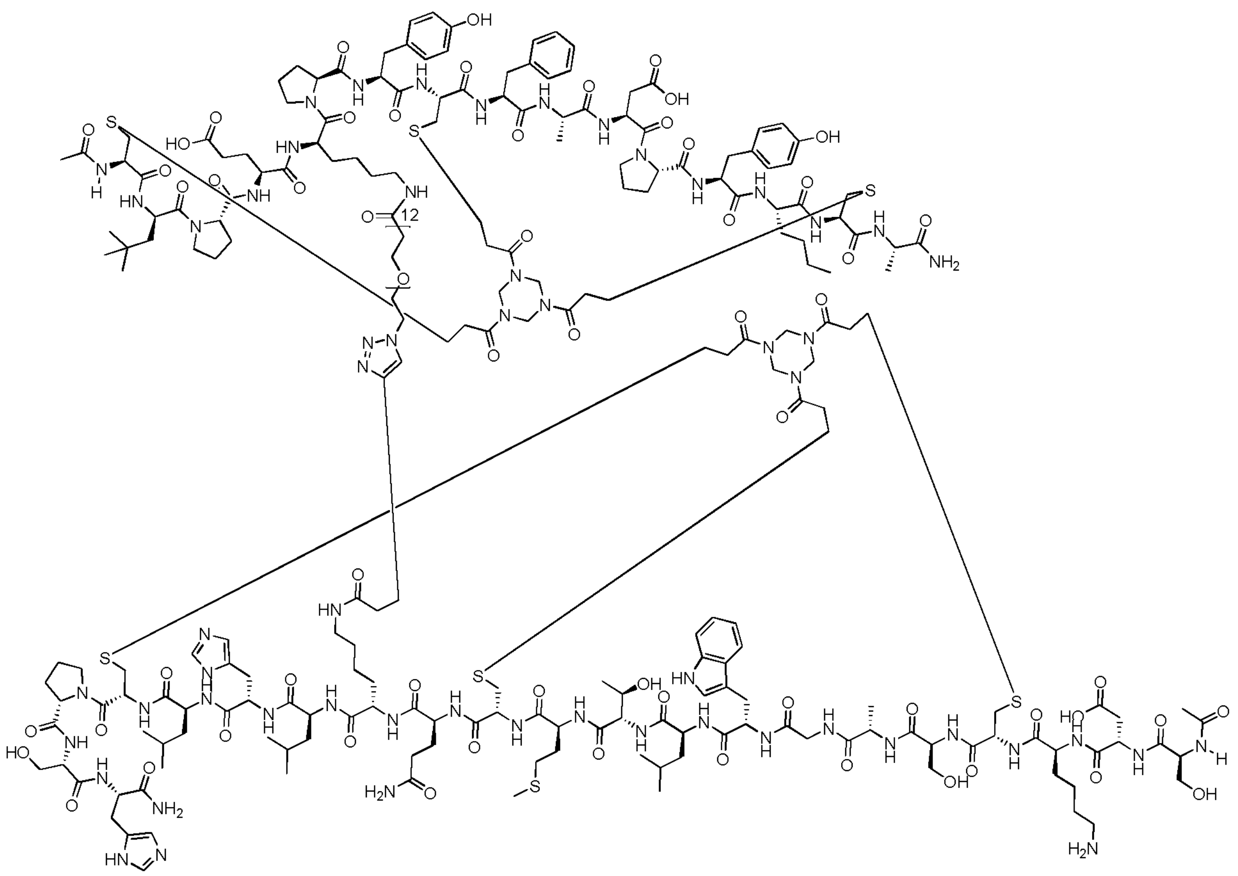
Procedure for preparation of BCY11017



2

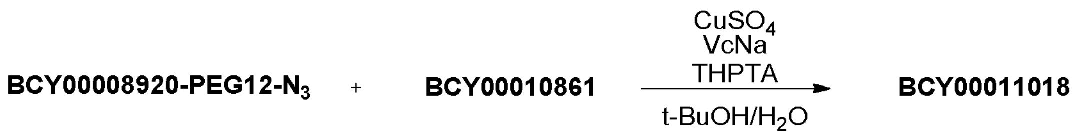
Compound **2** (which may be prepared as described in the procedure for preparing BCY10567; 7.0 mg, 2.59 µmol, 1.0 eq) and **BCY10861** (7.03 mg, 2.59 µmol, 1.0 eq) , were first dissolved in 2 mL of t-BuOH/H_2O (1:1), and then $CuSO_4$ (0.4 M, 13.0 µL, 2.0 eq), VcNa (1.0 mg, 5.03 µmol, 2.0 eq) and THPTA (1.1 mg, 2.53 µmol, 1.0 eq) were added. Finally 1 M NH_4HCO_3 was added to adjust pH to 8. All solvents here were degassed and purged with N_2 for 3 times. The reaction mixture was stirred at 35°C for 16 hr under N_2 atmosphere. LC-MS showed compound **2** was consumed completely and one main peak with desired m/z (calculated MW: 5421.30, observed *m/z*: 1084.7([M/5+H]$^+$)). The reaction mixture was purified by prep-HPLC (TFA condition) and **BCY11017** (6.6 mg, 1.17 µmol, 45.24% yield, 96.16% purity) was obtained as a white solid.

BCY11018



BCY00011018

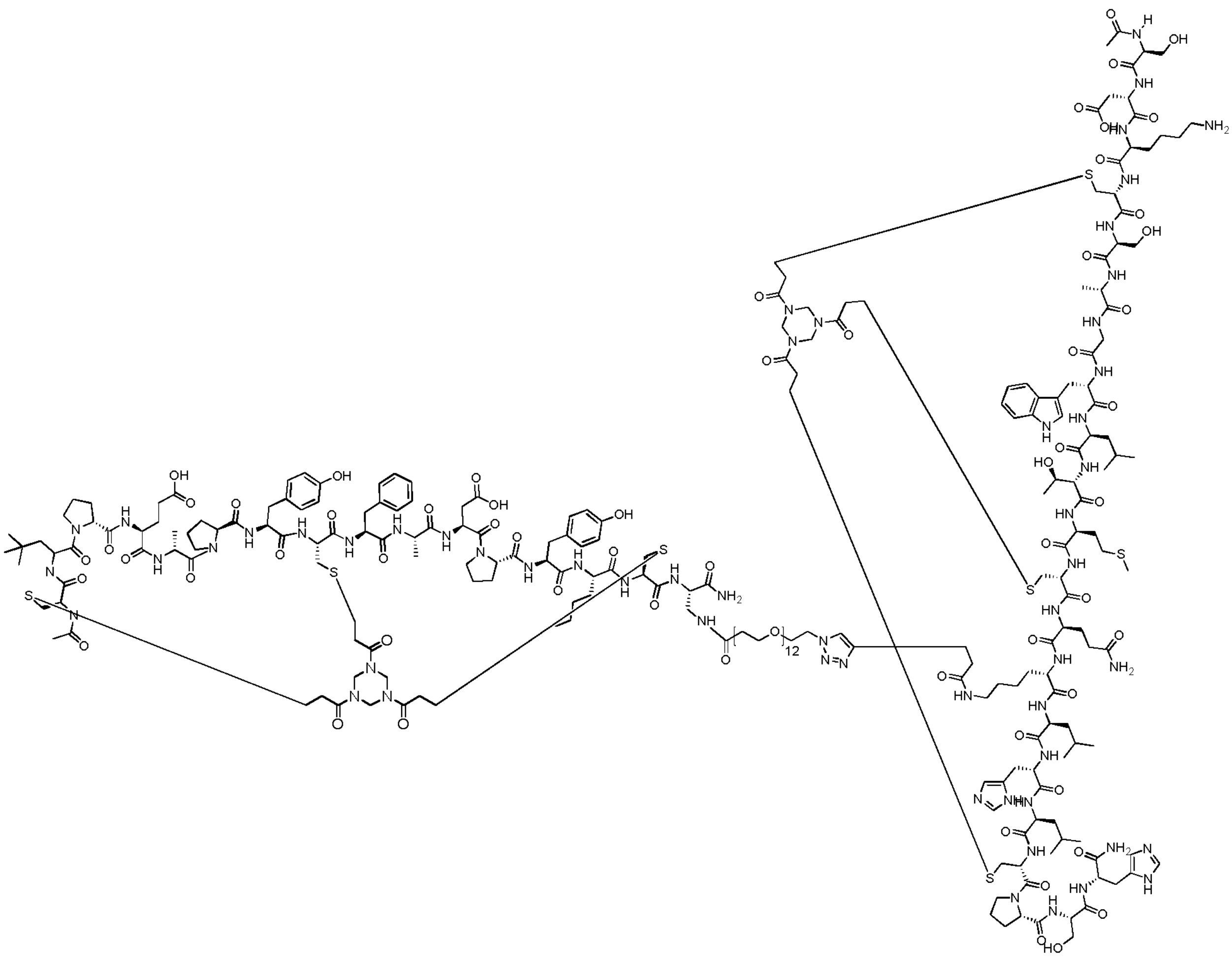
Procedure for preparation of BCY11018



2

Compound **2** (which may be prepared as described in the procedure for preparing BCY10570; 6.0 mg, 2.17 μmol, 1.0 eq) and **BCY10861** (5.9 mg, 2.17 μmol, 1.0 eq) , were first dissolved in 2 mL of t-BuOH/H_2O (1:1), and then $CuSO_4$ (0.4 M, 11.0 μL, 2.0 eq), VcNa (1.0 mg, 2.3 eq) and THPTA (1.1 mg, 1.0 eq) was added. Finally 1 M NH_4HCO_3 was added to adjust pH to 8. All solvents here were degassed and purged with N_2 for 3 times. The reaction mixture was stirred at 35°C for 16 hr under N_2 atmosphere. LC-MS showed compound **2** was consumed completely and one main peak with desired m/z (calculated MW: 5479.34, observed *m/z*: 1096.40([M/5+H]$^+$)). The reaction mixture was purified by prep-HPLC (TFA condition) and **BCY11018** (2.3 mg, 0.40 μmol, 18.31% yield, 94.73% purity) was obtained as a white solid.

BCY11019



BCY00011019

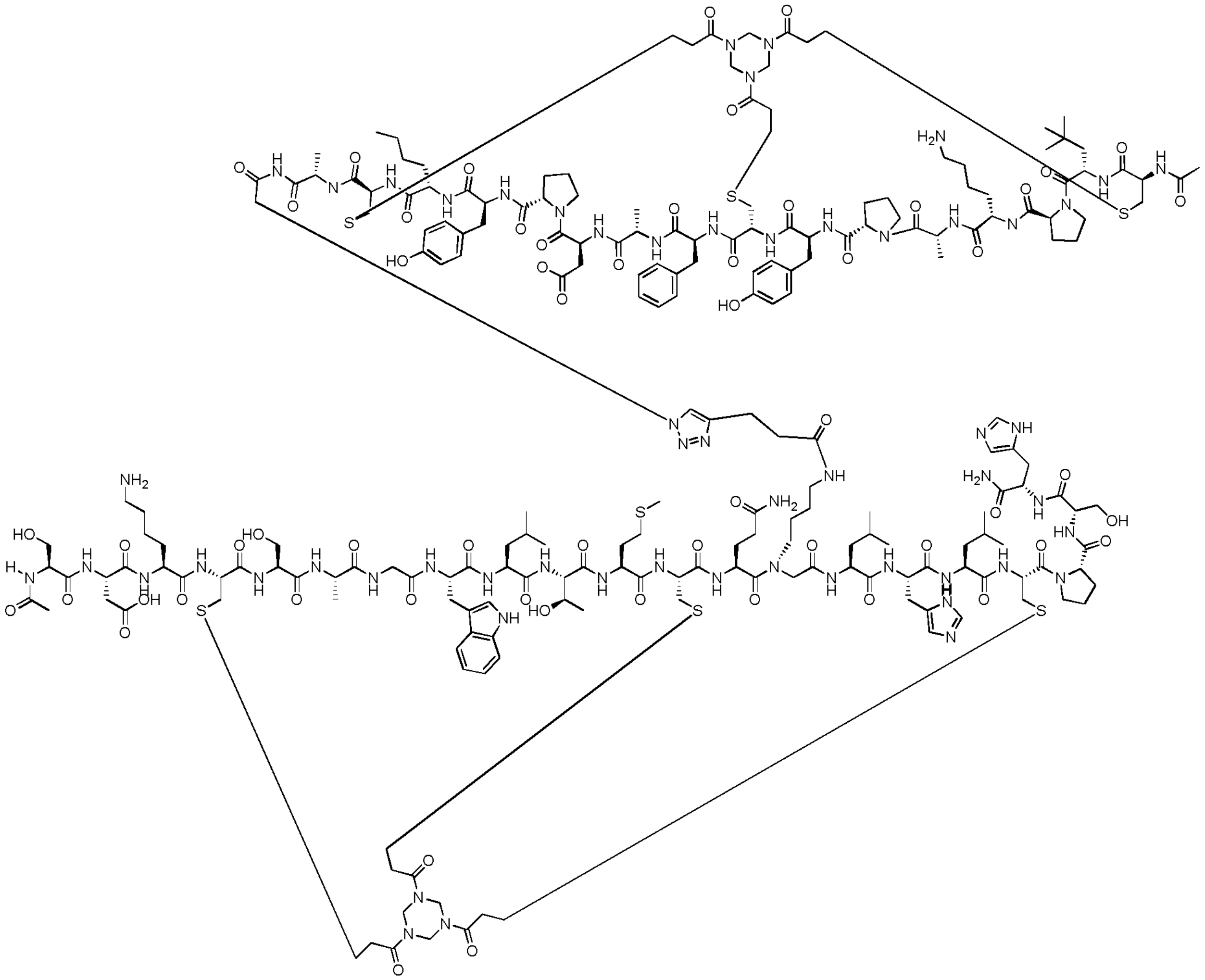
Procedure for preparation of BCY11019

BCY00009172-PEG12-N$_3$ + **BCY00010861** —($CuSO_4$, VcNa, THPTA; t-BuOH/H_2O)→ **BCY00011019**

2

Compound **2** (which may be prepared as described in the procedure for preparing BCY10581; 8.0 mg, 2.94 µmol, 1.0 eq) and **BCY10861** (8.0 mg, 2.95 µmol, 1.0 eq) , were first dissolved in 2 mL of t-BuOH/H_2O (1:1), and then $CuSO_4$ (0.4 M, 14.7 µL, 2.0 eq), VcNa (1.2 mg, 6.05 µmol, 2.0 eq) and THPTA (1.3 mg, 2.99 µmol, 1.0 eq) was added. Finally 1 M NH_4HCO_3 was added to adjust pH to 8. All solvents here were degassed and purged with N_2 for 3 times. The reaction mixture was stirred at 35°C for 16 hr under N_2 atmosphere. LC-MS showed compound **2** was consumed completely and one main peak with desired m/z (calculated MW: 5437.26, observed *m/z*: 1088.09([M/5+H]$^+$) and 1360.19([M/4+H]$^+$)). The reaction mixture was purified by prep-HPLC (TFA condition) and **BCY11019** (7.6 mg, 1.36 µmol, 46.09% yield, 96.95% purity) was obtained as a white solid.

BCY11376



BCY00011376

Procedure for preparation of Compound 2

$$\textbf{BCY00008919} + \underset{\mathbf{1}}{\mathbf{N_3\text{-}CH_2\text{-}COOH}} \xrightarrow[\text{2.DIEA, DMF}]{\text{1. EDCI, HOSu}} \underset{\mathbf{2}}{\mathbf{BCY00008919\text{-}CH_2\text{-}N_3}}$$

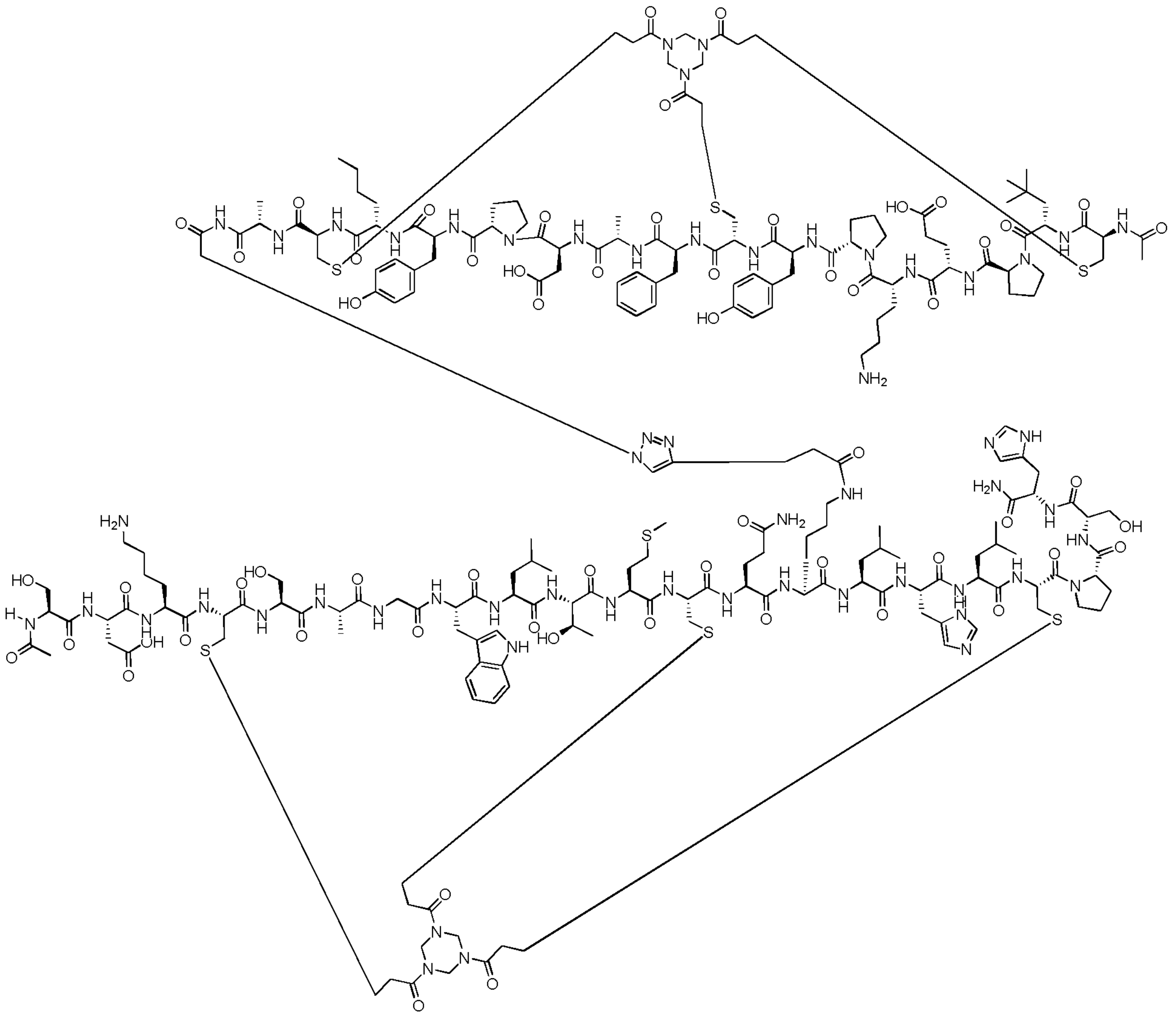
To a solution of compound **1** (5.0 mg, 49.5 µmol, 1.0 eq) in DMF (1 mL) was added EDCI (8.5 mg, 54.8 µmol, 1.1 eq) and HOSu (5.7 mg, 49.5 µmol, 1.0 eq)**.** The mixture was stirred at 25-30 °C for 30 min. TLC indicated compound **1** was consumed completely and one new spot formed. Then 0.2 mL of this mixture was added with **BCY8919** (20.0 mg, 9.62 µmol) and DIEA (1.7 µL, 9.62 µmol). The mixture was stirred at 25-30 °C for 2 hr. LC-MS showed **BCY8919** was consumed completely and one main peak with desired m/z (calculated MW:2162.51, observed *m/z*: 1081.8 ($[M/2+H]^+$)) was detected. The reaction mixture was then concentrated under reduced pressure to remove solvent and produced a residue, following by purification by prep-HPLC (TFA condition). Compound 2 (12 mg, 5.55 µmol, 56.28% yield, 97.54% purity) was obtained as a white solid.

Procedure for preparation of BCY11376

$$\underset{\mathbf{2}}{\text{BCY00008919-CH}_2\text{-N}_3} + \text{BCY00010861} \xrightarrow[\text{t-BuOH/H}_2\text{O}]{\text{CuSO}_4,\ \text{VcNa},\ \text{THPTA}} \text{BCY00011376}$$

A mixture of compound **2** (3 mg, 1.39 µmol, 1.0 eq.), **BCY10861** (3.8 mg, 1.40 µmol, 1.0 eq.), and THPTA (1.2 mg, 2.76 µmol, 2.0 eq.) was dissolved in t-BuOH/H_2O (1:1, 1 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 3.5 µL, 1.0 eq.) and VcNa (0.4 M, 3.5 µL, 1.0 eq.) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 40 °C for 2 hr under N_2 atmosphere. LC-MS showed **BCY10861** was consumed completely and one main peak with desired m/z (calculated MW: 4878.64, observed *m/z*: 1220.8($[M/4+H]^+$) was detected. The reaction mixture was filtered and concentrated under reduced pressure to give a residue. The crude product was purified by prep-HPLC (TFA condition), and **BCY11376** (1.9 mg, 1.0 µmol, 27.01% yield, 96.2% purity) was obtained as a white solid.

<u>**BCY11377**</u>



BCY00011377

Procedure for preparation of Compound 2

BCY00008920 + **N_3-CH_2-COOH** $\xrightarrow[\textbf{2.DIEA, DMF}]{\textbf{1. EDCI, HOSu}}$ **BCY00008920-CH_2-N_3**

1 → **2**

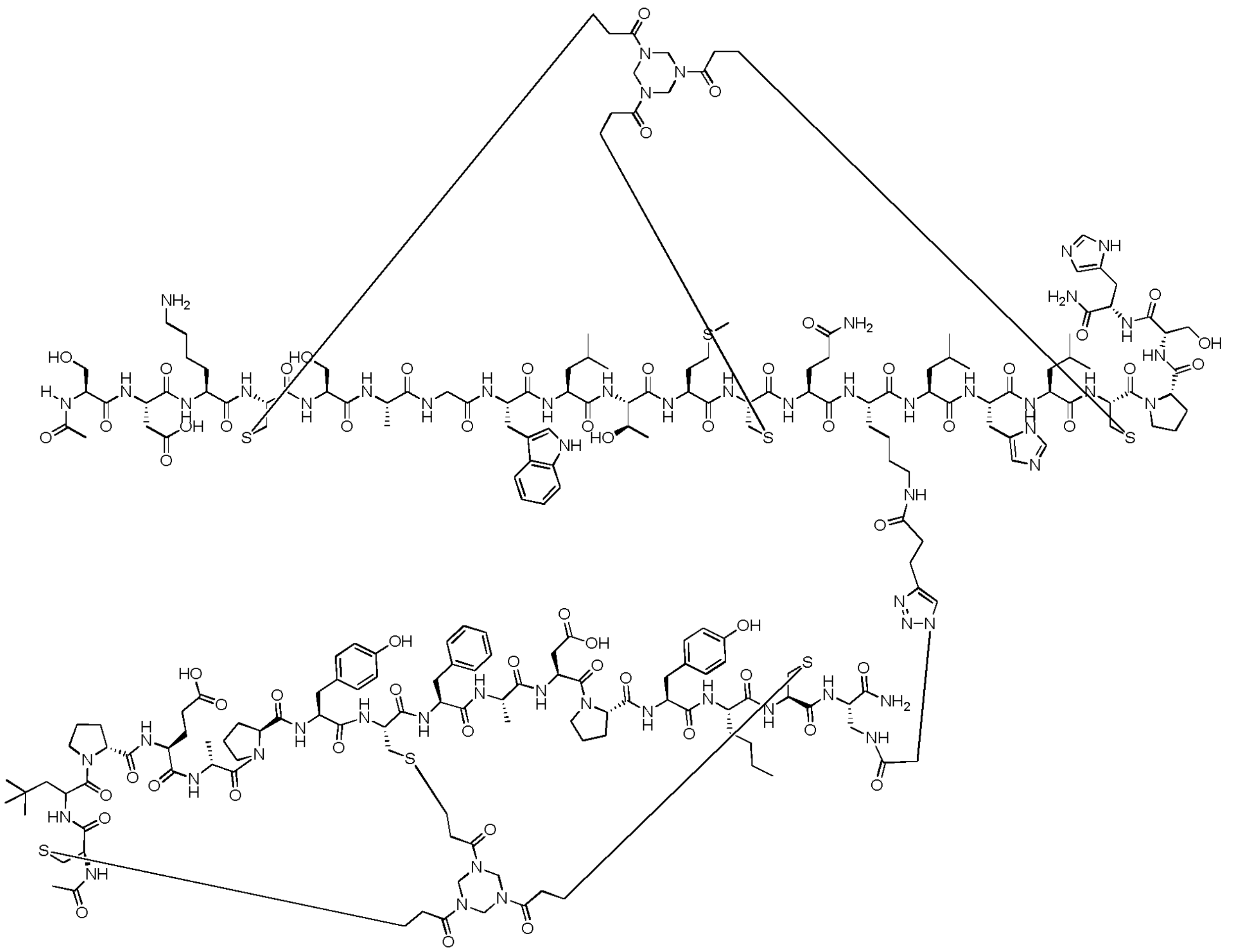
To a solution of compound **1** (5.0 mg, 49.5 µmol, 1.0 eq) in DMF (1 mL) was added EDCI (8.5 mg, 54.8 µmol, 1.1 eq) and HOSu (5.7 mg, 49.5 µmol, 1.0 eq). The mixture was stirred at 25-30 °C for 30 min. TLC indicated compound **1** was consumed completely and one new spot formed. Then 0.2 mL of this mixture was added with **BCY8920** (20.0 mg, 9.36 µmol) and DIEA (1.2 mg, 9.36 µmol). The mixture was stirred at 25-30 °C for 2 hr. LC-MS showed **BCY8920** was consumed completely and one main peak with desired m/z (calculated MW:2220.54, observed *m/z*: 1110.90 ($[M/2+H]^+$) was detected. The reaction mixture was then concentrated under reduced pressure to remove solvent and produced a residue, following by purification by prep-HPLC (TFA condition). Compound **2** (12 mg, 5.15 µmol, 56.28% yield, 95.3% purity) was obtained as a white solid.

Procedure for preparation of BCY11377

$$\text{BCY00008920-CH}_2\text{-N}_3 \; (\mathbf{2}) + \text{BCY00010861} \xrightarrow[\text{t-BuOH/H}_2\text{O}]{\text{CuSO}_4,\ \text{VcNa},\ \text{THPTA}} \text{BCY00011377}$$

A mixture of compound 2 (3 mg, 1.35 μmol, 1.0 eq.), **BCY10861** (3.8 mg, 1.35 μmol, 1.0 eq.), and THPTA (0.6 mg, 1.0 eq.) was dissolved in t-BuOH/H_2O (1:1, 1 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 3.4 μL, 1 eq.) and VcNa (0.4 M, 3.4 μL, 1 eq.) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 40 °C for 2 hr under N_2 atmosphere. LC-MS showed one main peak with desired m/z (calculated MW: 4936.68, observed *m/z*: 1234.9($[M/4+H]^+$) was detected. The reaction mixture was filtered and concentrated under reduced pressure to give a residue. The crude product was purified by prep-HPLC (TFA condition), and **BCY11377** (3.5 mg, 0.66 μmol, 48.86% yield, 93.1% purity) was obtained as a white solid.

BCY11378



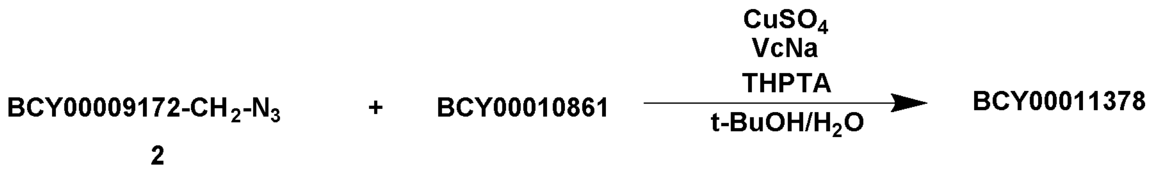
BCY00011378

Procedure for preparation of Compound 2

BCY00009172 + **N_3-CH_2-COOH** (**1**) —(**1. EDCI, HOSu**; **2.DIEA, DMF**)→ **BCY00009172-CH_2-N_3** (**2**)

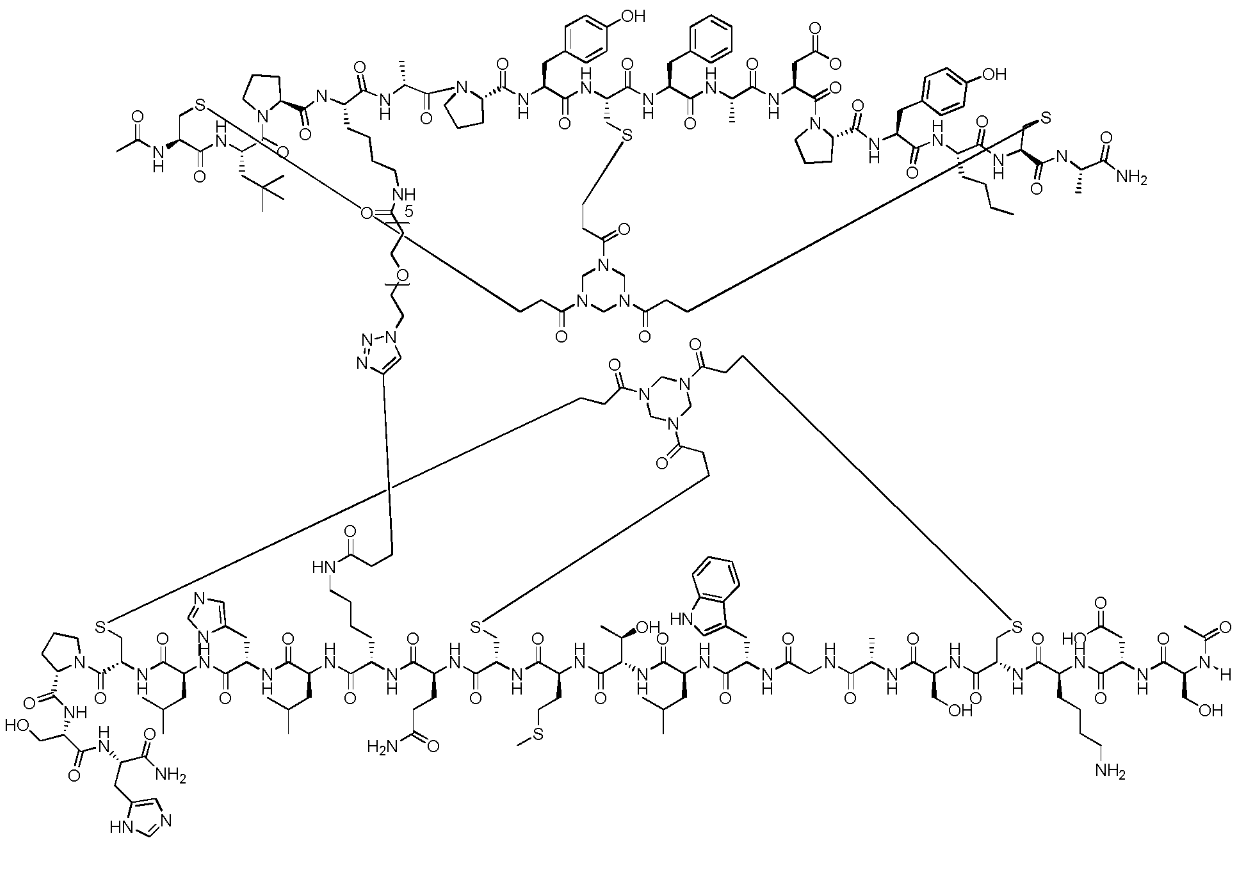
To a solution of compound **1** (5.0 mg, 49.5 µmol, 1.0 eq) in DMF (1 mL) was added EDCI (8.5 mg, 54.8 µmol, 1.1 eq) and HOSu (5.7 mg, 49.5 µmol, 1.0 eq)**.** The mixture was stirred at 25-30 °C for 30 min. TLC indicated compound **1** was consumed completely and **one new spot** formed. Then 0.2 mL of this mixture was added to **BCY9172** (20.0 mg, 9.54 µmol) and DIEA (1.7 µL, 9.62 µmol). The mixture was stirred at 25-30 °C for 2 hr. LC-MS showed compound **1** was consumed completely and one main peak with desired m/z (calculated MW:2176.49, observed *m/z*: 1090.0 ($[M/2+H]^+$) was detected. The reaction mixture was then concentrated under reduced pressure to remove solvent and produced a residue, following by purification by prep-HPLC (TFA condition). Compound **2** (20.2 mg, 7.48 µmol, 78.34% yield, 80.57% purity) was obtained as a white solid.

Procedure for preparation of BCY11378



A mixture of compound 2 (5 mg, 2.30 μmol, 1.0 eq.), **BCY10861** (6.24 mg, 2.30 μmol, 1.0 eq.), and THPTA (1.0 mg, 1.0 eq.) was dissolved in t-BuOH/H_2O (1:1, 1 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 5.8 μL, 1.0 eq.) and VcNa (0.4 M, 5.8 μL, 1.0 eq.) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 40 °C for 2 hr under N_2 atmosphere. LC-MS showed compound **3** was consumed completely and one main peak with desired m/z (calculated MW: 4894.61, observed *m/z*: 1224.3($[M/4+H]^+$) was detected. The reaction mixture was filtered and concentrated under reduced pressure to give a residue. The crude product was purified by prep-HPLC (TFA condition), and **BCY11378** (1.2 mg, 0.34 μmol, 10.07% yield, 94.3% purity) was obtained as a white solid.

__BCY11379__



BCY00011379

Procedure for preparation of BCY8919-PEG5-N_3

BCY00008919 + **NHS-PEG5-N_3** —($NaHCO_3$; MeCN/H_2O)→ **BCY00008919-PEG5-N_3**

1 **2**

BCY8919 (30.0 mg, 14.43 µmol, 1.0 eq) and compound **1** (6.3 mg, 14.57 µmol, 1.01 eq), were dissolved in a mixture of MeCN (1 mL) and H_2O (1 mL). The solution was added with 1 M $NaHCO_3$ to adjust pH to 8, and then the mixture was stirred at 35°C for 2 hr. LC-MS showed **BCY8919** was consumed completely and one main peak with desired m/z (calculated MW: 2396.79, observed *m/z*: 1198.74($[M/2+H]^+$) and 799.50($[M/4+H]^+$)) was detected. The reaction mixture was purified by prep-HPLC (TFA condition) and compound **2** (20 mg, 8.07 µmol, 55.92% yield, 96.68% purity) was obtained as a white solid.

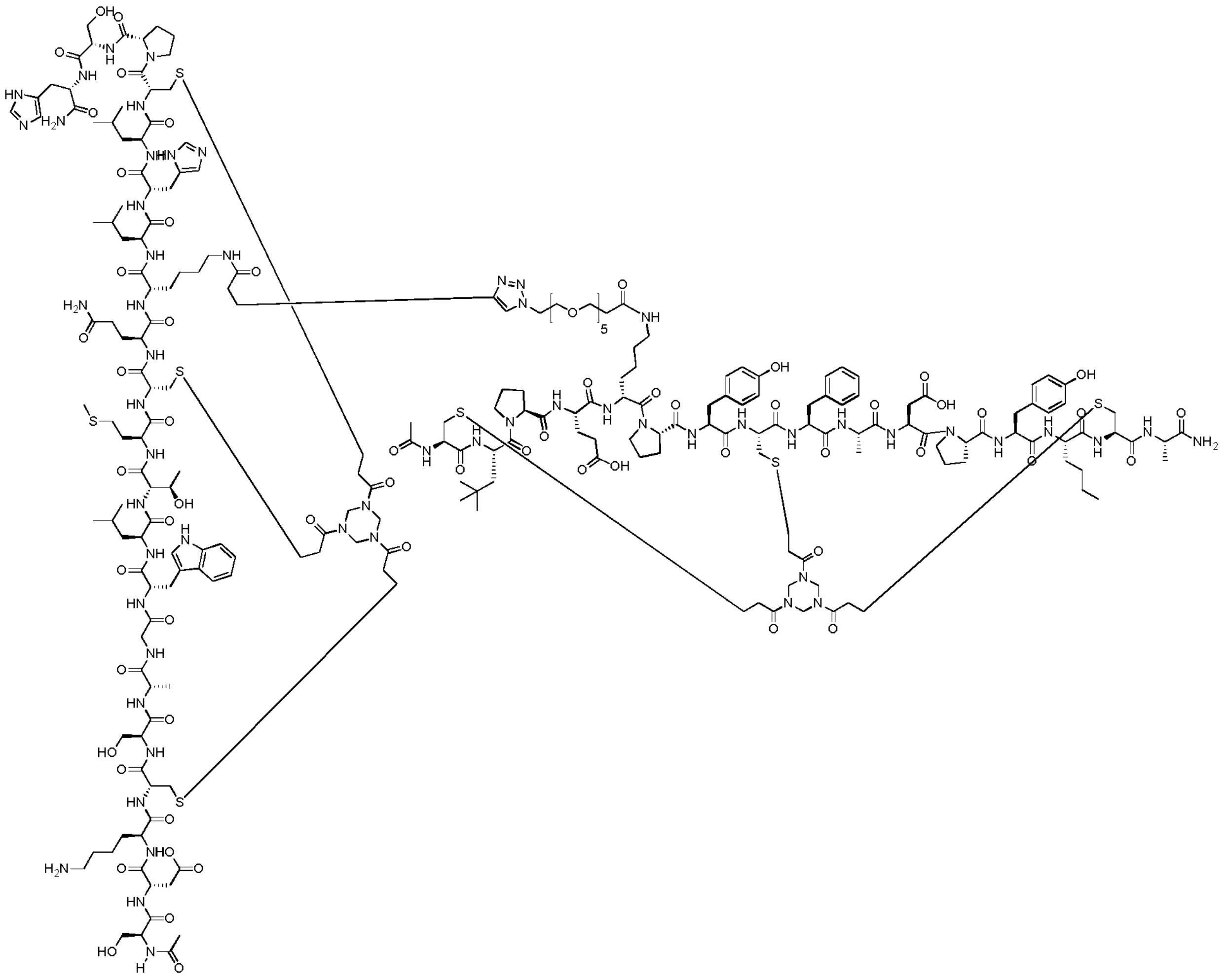
Procedure for preparation of BCY11379

BCY00008919-PEG5-N_3 + **BCY00010861** —($CuSO_4$, VcNa, THPTA; t-BuOH/H_2O)→ **BCY00011379**

2

Compound **2** (3.0 mg, 1.25 µmol, 1.0 eq) and **BCY10861** (3.4 mg, 1.25 µmol, 1.0 eq) were first dissolved in 2 mL of t-BuOH/H_2O (1:1), and then $CuSO_4$ (0.4 M, 7 µL, 2.24 eq), VcNa (1 mg, 5.04 µmol, 4.03 eq) and THPTA (1 mg, 2.30 µmol, 1.84 eq) was added. Finally 1 M NH_4HCO_3 was added to adjust pH to 8. All solvents here were degassed and purged with N_2 for 3 times. The reaction mixture was stirred at 25-30°C for 16 hr under N_2 atmosphere. LC-MS showed compound 2 was consumed completely and one main peak with desired m/z (calculated MW: 5112.93 observed *m/z*: 1022.96($[M/5+H]^+$) and 1278.74($[M/4+H]^+$)). The reaction mixture was purified by prep-HPLC (TFA condition) and **BCY11379** (3.4 mg, 0.615 µmol, 52.00% yield, 97.88% purity) was obtained as a white solid.

<u>BCY11380</u>



BCY00011380
Molecular Weight: 4994.77

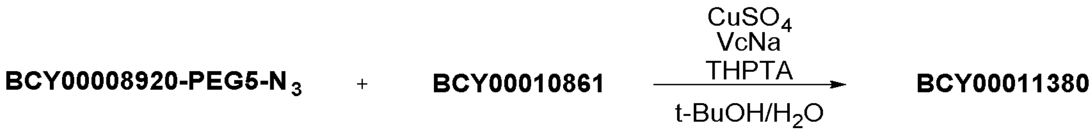
Procedure for preparation of BCY8920-PEG5-N_3

BCY00008920 + **NHS-PEG5-N_3** —($NaHCO_3$, MeCN/H_2O)→ **BCY00008920-PEG5-N_3**

1 **2**

BCY8920 (30.0 mg, 14.04 µmol, 1.0 eq) and compound **1** (6.1 mg, 14.11 µmol, 1.01 eq), were dissolved in a mixture of MeCN (1 mL) and H_2O (1 mL). The solution was added with 1 M $NaHCO_3$ to adjust pH to 8, and then the mixture was stirred at 35°C for 2 hr. LC-MS showed **BCY8920** was consumed completely and one main peak with desired m/z (calculated MW: 2454.83, observed *m/z*: 1227.63($[M/2+H]^+$) and 818.66($[M/3+H]^+$)) was detected. The reaction mixture was purified by prep-HPLC (TFA condition) and compound **2** (20 mg, 8.03 µmol, 57.21% yield, 98.56% purity) was obtained as a white solid.

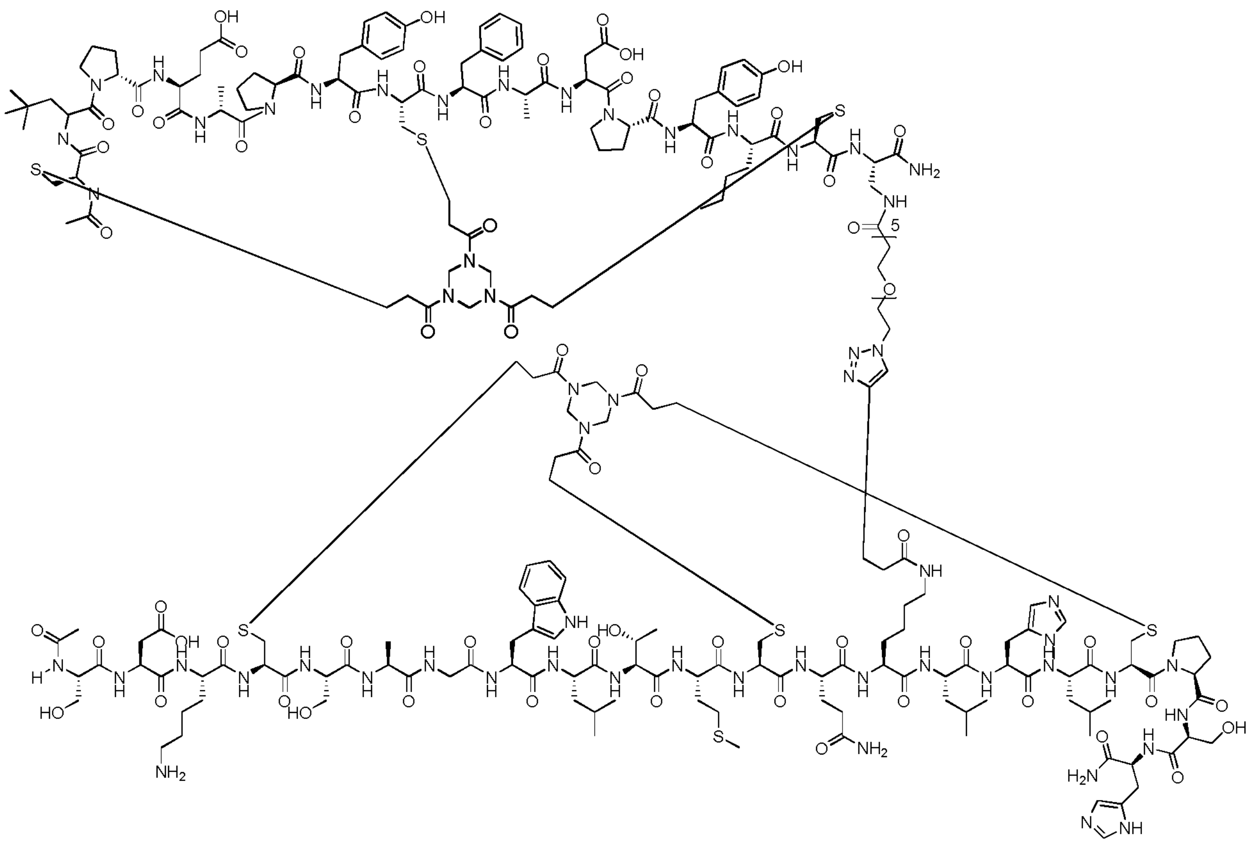
Procedure for preparation of BCY11380



2

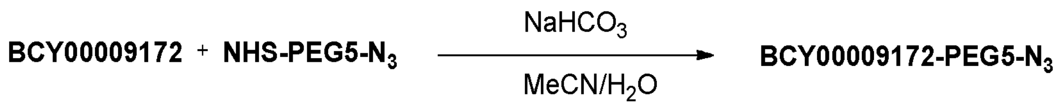
Compound **2** (3.5 mg, 1.43 µmol, 1.0 eq) and **BCY10861** (3.9 mg, 1.44 µmol, 1.0 eq) were first dissolved in 2 mL of t-BuOH/H_2O (1:1), and then $CuSO_4$ (0.4 M, 8 µL, 2.24 eq), VcNa (1 mg, 5.04 µmol, 3.52 eq) and THPTA (1 mg, 2.30 µmol, 1.61 eq) were added. Finally 1 M NH_4HCO_3 was added to adjust pH to 8. All solvents here were degassed and purged with N_2 for 3 times. The reaction mixture was stirred at 25-30°C for 16 hr under N_2 atmosphere. LC-MS showed majority of compound **2** was consumed and one main peak with desired m/z (calculated MW: 5170.97, observed *m/z*: 1034.28([M/5+H]$^+$) and 1293.10([M/4+H]$^+$)). The reaction mixture was purified by prep-HPLC (TFA condition) and **BCY11380** (1.6 mg, 0.296 µmol, 20.77% yield, 96.77% purity) was obtained as a white solid.

<u>**BCY11381**</u>



BCY00011381

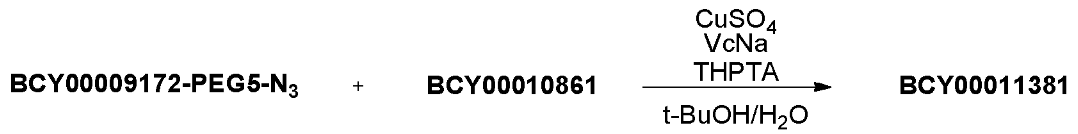
Procedure for preparation of BCY8920-PEG5-N$_3$



1 **2**

BCY9172 (30.0 mg, 14.32 µmol, 1.0 eq) and compound **1** (6.2 mg, 14.34 µmol, 1.0 eq), were dissolved in a mixture of MeCN (1 mL) and H_2O (1 mL). The solution was added with 1 M $NaHCO_3$ to adjust pH to 8, and then the mixture was stirred at 35°C for 2 hr. LC-MS showed **BCY9172** was consumed completely and one main peak with desired m/z (calculated MW: 2412.75, observed *m/z*: 1206.72($[M/2+H]^+$)) was detected. The reaction mixture was purified by prep-HPLC (TFA condition) and compound **2** (15 mg, 6.14 µmol, 42.87% yield, 98.75% purity) was obtained as a white solid.

Procedure for preparation of BCY11381



2

Compound **2** (3.0 mg, 1.24 µmol, 1.0 eq) and **BCY10861** (3.4 mg, 1.25 µmol, 1.01 eq) were first dissolved in 2 mL of t-BuOH/H_2O (1:1), and then $CuSO_4$ (0.4 M, 7 µL, 2.25 eq), VcNa (1 mg, 5.04 µmol, 4.06 eq) and THPTA (1 mg, 2.30 µmol, 1.85 eq) were added. Finally 1 M NH_4HCO_3 was added to adjust pH to 8. All solvents here were degassed and purged with N_2 for 3 times. The reaction mixture was stirred at 25-30°C for 16 hr under N_2 atmosphere. LC-MS showed one peak with desired m/z (calculated MW: 5128.89, observed *m/z*: 1026.05($[M/5+H]^+$) and 1282.50($[M/4+H]^+$)). The reaction mixture was purified by prep-HPLC (TFA condition) and **BCY11381** (1.6 mg, 0.295 µmol, 23.73% yield, 94.59% purity) was obtained as a white solid.

Example 5: Production of CD137 monoclonal antibody agonist

The sequence of the CD137 monoclonal antibody agonist that was used for comparison to CD137 multimers in the experiments presented herein was disclosed in US Patent Number US 7,288,638. The IgG4 isotype antibody was expressed using the ExpiCHO Expression System (Thermo Fisher Scientific) following transient transfection of the DNA expression construct. The antibody was purified by Protein A affinity chromatography and formulated in phosphate-buffered solution (PBS) pH 7.2. Purity analysis using HPLC-SEC (column GF-250, Agilent) indicated that the monomer rate of CD137 monoclonal antibody is approximately 95%. Binding activity analysis indicated that the CD137 monoclonal antibody with a concentration higher than 1µg/ml can bind to CHO cells expressing CD137. Endotoxin analysis using the ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (Genscript) indicated that the CD137 monoclonal antibody preparation contained <7 EU/mg of endotoxin.

BIOLOGICAL DATA

1. CD137 Biacore Experimental Description

Biacore experiments were performed to determine k_a ($M^{-1}s^{-1}$), k_d (s^{-1}), K_D (nM) values of heterotandem peptides binding to human CD137 protein. Recombinant human CD137 (R&D systems) was resuspended in PBS and biotinylated using EZ-Link™ Sulfo-NHS-LC-LC-Biotin reagent (Thermo Fisher) as per the manufacturer's suggested protocol. The protein was desalted to remove uncoupled biotin using spin columns into PBS.

For analysis of peptide binding, a Biacore T200 or a Biacore 3000 instrument was used with a XanTec CMD500D chip. Streptavidin was immobilized on the chip using standard amine-coupling chemistry at 25°C with HBS-N (10 mM HEPES, 0.15 M NaCl, pH 7.4) as the running buffer. Briefly, the carboxymethyl dextran surface was activated with a 7 min injection of a 1:1 ratio of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)/0.1 M *N*-hydroxy succinimide (NHS) at a flow rate of 10 µl/min. For capture of streptavidin, the protein was diluted to 0.2 mg/ml in 10 mM sodium acetate (pH 4.5) and captured by injecting 120µl of onto the activated chip surface. Residual activated groups were blocked with a 7 min injection of 1 M ethanolamine (pH 8.5) and biotinylated CD137 captured to a level of 270-1500 RU. Buffer was changed to PBS/0.05% Tween 20 and a dilution series of the peptides was prepared in this buffer with a final DMSO concentration of 0.5%. The top peptide concentration was 500nM with 6 further 2-fold or 3-fold dilutions. The SPR analysis was run at 25°C at a flow rate of 90µl/min with 60 seconds association and 900 seconds dissociation. After each cycle a regeneration step (10µl of 10mM glycine pH 2) was employed. Data were corrected for DMSO excluded volume effects as needed. All data were double-referenced for blank injections and reference surface using standard processing procedures and data processing and kinetic fitting were performed using Scrubber software, version 2.0c (BioLogic Software). Data were fitted using simple 1:1 binding model allowing for mass transport effects where appropriate.

Certain heterotandem peptides were tested in this assay and the results are shown in Table 1 below

Table 1: CD137 Biacore Assay Data with Heterotandem Peptides

Complex ID	SPR (K_D)(nM)

BCY9173	7.98
BCY7985	143
BCY8942	853
BCY8943	156
BCY9647	206
BCY9648	202
BCY9655	199
BCY9656	159
BCY9657	256
BCY9658	152
BCY9659	88.1
BCY9758	189
BCY8854	108
BCY9350	69.4
BCY9351	3640
BCY9399	73
BCY9400	53
BCY9408	105
BCY9409	97.7
BCY9410	65.8
BCY9411	71.1
BCY9759	44.3
BCY10000	6.19
BCY10571	12.03
BCY10572	5.00
BCY10573	3.39

2. Nectin-4 Biacore Experimental Description

Biacore experiments were performed to determine k_a ($M^{-1}s^{-1}$), k_d (s^{-1}), K_D (nM) values of heterotandem peptides binding to human Nectin-4 protein (obtained from Charles River). Human Nectin-4 (residues Gly32-Ser349; NCBI RefSeq: NP_112178.2) with a gp67 signal sequence and C-terminal FLAG tag was cloned into pFastbac-1 and baculovirus made using standard Bac-to-Bac™ protocols (Life Technologies). Sf21 cells at 1 x $10^6 ml^{-1}$ in Excell-420 medium (Sigma) at 27°C were infected at an MOI of 2 with a P1 virus stock and the supernatant harvested at 72 hours. The supernatant was batch bound for 1 hour at 4°C with Anti-FLAG M2 affinity agarose resin (Sigma) washed in PBS and the resin subsequently transferred to a column and washed extensively with PBS. The protein was eluted with 100µg/ml FLAG peptide. The eluted protein was concentrated to 2ml and loaded onto an S-200 Superdex column (GE Healthcare) in PBS at 1ml/min. 2ml fractions were collected and the fractions containing Nectin-4 protein were concentrated to 16mg/ml.

The protein was randomly biotinylated in PBS using EZ-Link™ Sulfo-NHS-LC-LC-Biotin reagent (Thermo Fisher) as per the manufacturer's suggested protocol. The protein was extensively desalted to remove uncoupled biotin using spin columns into PBS.

For analysis of peptide binding, a Biacore 3000 instrument was used with a CM5 chip (GE Healthcare). Streptavidin was immobilized on the chip using standard amine-coupling chemistry at 25°C with HBS-N (10 mM HEPES, 0.15 M NaCl, pH 7.4) as the running buffer. Briefly, the carboxymethyl dextran surface was activated with a 7 minute injection of a 1:1 ratio of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)/0.1 M *N*-hydroxy succinimide (NHS) at a flow rate of 10 µl/min. For capture of streptavidin, the protein was diluted to 0.2 mg/ml in 10 mM sodium acetate (pH 4.5) and captured by injecting 120µl of streptavidin onto the activated chip surface. Residual activated groups were blocked with a 7 minute injection of 1 M ethanolamine (pH 8.5) and biotinylated Nectin-4 captured to a level of 1,200-1,800 RU. Buffer was changed to PBS/0.05% Tween 20 and a dilution series of the peptides was prepared in this buffer with a final DMSO concentration of 0.5%. The top peptide concentration was 100nM with 6 further 2-fold dilutions. The SPR analysis was run at 25°C at a flow rate of 50µl/min with 60 seconds association and dissociation between 400 and 1,200 seconds depending upon the individual peptide. Data were corrected for DMSO excluded volume effects. All data were double-referenced for blank injections and reference surface using standard processing procedures and data processing and kinetic fitting were performed using Scrubber software, version 2.0c (BioLogic Software). Data were fitted using simple 1:1 binding model allowing for mass transport effects where appropriate.

Certain heterotandem peptides of the invention were tested in the above mentioned Nectin-4 binding assays and the results are shown in Table 2 below:

Table 2: Nectin-4 Biacore Assay Data with Heterotandem Peptides

Complex ID	SPR K_D(nM)
BCY8854	2.76
BCY9350	> 200 nM
BCY9351	2.47
BCY9399	1.67

BCY9400	1.8
BCY9408	1.57
BCY9409	1.66
BCY9410	1.49
BCY9411	1.48
BCY9759	2.14
BCY10000	2.26

3. EphA2 Biacore Experimental Description

Biacore experiments were performed to determine k_a ($M^{-1}s^{-1}$), k_d (s^{-1}), K_D (nM) values of heterotandem peptides binding to human EphA2 protein.

EphA2 were biotinylated with EZ-Link™ Sulfo-NHS-LC-Biotin for 1 hour in 4mM sodium acetate, 100mM NaCl, pH 5.4 with a 3x molar excess of biotin over protein. The degree of labelling was determined using a Fluorescence Biotin Quantification Kit (Thermo) after dialysis of the reaction mixture into PBS. For analysis of peptide binding, a Biacore T200 instrument was used with a XanTec CMD500D chip. Streptavidin was immobilized on the chip using standard amine-coupling chemistry at 25°C with HBS-N (10 mM HEPES, 0.15 M NaCl, pH 7.4) as the running buffer. Briefly, the carboxymethyl dextran surface was activated with a 7 min injection of a 1:1 ratio of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)/0.1 M N-hydroxy succinimide (NHS) at a flow rate of 10 µl/min. For capture of streptavidin, the protein was diluted to 0.2 mg/ml in 10 mM sodium acetate (pH 4.5) and captured by injecting 120µl onto the activated chip surface. Residual activated groups were blocked with a 7 min injection of 1 M ethanolamine (pH 8.5):HBS-N (1:1). Buffer was changed to PBS/0.05% Tween 20 and biotinylated EphA2 was captured to a level of 500-1500 RU using a dilution of protein to 0.2µM in buffer. A dilution series of the peptides was prepared in this buffer with a final DMSO concentration of 0.5% with a top peptide concentration was 50 or 100nM and 6 further 2-fold dilutions. The SPR analysis was run at 25°C at a flow rate of 90µl/min with 60 seconds association and 900-1200 seconds dissociation. Data were corrected for DMSO excluded volume effects. All data were double-referenced for blank injections and reference surface using standard processing procedures and data processing and kinetic fitting were performed using Scrubber software, version 2.0c (BioLogic Software). Data were fitted using simple 1:1 binding model allowing for mass transport effects where appropriate.

Certain heterotandem peptides of the invention were tested in the EphA2 binding assays and the results are shown in Table 3 below:

Table 3: EphA2 Biacore Assay Data with Heterotandem Peptides

Complex ID	SPR K_D(nM)
BCY9173	2.1
BCY7985	2
BCY8942	1.7
BCY8943	> 200 nM
BCY9647	1.69
BCY9648	1.75
BCY9655	1.33
BCY9656	0.75
BCY9657	1.1
BCY9658	1.9
BCY9659	1.03
BCY9758	1.5

4. CD137 reporter assay co-culture with tumour cells

Culture medium referred to as R1 media is prepared by adding 1 % FBS to RPMI-1640 (component of Promega kit CS196005). Serial dilutions of test articles in R1 are prepared in a sterile 96 well-plate. Use 25 µl per well of test articles or R1 (as a background control) to designated wells in white cell culture plate. Tumour cells* are harvested and resuspended at a concentration of 400,000 cells/mL in R1 media. Twenty five (25) µL/well tumour cells are used in white cell culture plate. Jurkat cells (Promega kit CS196005, 0.5 mL) are thawed in the water bath and then added to 5 ml pre-warmed R1 medium. Twenty five (25) µL/well Jurkat cells are used in white cell culture plate. Incubate the cells and test articles for 6h at 37°C, 5 % CO_2. At the end of 6h, add 75 µl/well Bio-Glo™ (Promega) and incubate for 10 min before reading luminescence in a plate reader (Clariostar, BMG). The fold change relative to cells (Jurkat cells + Cell line used in co-culture) is calculated and plotted in GraphPad Prism as log(agonist) vs response to determine EC50(nM) and Fold Induction over background (Max)

The tumour cell type used in co-culture is dependent on the tumour target specific for heterotandem as is shown in Table 4 below:

Table 4: Cell Lines Used for each Tumour Target

Tumour target	Cell line used in co-culture
EphA2	A549, SC-OV-3, PC3, LNCaP
Nectin-4	HT1376, NCI-H292
PD-L1	RKO

Data is presented in Figure 3 which shows that the EphA2-CD137 heterotandem BCY7985 showed strong induction of CD137 cell activity in the Promega CD137 luciferase reporter assay in the presence of EphA2-expressing HT1080 cells. There is no CD137 induction by the heterotandem in the absence of HT1080 cells.

Data is presented in Figure 4 which shows that EphA2/CD137 heterotandems induce strong CD137 activation in CD137 reporter assay and the fold induction of activation is dependent on tumour target expression level on the cell line (A549 and SC-OV-3:EphA2 High and LNCaP:EphA2 Low) used in co-culture.

Data is presented in Figure 6 which shows that Nectin-4/CD137 heterotandems induce strong CD137 activation in CD137 reporter assay and the fold induction of activation is dependent on tumour target expression level on the cell line (HT1376:Nectin-4 high and NCI-H292: Nectin-4 Medium) used in co-culture.

Data is presented in Figure 9 which shows that PD-L1/CD137 heterotandems induce strong activation of CD137 in the CD137 reporter assay in presence of PD-L1 expressing cell line.
A summary of the EC50(nM) and Fold Induction induced by heterotandem peptides in CD137 reporter assay in co-culture with different cells lines are reported in Table 5 below:

Table 5: Fold Induction induced by Heterotandem Peptides in CD137 Reporter Assay

Complex ID	Tumour Target	Cell Line used in Coculture	EC50 (nM)	Fold Induction over Background
BCY9173	EphA2	SC-OV-3	0.94	21
BCY7985	EphA2	SC-OV-3	4.0	15
BCY8942	EphA2	PC3	-	< 2 fold induction at 100 nM
BCY8943	EphA2	PC3	-	< 2 fold induction at 100 nM
BCY9647	EphA2	SC-OV-3	7.2	24

BCY9648	EphA2	SC-OV-3	9.3	20
BCY9655	EphA2	SC-OV-3	4.1	6
BCY9656	EphA2	SC-OV-3	1.1	3
BCY9657	EphA2	SC-OV-3	9.0	26
BCY9658	EphA2	SC-OV-3	6.2	11
BCY9659	EphA2	SC-OV-3	9.9	7
BCY9758	EphA2	SC-OV-3	1.2	7
BCY10568	EphA2	PC3	0.25	32
BCY10570	EphA2	PC3	0.41	38
BCY10574	EphA2	PC3	1.0	32
BCY10575	EphA2	PC3	0.62	38
BCY10576	EphA2	PC3	0.51	38
BCY10577	EphA2	PC3	0.28	37
BCY8854	Nectin4	H1376	1.2	30
BCY9350	Nectin4	H1376	-	< 2 fold induction at 100 nM
BCY9351	Nectin4	H1376	-	< 2 fold induction at 100 nM
BCY9399	Nectin4	H1376	11	13
BCY9400	Nectin4	H1376	2.9	13
BCY9401	Nectin4	H1376	18	70
BCY9407	Nectin4	H1376	3.4	29
BCY9408	Nectin4	H1376	1.1	20
BCY9409	Nectin4	H1376	1.2	24
BCY9410	Nectin4	H1376	1.3	24
BCY9411	Nectin4	H1376	14	41
BCY9759	Nectin4	H1376	2.7	15
BCY10000	Nectin4	H1376	0.58	61
BCY10567	Nectin4	H1376	1.7	45
BCY10569	Nectin4	H1376	1.2	52
BCY10571	Nectin4	H1376	3.5	60
BCY10572	Nectin4	H1376	0.44	55
BCY10573	Nectin4	H1376	0.90	55
BCY10578	Nectin4	H1376	0.42	58
BCY10917	Nectin4	H1376	0.27	54

BCY11020	Nectin4	H1376	0.26	47
BCY11373	Nectin4	H1376	0.16	74
BCY11374	Nectin4	H1376	0.091	72
BCY11375	Nectin4	H1376	0.23	72
BCY8939	mouse PD-L1	MC38	-	< 2 fold induction at 100 nM
BCY10580	PD-L1	RKO	28	3
BCY10581	PD-L1	RKO	18	6
BCY10582	PD-L1	RKO	28	4
BCY11017	PD-L1	RKO	66	4
BCY11018	PD-L1	RKO	27	7
BCY11019	PD-L1	RKO	18	6
BCY11376	PD-L1	RKO	127	9
BCY11377	PD-L1	RKO	40	6
BCY11378	PD-L1	RKO	80	3
BCY11379	PD-L1	RKO	68	6
BCY11380	PD-L1	RKO	34	7
BCY11381	PD-L1	RKO	105	7

5. Primary human T cells-A549 co-culture (Tumour cell killing)

PBMC were isolated from three healthy donors and added to Nuclight Red labelled tumour target cells (human lung carcinoma cells A549®, ATCC CLL-185™) at two defined ratios in the presence of anti-CD3 stimulation at two concentrations. Tumour cell: PBMC co-cultures were incubated with the lead bicycles at three concentrations. All test conditions were also plated onto tumour cells in the absence of stimulated PBMC in order to detect direct tumour cell cytotoxicity. Tumour killing was evaluated by counting viable Nuclight red positive tumour cells over time. In addition, a Caspase 3/7 dye was used to identify apoptotic tumour cells. Cultures were analysed using an IncuCyte S3 machine which allows real-time live cell fluorescence imaging. Co-cultures were imaged for 72 hours. Each condition was established in triplicate.

Data is presented in Figure 5 which demonstrates that EphA2/CD137 heterotandems induce tumour cell killing in primary human T-cell and cancer cell co-culture assay. Anti-CD137 mAb agonist is used as a control.

6. Human PBMC-4T1 co-culture (cytokine release) assay

Mouse mammary gland tumor cell line 4T1-1 (4T1-Parental) and murine Nectin-4 overexpressing 4T1 (4T1-D02) were cultured in RPMI1640 supplemented with 10% heat-inactivated Fetal Bovine Serum, 100 I.U/ml Penicillin and 100 I.U/Streptomycin, 20 mM HEPES, 1X Non-Essential Amino Acids, and 2 mM L-Glutamine (RPMI working medium). Frozen PBMCs from healthy human donors were thawed and washed one time in room temperature PBS, and then resuspended in RPMI working medium. For tumor cell and PBMC co-culture, 10000 PBMCs and 2000 tumor cells (5:1) were mixed and plated in each well of a 384 well plate. For stimulating human PBMCs, 125 ng/ml of soluble anti-CD3 mAb (clone OKT3) was added to the culture on day 0. Test, control compounds or vehicle controls were added to respective wells and brought the final volume per well to 100ul. Plates were incubated in a 37ºC cell culture incubator with 5% CO_2 for up to three days. Supernatants were collected 48 hours after stimulation, and human IL-2 and IFNγ were detected using HTRF assays. Raw data were analyzed using Excel or Prism software to generate standard curves to interpolate protein concentrations. Data represents one study with three different donor PBMC tested in experimental duplicates.

Data presented in Figure 7 demonstrates that Nectin-4/CD137 heterotandems induce robust IL-2 and IFN-γ cytokine secretion in a PBMC-4T1 co-culture assay. BCY9350 and BCY9351 are non-binding controls for Nectin-4 and CD137 respectively.

A summary of the EC50(nM) and maximum IFN-y cytokine secretion (pg/ml) induced by selected Nectin-4/CD137 heterotandem peptides in Human PBMC-4T1 co-culture (cytokine release) assay is reported in Table 6 below:

Table 6: EC50 and maximum IFN-y cytokine secretion induced by selected Nectin-4/CD137 heterotandem peptides in Human PBMC-4T1 co-culture (cytokine release) assay

Complex ID	Cell line	EC50 (nM)	max IFN-y (pg/ml)
BCY8854	4T1-D02(Nectin4+)	0.89	15962
BCY9350	4T1-D02(Nectin4+)	-	No Activity up to 1 μM
BCY9351	4T1-D02(Nectin4+)	-	No Activity up to 1 μM

BCY10000	4T1-D02(Nectin4+)	0.21	19642
BCY10571	4T1-D02(Nectin4+)	0.44	18349
BCY10572	4T1-D02(Nectin4+)	0.25	17915

7. *Ex vivo* culture protocol

Primary patient derived tumour cells from Discovery Life Sciences (DLS) are thawed gently in 10mL pre-warmed wash medium spiked fresh with Benzonase. The 3D spheroid kit from Greiner (cat# 655840) is used to maintain cells in culture for 2 days. Briefly, tumour cells are counted with trypan blue using a haemocytometer. The cells are centrifuged at 1500rpm for 5min to wash, and the pellet is resuspended in 100µL per $1X10^6$ cells N3D nanoshuttle. To make them magnetic, cells are spun down at 1500rpm for 5 min and resuspended; this process is repeated for a total of 4 times. After the final spin, cells are resuspended in the appropriate amount of fresh Lung DTC medium (DLS) to give 50,000-100,000 cells per well in 100µL/well. Greiner cell-repellent, 96-well plates (cat #655976) are used for this experiment. If there are cell clumps or debris visible, sample is applied to a 70-100µm filter before plating. At least 50,000 cells per sample are reserved for a Day 0 flow cytometry panel, these cells are stained, fixed, and stored at 4°C for later flow analysis. Control/test compound dilutions are prepared in a separate plate at 2x in Lung DTC medium, and 100µL/well of these 2X drug solutions are added to the wells as described by the plate map. The assay plate is then placed onto the 96-well magnetic spheroid drive in a humidified chamber at 37C, 5% CO_2. At 24h, the magnetic spheroid drive is removed. At 48h, medium is collected for cytokine analysis and cells are collected for a Day 2 flow cytometry panel. Cytokines are quantified using a custom-built cytokine/chemokine panel (IP-10, Granzyme B, IFNγ, IL-2, IL-6, TNFα, IL-8, MIP-1a, MIP-1b, MCP-1, IL-10, MIG) from R&D systems on a Luminex reader. Flow panels: Day 0 = Live/Dead, CD45, EpCAM, Nectin4, CD3, CD4, CD8, CD137; Day 2 = Live/Dead, CD45, EpCAM, Nectin4, CD3, CD8, Ki67, and counting beads. Flow data is analysed with Flowjo software.

Data shown in Figure 8 demonstrate that Nectin-4/CD137 heterotandems induce target dependent cytokine release in ex-vivo cultures of primary patient-derived lung tumours. Treatment of BCY10572 induced Nectin-4 dependent change in several immune markers (normalized to vehicle) and in $\%CD8^{+}ki67^{+}$ T cells in patient-derived samples.

8. Pharmacokinetics of CD137 Bispecifics in SD Rats

Male SD Rats were dosed with 2 mg/kg of each Bicycle multimer formulated in 25 mM Histidine HCl, 10% sucrose pH 7. Serial bleeding (about 80 µL blood/time point) was performed via submadibular 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 was immediately added into the plasma, mixed well and centrifuged at 12,000 rpm, 4°C for 10 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. 7.5 µL of the supernatant samples were directly injected for LC-MS/MS analysis using an Orbitrap Q Exactive in positive ion mode to determine the concentrations of Bicycle multimer. Plasma concentration versus time data were analyzed by non-compartmental approaches using the Phoenix WinNonlin 6.3 software program. C0, Cl, Vdss, T½, AUC(0-last), AUC(0-inf), MRT(0-last) , MRT(0-inf) and graphs of plasma concentration versus time profile were reported.

Figure 10 shows the plasma concentration vs time curve of BCY10572 and BCY10000 from a 2 mg/kg IV dose in SD Rat (n =3). The pharmacokinetic parameters from the experiment are as shown in Table 7:

Table 7: Pharmacokinetic Parameters of plasma concentration vs time curve of BCY10572 and BCY10000

Compound	T1/2(h)	Clp (ml/min/kg)	Vdss (L/kg)
BCY10000	0.357	16.1	0.395
BCY10572	0.926	15.6	0.882

CLAIMS

1. A heterotandem bicyclic peptide complex comprising:

(a) a first peptide ligand which binds to a component present on an immune cell; conjugated via a linker to

(b) a second peptide ligand which binds to a component present on a cancer cell;

wherein each of said peptide ligands comprises a polypeptide comprising at least three cysteine residues, separated by at least two loop sequences, and a molecular scaffold which forms covalent bonds with the cysteine residues of the polypeptide such that at least two polypeptide loops are formed on the molecular scaffold.

2. The heterotandem bicyclic peptide complex as defined in claim 1, wherein the immune cell is selected from: white blood cells; lymphocytes (e.g. T lymphocytes or T cells, B cells or natural killer cells); CD8 or CD4; CD8; dendritic cells, follicular dendritic cells and granulocytes.

3. The heterotandem bicyclic peptide complex as defined in claim 1 or claim 2, wherein the component present on an immune cell is CD137.

4. The heterotandem bicyclic peptide complex as defined in any one of claims 1 to 3, wherein the first peptide ligand comprises a CD137 binding bicyclic peptide ligand.

5. The heterotandem bicyclic peptide complex as defined in claim 4, wherein the CD137 binding bicyclic peptide ligand comprises an amino acid sequence selected from:

C_iIEEGQYC_{ii}FADPY[Nle]C_{iii} (SEQ ID NO: 1);

C_i[tBuAla]PE[D-Ala]PYC_{ii}FADPY[Nle]C_{iii} (SEQ ID NO: 3);

C_iIEEGQYC_{ii}F[D-Ala]DPY[Nle]C_{iii} (SEQ ID NO: 4);

C_i[tBuAla]PK[D-Ala]PYC_{ii}FADPY[Nle]C_{iii} (SEQ ID NO: 5);

C_i[tBuAla]PE[D-Lys]PYC_{ii}FADPY[Nle]C_{iii} (SEQ ID NO: 6);

C_i[tBuAla]P[K(PYA)][D-Ala]PYC_{ii}FADPY[Nle]C_{iii} (SEQ ID NO: 7);

C_i[tBuAla]PE[D-Lys(PYA)]PYC_{ii}FADPY[Nle]C_{iii} (SEQ ID NO: 8);

C_iIEE[D-Lys(PYA)]QYC_{ii}FADPY(Nle)C_{iii} (SEQ ID NO: 9); and

[dC_i][dI][dE][dE][K(PYA)][dQ][dY][dC_{ii}][dF][dA][dD][dP][dY][dNle][dC_{iii}] (SEQ ID NO: 10);

wherein C_i, C_{ii} and C_{iii} represent first, second and third cysteine residues, respectively, Nle represents norleucine, tBuAla represents t-butyl-alanine, PYA represents 4-pentynoic acid, or a pharmaceutically acceptable salt thereof.

6. The heterotandem bicyclic peptide complex as defined in claim 5, wherein the CD137 binding bicyclic peptide ligand comprises N- and C-terminal modifications and comprises:

Ac-A-(SEQ ID NO: 1)-Dap (hereinafter referred to as BCY7732);

Ac-A-(SEQ ID NO: 1)-Dap(PYA) (hereinafter referred to as BCY7741);

Ac-(SEQ ID NO: 3)-Dap (hereinafter referred to as BCY9172);

Ac-(SEQ ID NO: 3)-Dap(PYA) (hereinafter referred to as BCY11014);

Ac-A-(SEQ ID NO: 4)-Dap (hereinafter referred to as BCY8045);

Ac-(SEQ ID NO: 5)-A (hereinafter referred to as BCY8919);

Ac-(SEQ ID NO: 6)-A (hereinafter referred to as BCY8920);

Ac-(SEQ ID NO: 7)-A (hereinafter referred to as BCY8927);

Ac-(SEQ ID NO: 8)-A (hereinafter referred to as BCY8928);

Ac-A-(SEQ ID NO: 9)-A (hereinafter referred to as BCY7744); and

Ac-[dA]-(SEQ ID NO: 10)-[dA]-NH_2 (hereinafter referred to as BCY11506);

wherein Ac represents an acetyl group, Dap represents diaminopropionic acid and PYA represents 4-pentynoic acid, or a pharmaceutically acceptable salt thereof.

7. The heterotandem bicyclic peptide complex as defined in any one of claims 1 to 6, wherein the cancer cell is selected from an HT1080, SC-OV-3, PC3, H1376, NCI-H292, LnCap, MC38 and RKO tumor cell.

8. The heterotandem bicyclic peptide complex as defined in any one of claims 1 to 7, wherein the component present on a cancer cell is EphA2.

9. The heterotandem bicyclic peptide complex as defined in any one of claims 1 to 8, wherein the second peptide ligand comprises an EphA2 binding bicyclic peptide ligand.

10. The heterotandem bicyclic peptide complex as defined in claim 9, wherein the EphA2 binding bicyclic peptide ligand comprises an amino acid sequence selected from:

C_i[HyP]LVNPLC_{ii}LHP[dD]W[HArg]C_{iii} (SEQ ID NO: 2); and

C_iLWDPTPC_{ii}ANLHL[HArg]C_{iii} (SEQ ID NO: 11);

wherein C_i, C_{ii} and C_{iii} represent first, second and third cysteine residues, respectively, HyP represents hydroxyproline, dD represents aspartic acid in D-configuration and HArg represents homoarginine, or a pharmaceutically acceptable salt thereof.

11. The heterotandem bicyclic peptide complex as defined in claim 10, wherein the EphA2 binding bicyclic peptide ligand comprises N-terminal modifications and comprises:

A-HArg-D-(SEQ ID NO: 2) (hereinafter referred to as BCY9594);

[B-Ala]-[Sar_{10}]-A-[HArg]-D-(SEQ ID NO: 2) (hereinafter referred to as BCY6099);

[PYA]-[B-Ala]-[Sar_{10}]-A-[HArg]-D-(SEQ ID NO: 2) (hereinafter referred to as BCY6169); and

[PYA]-[B-Ala]-[Sar_{10}]-VGP-(SEQ ID NO: 11) (hereinafter referred to as BCY8941);

wherein HArg represents homoarginine, PYA represents 4-pentynoic acid, Sar_{10} represents 10 sarcosine units, B-Ala represents beta-alanine, or a pharmaceutically acceptable salt thereof.

12. The heterotandem bicyclic peptide complex as defined in any one of claims 9 to 11 which is a CD137/EphA2 complex selected from: BCY9173, BCY7985, BCY8942, BCY8943, BCY9647, BCY9648, BCY9655, BCY9656, BCY9657, BCY9658, BCY9659, BCY9758, BCY10568, BCY10570, BCY10574, BCY10575, BCY10576 and BCY10577.

13. The heterotandem bicyclic peptide complex as defined in any one of claims 1 to 7, wherein the component present on a cancer cell is PD-L1.

14. The heterotandem bicyclic peptide complex as defined in any one of claims 1 to 7 and 13, wherein the second peptide ligand comprises PD-L1 binding bicyclic peptide ligand.

15. The heterotandem bicyclic peptide complex as defined in claim 14, wherein the PD-L1 binding bicyclic peptide ligand comprises an amino acid sequence selected from:

C_i[HArg]DWC_{ii}HWTFSHGHPC_{iii} (SEQ ID NO: 12);

C_iSAGWLTMC_{ii}QKLHLC_{iii} (SEQ ID NO: 13); and

C_iSAGWLTMC_{ii}Q[K(PYA)]LHLC_{iii} (SEQ ID NO: 14);

wherein C_i, C_{ii} and C_{iii} represent first, second and third cysteine residues, respectively, HArg represents homoarginine and PYA represents 4-pentynoic acid, or a pharmaceutically acceptable salt thereof.

16. The heterotandem bicyclic peptide complex as defined in claim 15, wherein the PD-L1 binding bicyclic peptide ligand comprises N-terminal and/or C-terminal modifications and comprises:

[PYA]-[B-Ala]-[Sar_{10}]-(SEQ ID NO: 12) (hereinafter referred to as BCY8938);

[PYA]-[B-Ala]-[Sar_{10}]-SDK-(SEQ ID NO: 13) (hereinafter referred to as BCY10043);

NH_2-SDK-(SEQ ID NO: 13)-[Sar_{10}]-[K(PYA)] (hereinafter referred to as BCY10044);

NH_2-SDK-(SEQ ID NO: 14) (hereinafter referred to as BCY10045); and

Ac-SDK-(SEQ ID NO: 14)-PSH (hereinafter referred to as BCY10861);

wherein PYA represents 4-pentynoic acid, B-Ala represents beta-alanine, Sar_{10} represents 10 sarcosine units, or a pharmaceutically acceptable salt thereof.

17. The heterotandem bicyclic peptide complex as defined in any one of claims 14 to 16 which is a CD137/PD-L1 complex selected from: BCY8939, BCY10580, BCY10581, BCY10582, BCY11017, BCY11018, BCY11019, BCY11376, BCY11377, BCY11378, BCY11379, BCY11380 and BCY11381.

18. The heterotandem bicyclic peptide complex as defined in any one of claims 1 to 7, wherein the component present on a cancer cell is Nectin-4.

19. The heterotandem bicyclic peptide complex as defined in any one of claims 1 to 7 and 18, wherein the second peptide ligand comprises Nectin-4 binding bicyclic peptide ligand.

20. The heterotandem bicyclic peptide complex as defined in claim 19, wherein the Nectin-4 binding bicyclic peptide ligand comprises an amino acid sequence selected from:

C_iP[1Nal][dD]C_{ii}M[HArg]DWSTP[HyP]WC_{iii} (SEQ ID NO: 15; hereinafter referred to as BCY8116);

C_iP[1Nal][dD]C_{ii}M[HArg]D[dW]STP[HyP][dW]C_{iii} (SEQ ID NO: 16; hereinafter referred to as BCY11415); and

C_iP[1Nal][dK](Sar_{10}-(B-Ala))C_{ii}M[HArg]DWSTP[HyP]WC_{iii} (SEQ ID NO: 17);

C_iPFGC_{ii}M[HArg]DWSTP[HyP]WC_{iii} (SEQ ID NO: 18; hereinafter referred to as BCY11414);

wherein C_i, C_{ii} and C_{iii} represent first, second and third cysteine residues, respectively, 1Nal represents 1-naphthylalanine, HArg represents homoarginine, HyP represents hydroxyproline, Sar_{10} represents 10 sarcosine units, B-Ala represents beta-alanine, or a pharmaceutically acceptable salt thereof.

21. The heterotandem bicyclic peptide complex as defined in claim 20, wherein the Nectin-4 binding bicyclic peptide ligand optionally comprises N-terminal modifications and comprises:

SEQ ID NO: 15 (hereinafter referred to as BCY8116);

[PYA]-[B-Ala]-[Sar_{10}]-(SEQ ID NO: 15) (hereinafter referred to as BCY8846);

SEQ ID NO: 16 (hereinafter referred to as BCY11415);

[PYA]-[B-Ala]-[Sar_{10}]-(SEQ ID NO: 16) (hereinafter referred to as BCY11942);

Ac-(SEQ ID NO: 17) (hereinafter referred to as BCY8831); and

SEQ ID NO: 18 (hereinafter referred to as BCY11414);

wherein PYA represents 4-pentynoic acid, B-Ala represents beta-alanine, Sar_{10} represents 10 sarcosine units, or a pharmaceutically acceptable salt thereof.

22. The heterotandem bicyclic peptide complex as defined in any one of claims 19 to 21 which is a CD137/Nectin-4 complex selected from: BCY8854, BCY9350, BCY9351, BCY9399, BCY9400, BCY9401, BCY9403, BCY9405, BCY9406, BCY9407, BCY9408, BCY9409, BCY9410, BCY9411, BCY9759, BCY10000, BCY10567, BCY10569, BCY10571, BCY10572, BCY10573, BCY10578, BCY10917, BCY11020, BCY11373, BCY11374, BCY11375, BCY11616, BCY11617, BCY11857, BCY11858 and BCY11859.

23. The heterotandem bicyclic peptide complex as defined in any one of claims 1 to 7, wherein the component present on a cancer cell is PSMA.

24. The heterotandem bicyclic peptide complex as defined in any one of claims 1 to 7 and 23, wherein the second peptide ligand comprises PSMA binding bicyclic peptide ligand.

25. The heterotandem bicyclic peptide complex as defined in any one of claims 1 to 24, wherein the linker is selected from: $-CH_2-$, $-PEG_5-$, $-PEG_{10}-$, $-PEG_{12}-$, $-PEG_{23}-$, $-PEG_{24}-$, $-PEG_{15}-Sar_5-$, $-PEG_{10}-Sar_{10}-$, $-PEG_5-Sar_{15}-$, $-PEG_5-Sar_5-$, -B-Ala-Sar_{20}-, -B-Ala-Sar_{10}-PEG_{10}-, -B-Ala-Sar_5-PEG_{15}- and -B-Ala-Sar_5-PEG_5-..

26. The heterotandem bicyclic peptide complex as defined in any one of claims 1 to 25, wherein the molecular scaffold is selected from 1,1',1''-(1,3,5-triazinane-1,3,5-triyl)triprop-2-en-1-one (TATA).

27. The heterotandem bicyclic peptide complex as defined in any one of claims 1 to 26, wherein the pharmaceutically acceptable salt is selected from the free acid or the sodium, potassium, calcium, ammonium salt.

28. A pharmaceutical composition which comprises the heterotandem bicyclic peptide complex of any one of claims 1 to 27 in combination with one or more pharmaceutically acceptable excipients.

29. The heterotandem bicyclic peptide complex as defined in any one of claims 1 to 27 for use in preventing, suppressing or treating cancer.

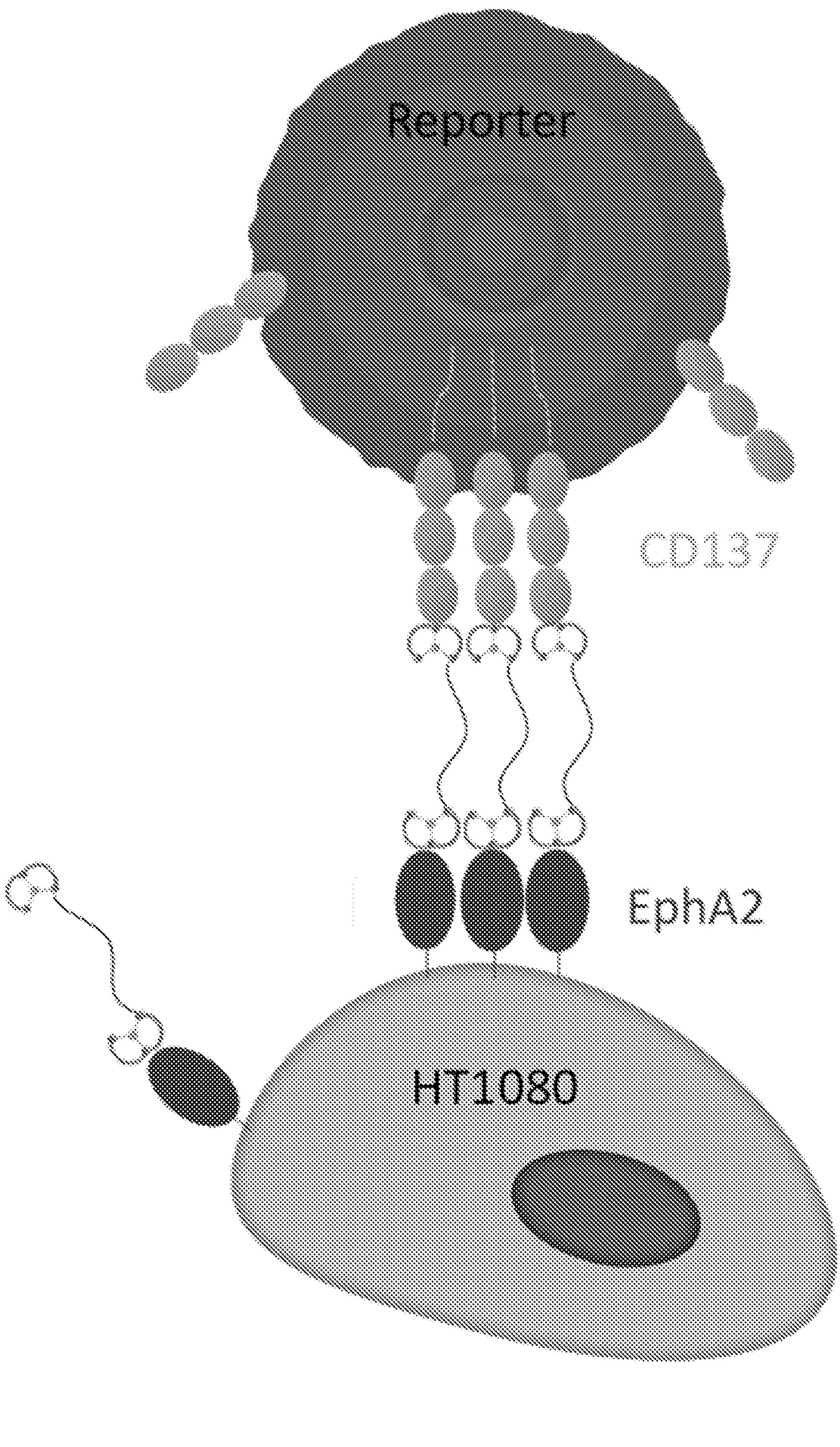


FIGURE 1

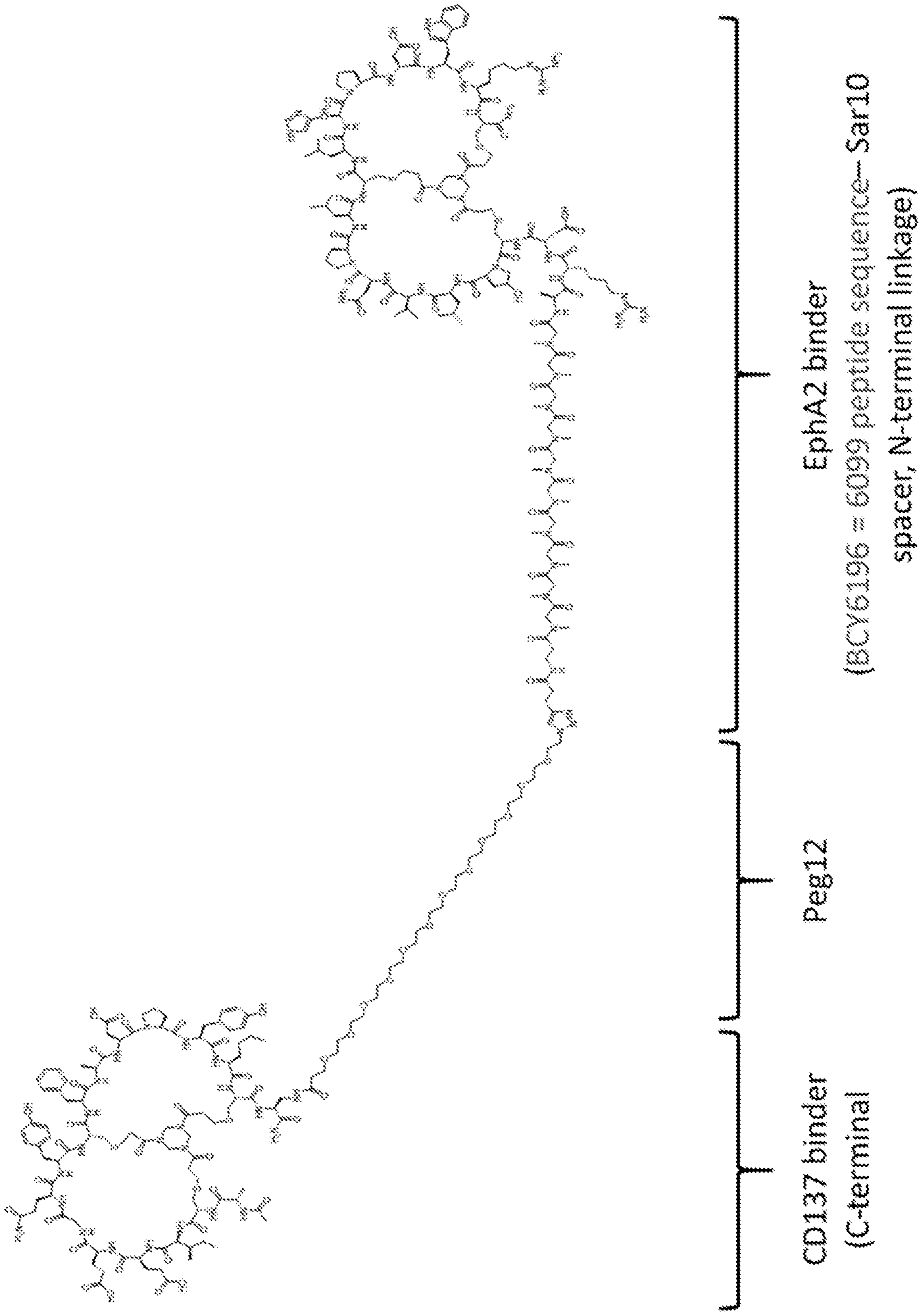

EphA2 binder
(BCY6196 = 6099 peptide sequence—Sar10
spacer, N-terminal linkage)
Peg12
CD137 binder
(C-terminal

FIGURE 2

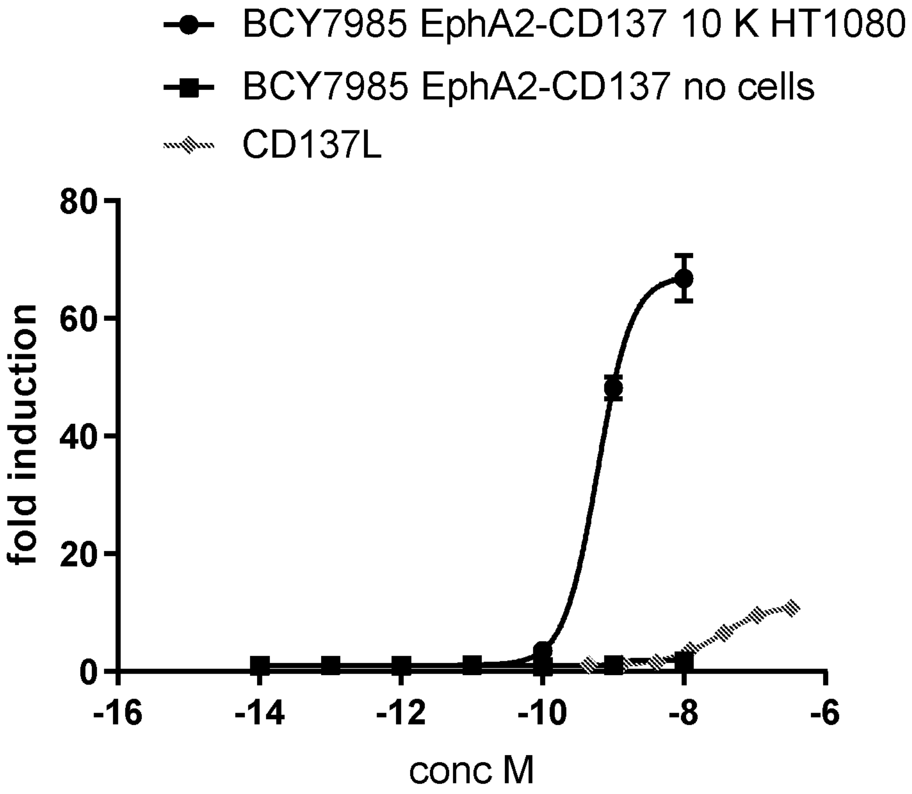

BCY7985 EphA2-CD137 10 K HT1080
BCY7985 EphA2-CD137 no cells
CD137L
80
60
40
20
0
fold induction
-16
-14
-12
-10
-8
-6
conc M

FIGURE 3

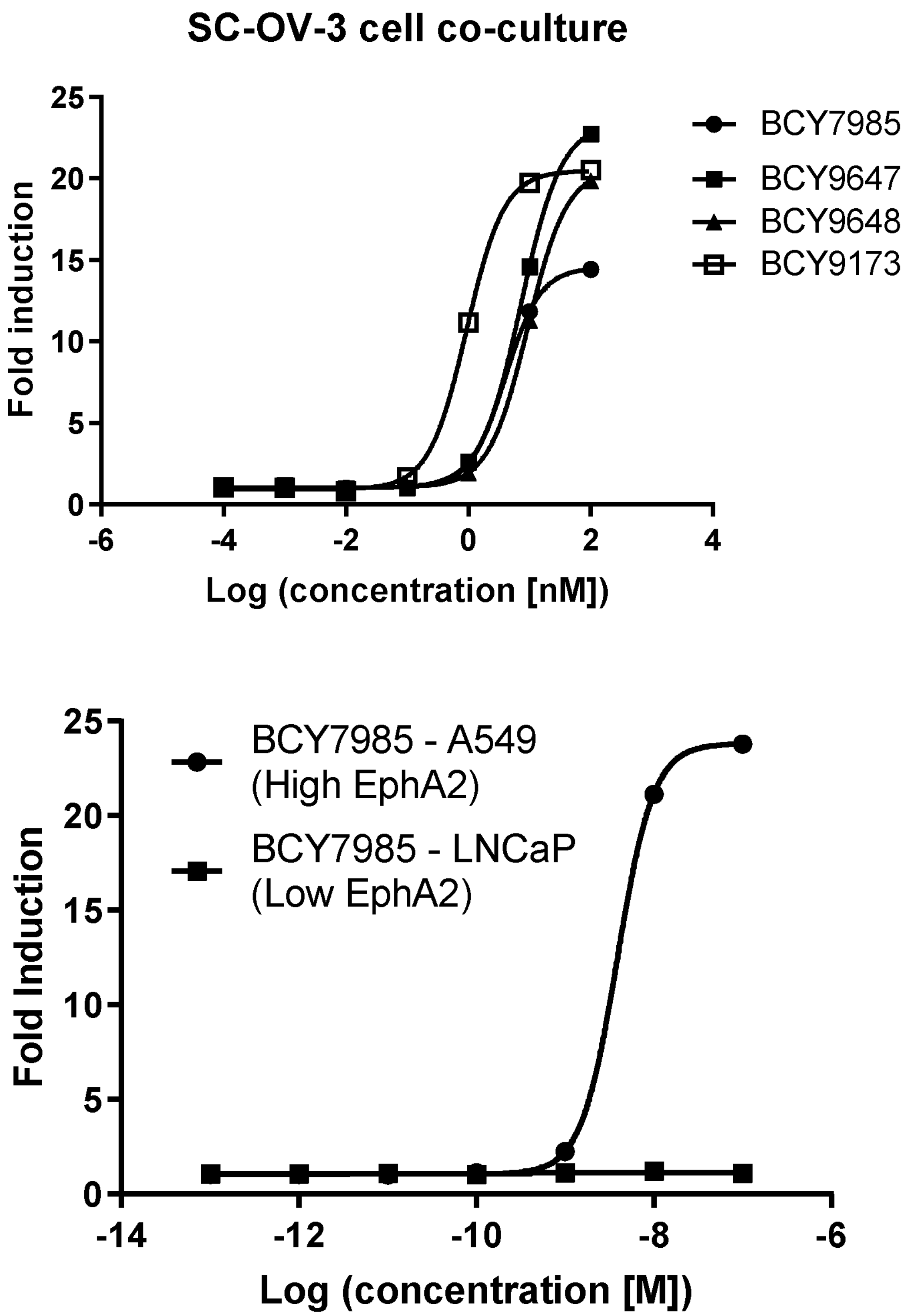

SC-OV-3 cell co-culture
Fold induction
Log (concentration [nM])
BCY7985
BCY9647
BCY9648
BCY9173
25
20
15
10
5
0
-6
-4
-2
0
2
4
BCY7985 - A549 (High EphA2)
BCY7985 - LNCaP (Low EphA2)
Fold Induction
Log (concentration [M])
-14
-12
-10
-8
-6

FIGURE 4

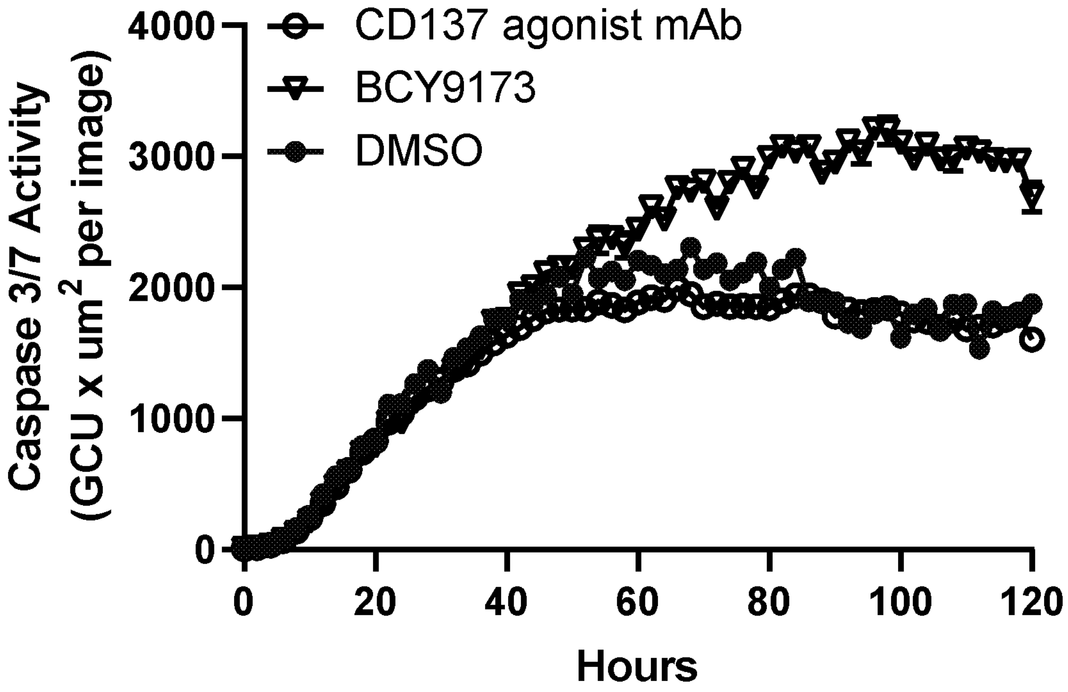

CD137 agonist mAb
BCY9173
DMSO
Caspase 3/7 Activity
(GCU x um2 per image)
4000
3000
2000
1000
0
0
20
40
60
80
100
120
Hours

FIGURE 5

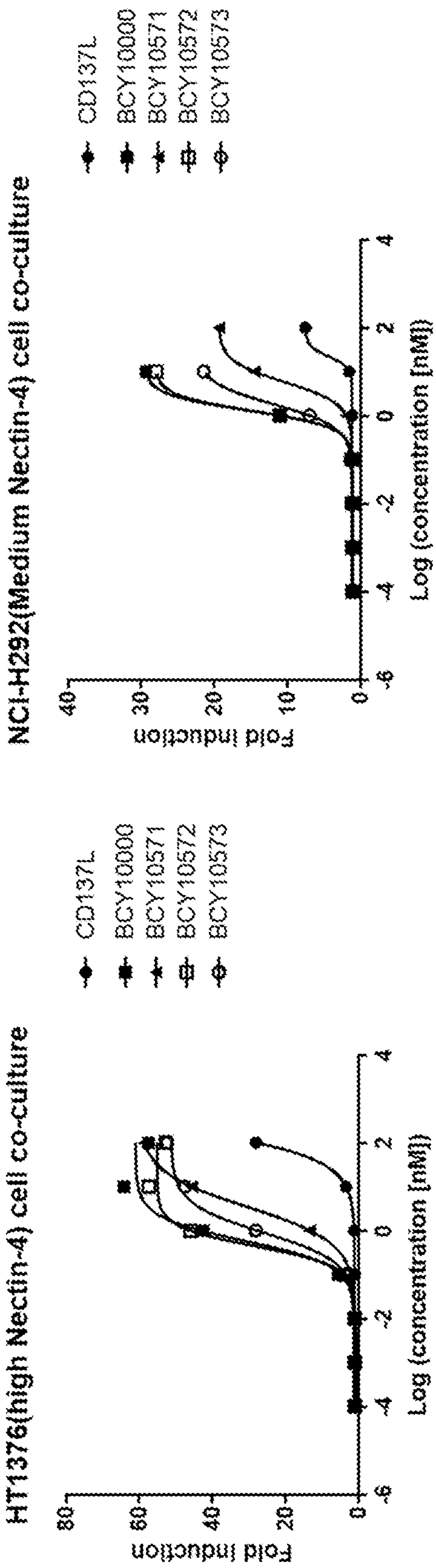
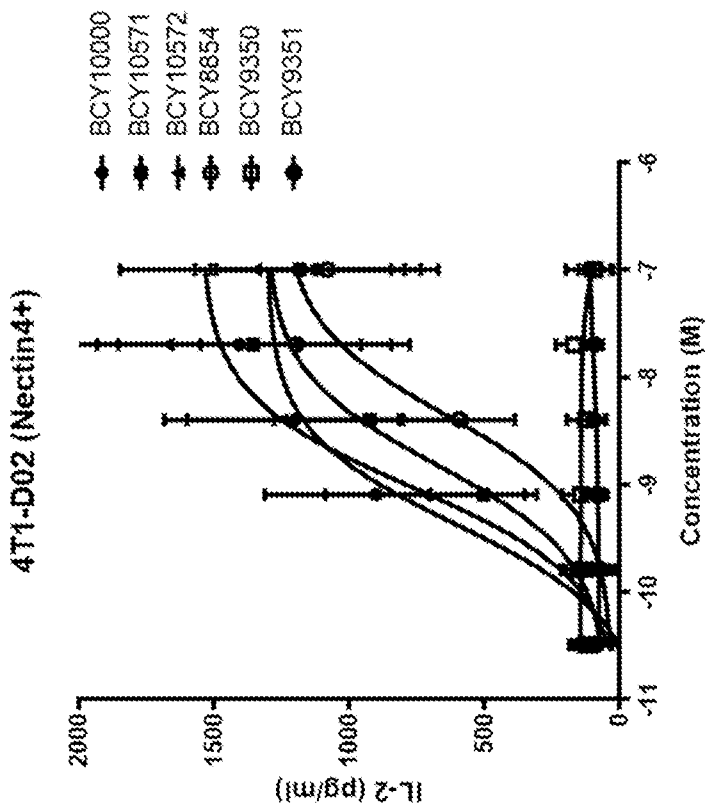

NCI-H292(Medium Nectin-4) cell co-culture
Fold induction
Log (concentration [nM])
CD137L
BCY10000
BCY10571
BCY10572
BCY10573
HT1376(high Nectin-4) cell co-culture
Fold induction
Log (concentration [nM])
CD137L
BCY10000
BCY10571
BCY10572
BCY10573

FIGURE 6


4T1-D02 (Nectin4+)
IL-2 (pg/ml)
2000
1500
1000
500
0
-11
-10
-9
-8
-7
-6
Concentration (M)
BCY10000
BCY10571
BCY10572
BCY8854
BCY9350
BCY9351

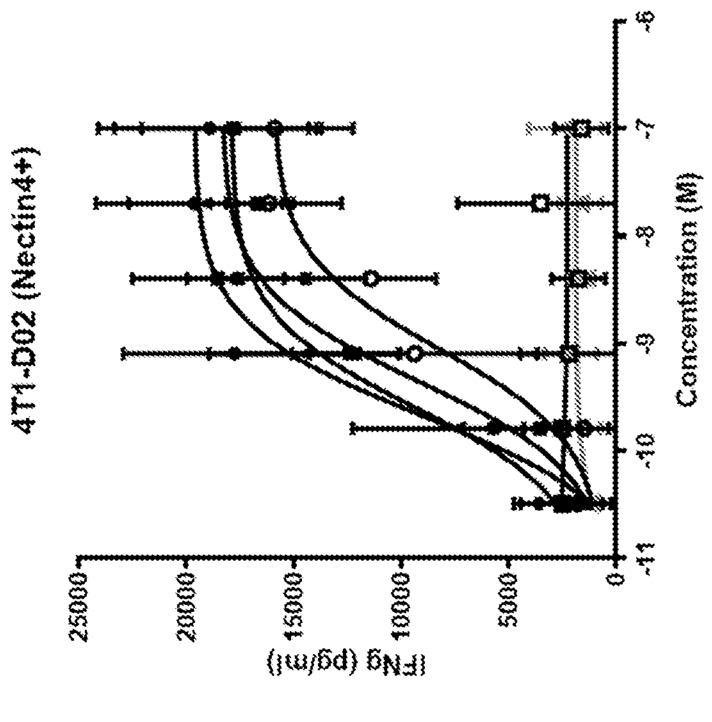
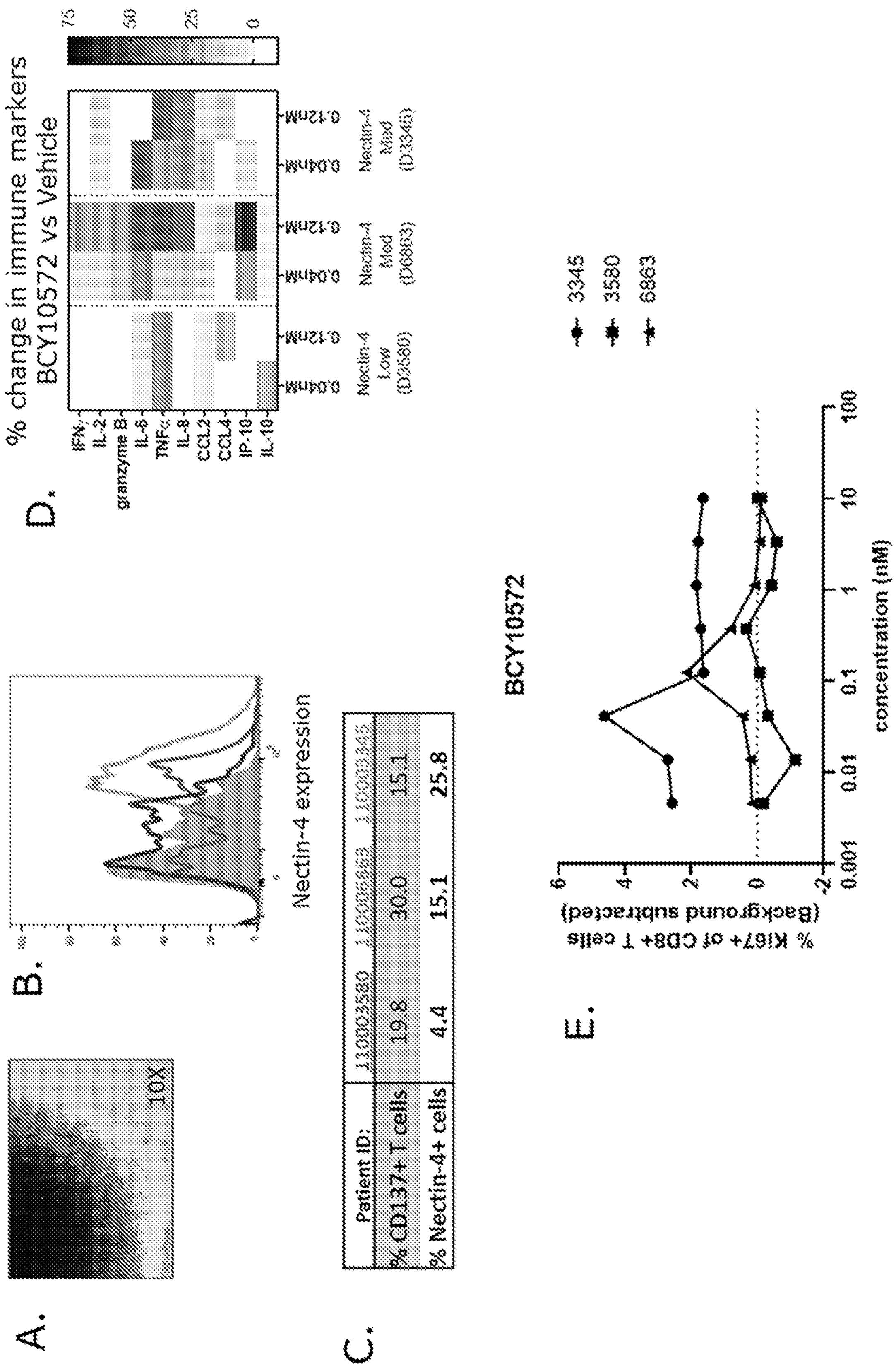

4T1-D02 (Nectin4+)
IFNg (pg/ml)
25000
20000
15000
10000
5000
0
-11
-10
-9
-8
-7
-6
Concentration (M)

FIGURE 7



Patient ID:	110003580	110006863	110003345
% CD137+ T cells	19.8	30.0	15.1
% Nectin-4+ cells	4.4	15.1	25.8

FIGURE 8

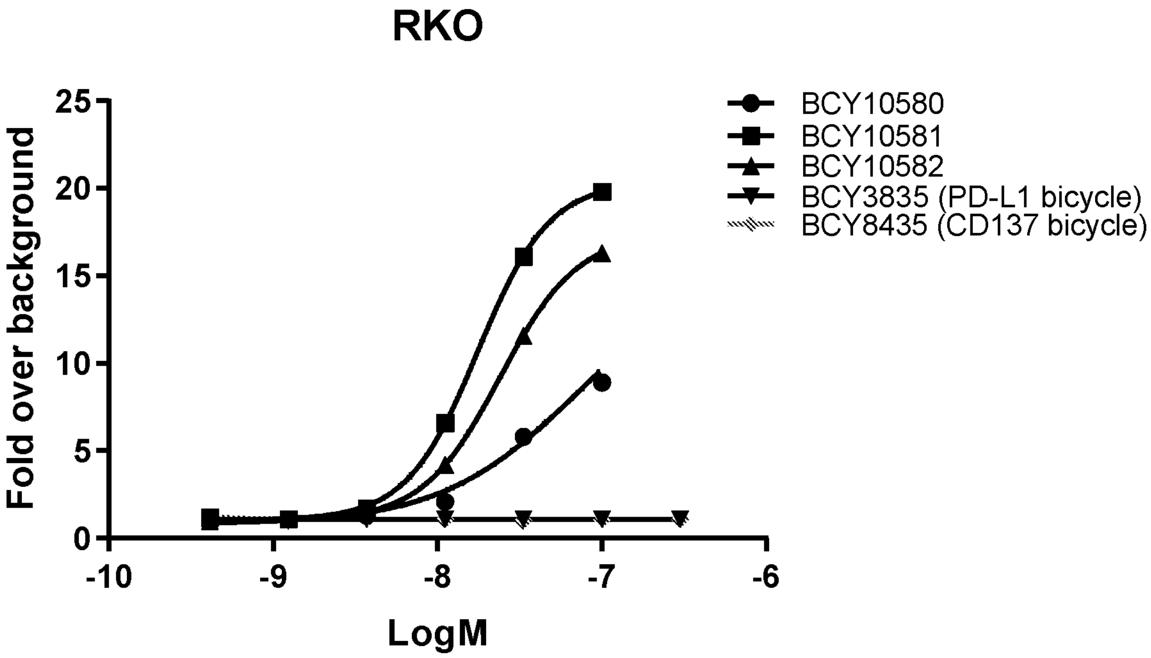


FIGURE 9

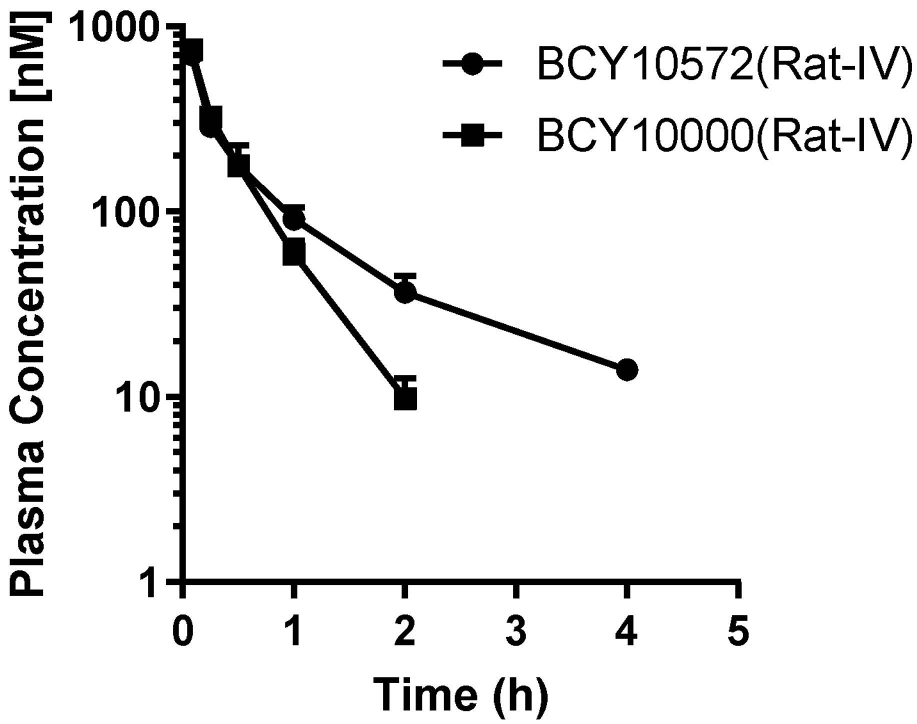


FIGURE 10

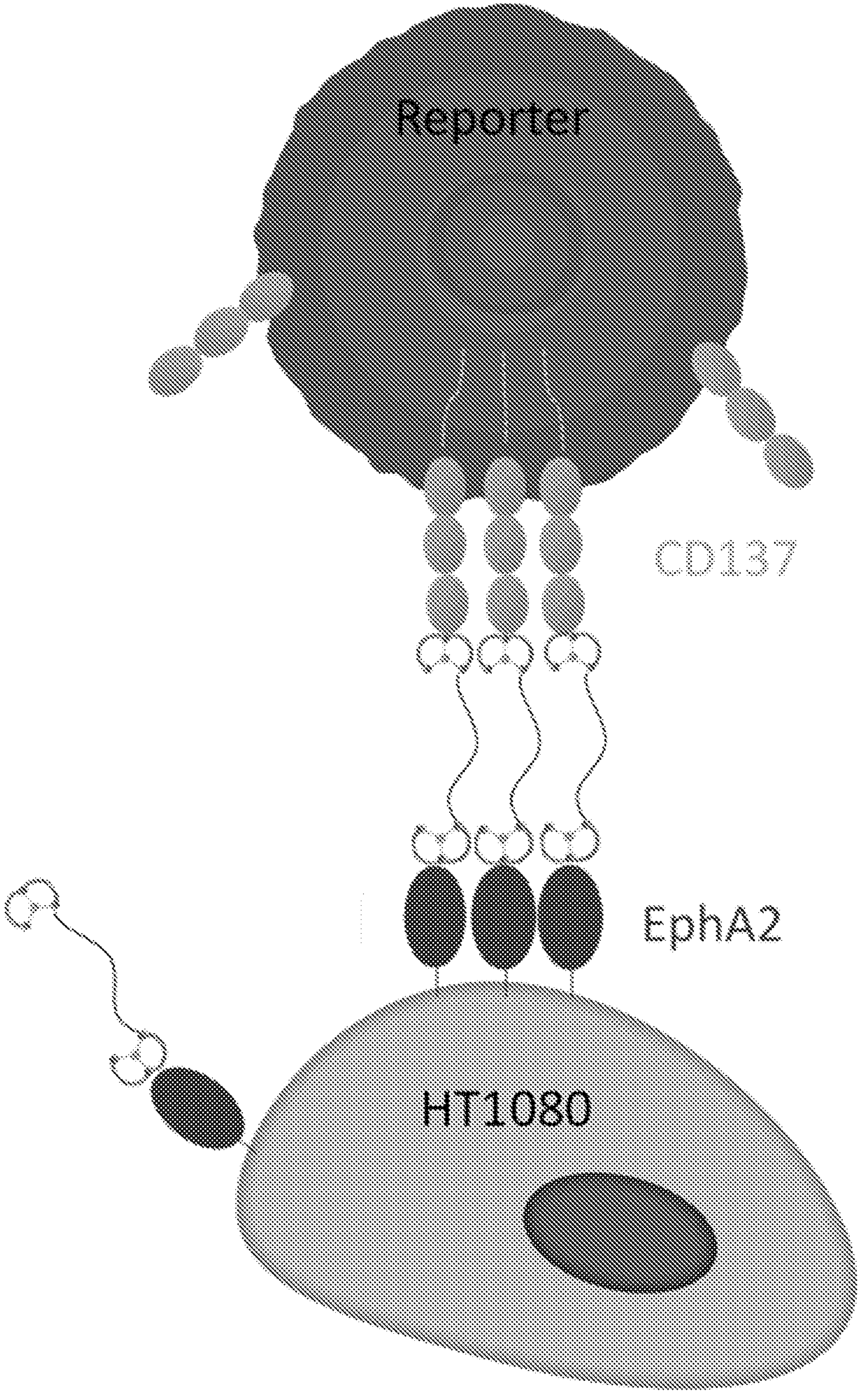


FIGURE 1