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(54) Title: IMMUNOMODULATORS

(57) Abstract: The present disclosure provides novel macrocyclic peptides which inhibit the PD-1/PD-L1 and PD-L1/CD80 protein/protein interaction, and thus are useful for the amelioration of various diseases, including cancer and infectious diseases.

IMMUNOMODULATORS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application Serial

5 Number 62/094,008 filed December 18, 2014 hereby incorporated by reference in its entirety.

The present disclosure provides novel macrocyclic peptides which inhibit the PD-1/PD-L1 and CD80/PD-L1 protein/protein interaction, and are thus useful for the amelioration of various diseases, including cancer and infectious diseases.

10 The protein Programmed Death 1 (PD-1) is an inhibitory member of the CD28 family of receptors, that also includes CD28, CTLA-4, ICOS and BTLA. PD-1 is expressed on activated B cells, T cells, and myeloid cells (Agata et al., *supra*; Okazaki et al., *Curr. Opin. Immunol.*, 14:779-782 (2002); Bennett et al., *J. Immunol.*, 170:711-718 (2003)).

15 The PD-1 protein is a 55 kDa type I transmembrane protein that is part of the Ig gene superfamily (Agata et al., *Int. Immunol.*, 8:765-772 (1996)). PD-1 contains a membrane proximal immunoreceptor tyrosine inhibitory motif (ITIM) and a membrane distal tyrosine-based switch motif (ITSM) (Thomas, M.L., *J. Exp. Med.*, 181:1953-1956 (1995); Vivier, E. et al., *Immunol. Today*, 18:286-291 (1997)).

20 Although structurally similar to CTLA-4, PD-1 lacks the MYPPY motif that is critical for CD80 CD86 (B7-2) binding. Two ligands for PD-1 have been identified, PD-L1 (B7-H1) and PD-L2 (b7-DC). The activation of T cells expressing PD-1 has been shown to be downregulated upon interaction with cells expressing PD-L1 or PD-L2 (Freeman et al., *J. Exp. Med.*, 192:1027-1034 (2000); Latchman et al., *Nat. Immunol.*, 2:261-268 (2001); Carter et al., *Eur. J. Immunol.*, 32:634-643 (2002)).

25 Both PD-L1 and PD-L2 are B7 protein family members that bind to PD-1, but do not bind to other CD28 family members. The PD-L1 ligand is abundant in a variety of human cancers (Dong et al., *Nat. Med.*, 8:787-789 (2002)). The interaction between PD-1 and PD-L1 results in a decrease in tumor infiltrating lymphocytes, a decrease in 30 T-cell receptor mediated proliferation, and immune evasion by the cancerous cells (Dong et al., *J. Mol. Med.*, 81:281-287 (2003); Blank et al., *Cancer Immunol. Immunother.*, 54:307-314 (2005); Konishi et al., *Clin. Cancer Res.*, 10:5094-5100

(2004)). Immune suppression can be reversed by inhibiting the local interaction of PD-1 with PD-L1, and the effect is additive when the interaction of PD-1 with PD-L2 is blocked as well (Iwai et al., *Proc. Natl. Acad. Sci. USA*, 99:12293-12297 (2002); Brown et al., *J. Immunol.*, 170:1257-1266 (2003)).

5 PD-L1 has also been shown to interact with CD80 (Butte MJ et al, *Immunity*, 21:111-122 (2007)). The interaction PD-L1/CD80 on expressing immune cells has been shown to be an inhibitory one. Blockade of this interaction has been shown to abrogate this inhibitory interaction (Paterson AM, et al., *J Immunol.*, 187:1097-1 105 (201 1); Yang J, et al. *J Immunol.* Aug 1;187(3): 1113-9 (201 1)).

10 When PD-1 expressing T cells contact cells expressing its ligands, functional activities in response to antigenic stimuli, including proliferation, cytokine secretion, and cytotoxicity, are reduced. PD-1/PD-L1 or PD-L2 interactions down regulate immune responses during resolution of an infection or tumor, or during the development of self tolerance (Keir, M.E. et a\., *Annu. Rev. Immunol.*, 26:Epup 15 (2008)). Chronic antigen stimulation, such as that which occurs during tumor disease or chronic infections, results in T cells that express elevated levels of PD-1 and are dysfunctional with respect to activity towards the chronic antigen (reviewed in Kim et al., *Curr. Opin. Imm.* (2010)). This is termed "T cell exhaustion". B cells also display PD-1/PD-ligand suppression and "exhaustion".

20 Blockade of PD-1/PD-L1 ligation using antibodies to PD-L1 has been shown to restore and augment T cell activation in many systems. Patients with advanced cancer benefit from therapy with a monoclonal antibody to PD-L1 (Brahmer et al., *New Engl. J. Med.* (2012)). Preclinical animal models of tumors and chronic infections have shown that blockade of the PD-1/PD-L1 pathway by monoclonal 25 antibodies can enhance the immune response and result in tumor rejection or control of infection. Antitumor immunotherapy via PD-1/PD-L1 blockade may augment therapeutic immune response to a number of histologically distinct tumors (Dong, H. et al., "B7-H1 pathway and its role in the evasion of tumor immunity", *J. Mol. Med.*, 81(5):281-287 (2003); Dong, H. et al., "Tumor-associated B7-H1 promotes T-cell 30 apoptosis: a potential mechanism of immune evasion", *Nat. Med.*, 8(8):793-800 (2002)).

Interference with the PD-1/PD-L1 interaction causes enhanced T cell activity in systems with chronic infection. Blockade of PD-L1 caused improved viral clearance and restored immunity in mice with chromoic lymphocytic chorio meningitis virus infection (Barber, D.L. et al., "Restoring function in exhausted CD8 T cells during chronic viral infection", *Nature*, 439(7077):682-687 (2006)). Humanized mice infected with HIV-1 show enhanced protection against viremia and viral depletion of CD4+ T cells (Palmer et al., *J. Immunol.* (2013)). Blockade of PD-1/PD-L1 through monoclonal antibodies to PD-L1 can restore *in vitro* antigen-specific functionality to T cells from HIV patients (Day, *Nature* (2006); Petrovas, *J. Exp. Med.* (2006); Trautman, *Nature Med.* (2006); D'Souza, *J. Immunol.* (2007); Zhang, *Blood* (2007); Kaufmann, *Nature Imm.* (2007); Kasu, *J. Immunol.* (2010); Porichis, *Blood* (2011)), HCV patients (Golden-Mason, *J. Virol.* (2007); Jeung, *J. Leuk. Biol.* (2007); Urbani, *J. Hepatol.* (2008); Nakamoto, *PLoS Path.* (2009); Nakamoto, *Gastroenterology* (2008)) and HBV patients (Boni, *J. Virol.* (2007); Fisicaro, *Gastro.* (2010); Fisicaro et al., *Gastroenterology* (2012); Boni et al., *Gastro.* (2012); Penna et al., *J. Hep.* (2012); Raziorrough, *Hepatology* (2009); Liang, *World J. Gastro.* (2010); Zhang, *Gastro.* (2008)).

Blockade of the PD-L1/CD80 interaction has also been shown to stimulate immunity (Yang J., et al., *J Immunol.* Aug 1;187(3): 1113-9 (2011)). Immune stimulation resulting from blockade of the PD-L1/CD80 interaction has been shown to be enhanced through combination with blockade of further PD-1/PD-L1 or PD-1/PD-L2 interactions.

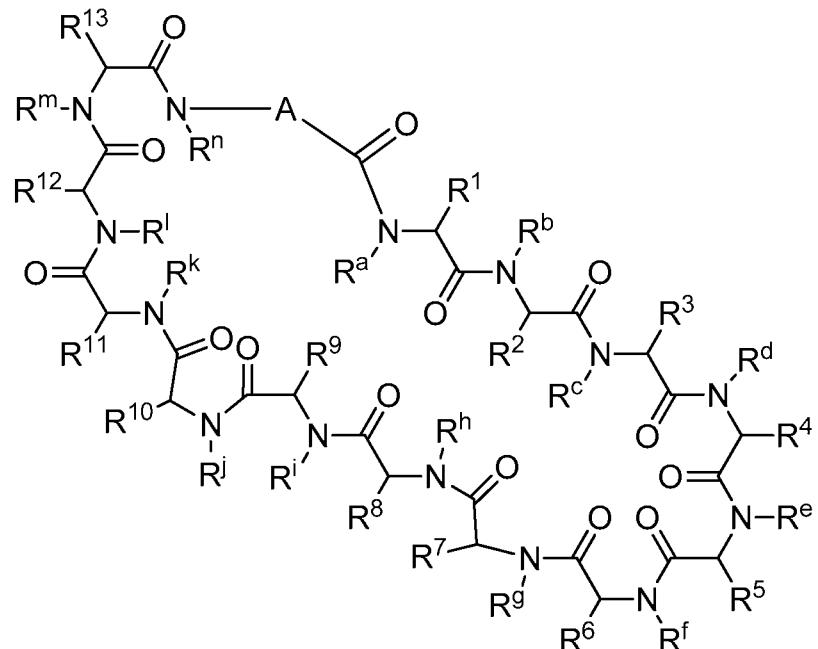
Alterations in immune cell phenotypes are hypothesized to be an important factor in septic shock (Hotchkiss, et al., *Nat Rev Immunol* (2013)). These include increased levels of PD-1 and PD-L1 (Guignant, et al, *Crit. Care* (2011)). Cells from septic shock patients with increased levels of PD-1 and PD-L1 exhibit an increased level of T cell apoptosis. Antibodies directed to PD-L1, can reduce the level of Immune cell apoptosis (Zhang et al, *Crit. Care* (2011)). Furthermore, mice lacking PD-1 expression are more resistant to septic shock symptoms than wildtype mice. Yang J., et al.. *J Immunol.* Aug 1;187(3):1 113-9 (2011)). Studies have revealed that blockade of the interactions of PD-L1 using antibodies can suppress inappropriate immune responses and ameliorate disease signs.

In addition to enhancing immunologic responses to chronic antigens, blockade of the PD-1/PD-L1 pathway has also been shown to enhance responses to vaccination, including therapeutic vaccination in the context of chronic infection (Ha, S.J. et al., "Enhancing therapeutic vaccination by blocking PD-1-mediated inhibitory signals during chronic infection", *J. Exp. Med.*, 205(3):543-555 (2008); Finnefrock, A.C. et al., "PD-1 blockade in rhesus macaques: impact on chronic infection and prophylactic vaccination", *J. Immunol.*, 182(2):980-987 (2009); Song, M.-Y. et al., "Enhancement of vaccine-induced primary and memory CD8+ t-cell responses by soluble PD-1", *J. Immunother.*, 34(3):297-306 (2011)).

10 The molecules described herein demonstrate the ability to block the interaction of PD-L1 with PD-1, in both biochemical and cell-based experimental systems. These results are consistent with a potential for therapeutic administration to enhance immunity in cancer or chronic infection, including therapeutic vaccine.

15 The macrocyclic peptides described herein are capable of inhibiting the interaction of PD-L1 with PD-1 and with CD80. These compounds have demonstrated highly efficacious binding to PD-L1, blockade of the interaction of PD-L1 with either PD-1 or CD80, and are capable of promoting enhanced T cell functional activity, thus making them candidates for parenteral, oral, pulmonary, nasal, buccal and sustained release formulations.

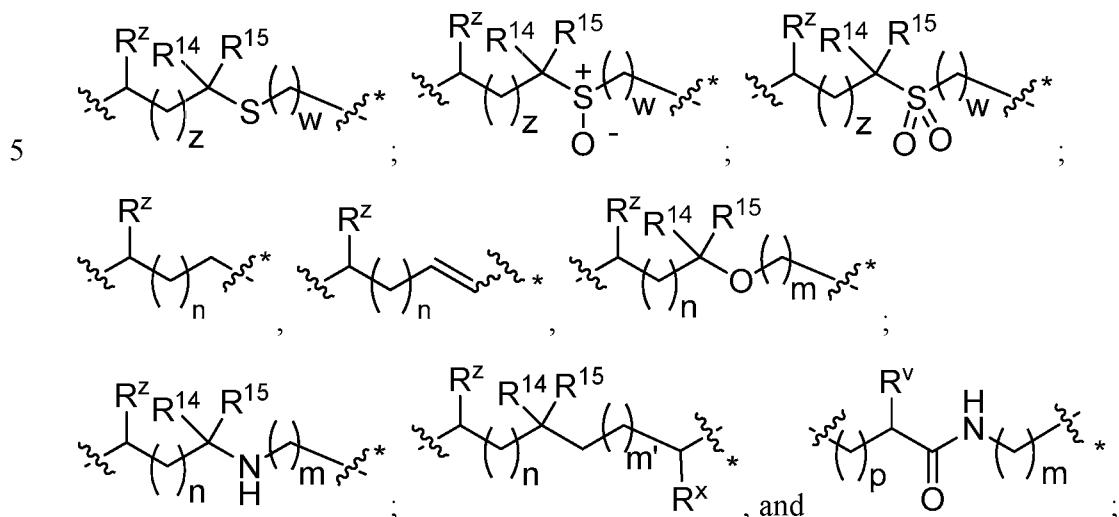
20 In one aspect the present disclosure provides a compound of formula (I).



(I),

or a pharmaceutically acceptable salt thereof, wherein:

A is selected from a bond,



wherein:

10 $\begin{array}{c} * \\ \text{~~~~~} \end{array}$ denotes the point of attachment to the carbonyl group and $\begin{array}{c} * \\ \text{~~~~~} \end{array}$ denotes the point of attachment to the nitrogen atom;

z is 0, 1, or 2;

w is 1 or 2;

n is 0 or 1;

m is 1 or 2;

m' is 0 or 1;

p is 0, 1, or 2;

R^x is selected from hydrogen, amino, hydroxy, and methyl;

5 R¹⁴ and R¹⁵ are independently selected from hydrogen and methyl; and

R^z is selected from hydrogen and -C(0)NHR¹⁶; wherein R¹⁶ is selected from hydrogen, -CHR¹⁷C(0)NH₂, -CHR¹⁷C(0)NHCHR¹⁸C(0)NH₂, and -CHR¹⁷C(0)NHCHR¹⁸C(0)NHCH₂C(0)NH₂; wherein R¹⁷ is selected from hydrogen and -CH₂OH and wherein R¹⁸ is selected from hydrogen and methyl;

10 R^v is hydrogen or a natural amino acid side chain;

R^c, R^f, R^h, Rⁱ, and R^m are hydrogen;

Rⁿ is hydrogen or methyl or R^v and Rⁿ form a pyrrolidine ring;

R^a is hydrogen or methyl;

R^j is selected from hydrogen, Ci-C₆alkoxyCi-C₆alkyl, Ci-C₆alkyl, carboxyCi-15 Cealkyl, haloCi-Cealkyl, hydroxyCi-Cealkyl, (NR^{a'}R^{b'})Ci-C₆alkyl wherein R^{a'} and R^{b'} are independently selected from hydrogen and Ci-C₆alkyl; and phenylCi-C₆alkyl wherein the phenyl is optionally substituted with one, two, three, four or five groups independently selected from Ci-C₆alkoxy, Ci-C₆alkyl, cyano, halo, and nitro;

R¹, R², R³, R⁴, R⁵, R⁶, R⁷, R⁸, R⁹, R¹⁰, R¹¹, R¹², and R¹³ are independently selected from a natural amino acid side chain and an unnatural amino acid side chain;

20 or

R¹, R², R³, R⁴, R⁵, R⁶, R⁷, R⁸, R⁹, R¹⁰, R¹¹, R¹², and R¹³ can each independently form a ring with the corresponding vicinal R group as described below;

25 R^b is selected from Ci-C₆alkoxyCi-C₆alkyl, Ci-C₆alkyl, carboxyCi-Cealkyl, haloCi-Cealkyl, hydroxyCi-Cealkyl, (NR^{a'}R^{b'})Ci-C₆alkyl wherein R^{a'} and R^{b'} are independently selected from hydrogen and Ci-C₆alkyl; and phenylCi-C₆alkyl wherein the phenyl is optionally substituted with one, two, three, four or five groups independently selected from Ci-C₆alkoxy, Ci-C₆alkyl, cyano, halo, and nitro; or, R^b and R², together with the atoms to which they are attached, form a ring selected from azetidine, pyrrolidine, morpholine, piperidine, piperazine, and tetrahydrothiazole;

wherein each ring is optionally substituted with one to four groups independently selected from amino, cyano, methyl, halo, and hydroxy;

R^d is selected from hydrogen, Ci-C₆alkoxyCi-C₆alkyl, Ci-C₆alkyl, carboxyCi-Cealkyl, haloCi-Cealkyl, hydroxyCi-Cealkyl, (NR^{a'}R^{b'})Ci-C₆alkyl wherein 5 R^{a'} and R^{b'} are independently selected from hydrogen and Ci-C₆alkyl; and phenylCi-C₆alkyl wherein the phenyl is optionally substituted with one, two, three, four or five groups independently selected from Ci-C₆alkoxy, Ci-C₆alkyl, cyano, halo, and nitro; or, R^d and R⁴, together with the atoms to which they are attached, can form a ring selected from azetidine, pyrrolidine, morpholine, piperidine, piperazine, and 10 tetrahydrothiazole; wherein each ring is optionally substituted with one to four groups independently selected from amino, cyano, methyl, halo, hydroxy, and phenyl;

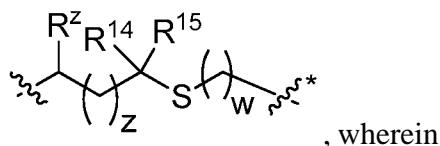
R^e is selected from hydrogen, Ci-C₆alkoxyCi-C₆alkyl, Ci-C₆alkyl, carboxyCi-Cealkyl, haloCi-Cealkyl, hydroxyCi-Cealkyl, (NR^{a'}R^{b'})Ci-C₆alkyl wherein R^{a'} and 15 R^{b'} are independently selected from hydrogen and Ci-C₆alkyl; and phenylCi-C₆alkyl wherein the phenyl is optionally substituted with one, two, three, four or five groups independently selected from Ci-C₆alkoxy, Ci-C₆alkyl, cyano, halo, and nitro; or R^e and R^{5'} together with the atoms to which they are attached, can form a ring selected from azetidine, pyrrolidine, morpholine, piperidine, piperazine, and 20 tetrahydrothiazole; wherein each ring is optionally substituted with one to four groups independently selected from amino, cyano, methyl, halo, and hydroxy;

R^g is selected from hydrogen, Ci-C₆alkoxyCi-C₆alkyl, Ci-C₆alkyl, carboxyCi-Cealkyl, haloCi-Cealkyl, hydroxyCi-Cealkyl, (NR^{a'}R^{b'})Ci-C₆alkyl wherein 25 R^{a'} and R^{b'} are independently selected from hydrogen and Ci-C₆alkyl; and phenylCi-C₆alkyl wherein the phenyl is optionally substituted with one, two, three, four or five groups independently selected from Ci-C₆alkoxy, Ci-C₆alkyl, cyano, halo, and nitro; or R^g and R⁷, together with the atoms to which they are attached, can form a ring selected from azetidine, pyrrolidine, morpholine, piperidine, piperazine, and tetrahydrothiazole; wherein each ring is optionally substituted with one to four 30 groups independently selected from amino, benzyl optionally substituted with a halo group, benzyloxy, cyano, cyclohexyl, methyl, halo, hydroxy, isoquinolinyloxy optionally substituted with a methoxy group, quinolinyloxy optionally substituted

with a halo group, and tetrazolyl; and wherein the pyrrolidine and the piperidine ring are optionally fused to a cyclohexyl, phenyl, or indole group;

provided that at least one of R^a , R^b , R^d , R^e , R^g , R^j , R^k , and R^l is selected from , $Ci-C_6alkoxyCi-C_6alkyl$, C_2-C_6alkyl , $carboxyCi-Cealkyl$, $haloCi-Cealkyl$, $hydroxyCi-C_6alkyl$, $(NR^{a'}R^{b'})Ci-C_6alkyl$ wherein $R^{a'}$ and $R^{b'}$ are independently selected from hydrogen and $Ci-C_6alkyl$; and $phenylCi-C_6alkyl$ wherein the phenyl is optionally substituted with one, two, three, four or five groups independently selected from $Ci-C_6alkoxy$, $Ci-C_6alkyl$, cyano, halo, and nitro.

In a first embodiment of the first aspect the present disclosure provides a compound of formula (I), or a pharmaceutically acceptable salt thereof, wherein R^e is selected from hydrogen and methyl, or R^e and R^5 together with the atoms to which they are attached, can form a ring selected from azetidine, pyrrolidine, morpholine, piperidine, piperazine, and tetrahydrothiazole; wherein each ring is optionally substituted with one to four groups independently selected from amino, cyano, methyl, halo, and hydroxy; and R^j is hydrogen or methyl. In a second embodiment of the first aspect the present disclosure R^k is selected from hydrogen and methyl, or R^k and R^{11} , together with the atoms to which they are attached, can form a ring selected from azetidine, pyrrolidine, morpholine, piperidine, piperazine, and tetrahydrothiazole; wherein each ring is optionally substituted with one to four groups independently selected from amino, cyano, methyl, halo, and hydroxy; and R^l is methyl, or, R^l and R^{12} , together with the atoms to which they are attached, form a ring selected from azetidine and pyrrolidine, wherein each ring is optionally substituted with one to four groups independently selected from amino, cyano, methyl, halo, and hydroxy. In a third aspect A is



z and w are each 1;

R^{14} and R^{15} are hydrogen; and

R^z is $C(0)NHR^{16}$; wherein R^{16} is $-CHR^{17}C(0)NH_2$;

30 R^{17} is hydrogen;

R¹ is benzyl optionally substituted with hydroxy;
R² is hydrogen or methyl;
R⁸ is -(CH₂)indolyl;
R¹⁰ is selected from -(CH₂)indolyl and -(CH₂)benzothienyl, each optionally
5 substituted with -CH₂C(=O)H;
R¹¹ is butyl; and
R¹² is butyl.

In another embodiment the present disclosure provides a method of enhancing, stimulating, and/or increasing the immune response in a subject in need
10 thereof, said method comprising administering to the subject a therapeutically effective amount of a compound of formula (I) or a therapeutically acceptable salt thereof. In another embodiment the method further comprises administering an additional agent prior to, after, or simultaneously with the compound of formula (I) or a therapeutically acceptable salt thereof. In another embodiment the additional
15 agent is an antimicrobial agent, an antiviral agent, a cytotoxic agent, and/or an immune response modifier. In another embodiment the additional agent is an HDAC inhibitor. In another embodiment the additional agent is a TLR7 and/or TLR8 agonist.

In another embodiment the present disclosure provides a method of inhibiting
20 growth, proliferation, or metastasis of cancer cells in a subject in need thereof, said method comprising administering to the subject a therapeutically effective amount of a compound of formula (I) or a therapeutically acceptable salt thereof. It should be understood that said inhibition can be direct or indirect. In another embodiment the cancer is selected from melanoma, renal cell carcinoma, squamous non-small cell
25 lung cancer (NSCLC), non-squamous NSCLC, colorectal cancer, castration-resistant prostate cancer, ovarian cancer, gastric cancer, hepatocellular carcinoma, pancreatic carcinoma, squamous cell carcinoma of the head and neck, carcinomas of the esophagus, gastrointestinal tract and breast, and a hematological malignancy.

In another embodiment the present disclosure provides a method of treating
30 an infectious disease in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of a compound of formula (I) or a therapeutically acceptable salt thereof. In another embodiment the

infectious disease is caused by a virus. In another embodiment the virus is selected from HIV, Hepatitis A, Hepatitis B, Hepatitis C, herpes virus, and influenza.

In another embodiment the present disclosure provides a method of treating septic shock in a subject in need thereof, the method comprising administering to the 5 subject a therapeutically effective amount of one or more macrocyclic peptides described herein.

In another embodiment the present disclosure provides a method blocking the interaction of PD-L1 with PD-1 and/or CD80 in a subject, said method comprising administering to the subject a therapeutically effective amount of at least one 10 macrocyclic peptide described herein.

In compounds of formula (I) where the R side chains are part of a ring that is substituted with methyl, it is understood that the methyl group may be on any substitutable carbon atom in the ring, including the carbon that is part of the macrocyclic parent structure.

15 In compounds of formula (I), preferred R¹ side chains are: phenylalanine, tyrosine, 3-thien-2-yl, 4-methylphenylalanine, 4-chlorophenylalanine, 3-methoxyphenylalanine, isotryptophan, 3-methylphenylalanine, 1-naphthylalanine, 3,4-difluorophenylalanine, 4-fluorophenylalanine, 3,4-dimethoxyphenylalanine, 3,4-dichlorophenylalanine, 4-difluoromethylphenylalanine, 2-methylphenylalanine, 2-naphthylalanine, tryptophan, 4-pyridinyl, 4-bromophenylalanine, 3-pyridinyl, 4-trifluoromethylphenylalanine, 4-carboxyphenylalanine, 4-methoxyphenylalanine, biphenylalanine, and 3-chlorophenylalanine; and 2,4-diaminobutane.

20 In compounds of formula (I) where R² is not part of a ring, preferred R² side chains are: alanine, serine, and glycine.

25 In compounds of formula (I), preferred R³ side chains are: asparagine, aspartic acid, glutamic acid, glutamine, serine, ornithine, lysine, histidine, threonine, leucine, alanine, 2,3-diaminopropane, and 2,4-diaminobutane.

In compounds of formula (I) where R⁴ is not part of a ring, preferred R⁴ side 30 chains are: valine, alanine, isoleucine, and glycine.

30 In compounds of formula (I), preferred R⁵ side chains are: aminomethane, histidine, asparagine, 2,3-diaminopropane, serine, glycine, 2,4-diaminobutane, threonine, alanine, lysine, aspartic acid, alanine, and 3-thiazolylalanine.

In compounds of formula (I), preferred R⁶ side chains are: leucine, aspartic acid, asparagine, glutamic acid, glutamine, serine, lysine, 3-cyclohexane, threonine, ornithine, 2,4-diaminobutane, alanine, arginine, and ornithine (COCH₃).

5 In compounds of formula (I) where R⁷ is not part of a ring, preferred R⁷ side chains are: glycine, 2,4-diaminobutane, serine, lysine, arginine, ornithine, histidine, asparagine, glutamine, alanine, and 2,4-diaminobutane (C(O)cyclobutane).

In compounds of formula (I) preferred R⁸ side chains are tryptophan and 1,2-benzisothiazolinylalanine.

10 In compounds of formula (I) preferred R⁹ side chains are: serine, histidine, lysine, ornithine, 2,4-dibutylamine, threonine, lysine, glycine, glutamic acid, valine, 2,3-diaminopropane, arginine, aspartic acid, and tyrosine.

In compounds of formula (I) preferred R¹⁰ side chains are: optionally substituted tryptophan, benzisothiazolylalanine, 1-naphthylalanine, and methionine.

15 In compounds of formula (I) preferred R¹¹ side chains are: norleucine, leucine, asparagine, phenylalanine, methionine, ethoxymethane, alanine, tryptophan, isoleucine, phenylpropane, glutamic acid, hexane, and heptane.

20 In compounds of formula (I) where R¹² is not part of a ring, preferred R¹² side chains are: norleucine, alanine, ethoxymethane, methionine, serine, phenylalanine, methoxyethane, leucine, tryptophan, isoleucine, glutamic acid, hexane, heptane, and glycine.

In compounds of formula (I) preferred R¹³ side chains : arginine, ornithine, alanine, 2,4-diaminobutane, 2,3-diaminopropane, leucine, aspartic acid, glutamic acid, serine, lysine, threonine, cyclopropylmethane, glycine, valine, isoleucine, histidine, and 2-aminobutane.

25 In accordance with the present disclosure, we have discovered peptides that specifically bind to PD-L1 and are capable of inhibiting the interaction of PD-L1 with PD-1 and CD80. These macrocyclic peptides exhibit *in vitro* immunomodulatory efficacy thus making them therapeutic candidates for the treatment of various diseases including cancer and infectious diseases.

30 The terms "specific binding" or "specifically bind" refer to the interaction between a protein and a binding molecule, such as a compound or ligand. The interaction is dependent upon the presence of a particular structure *{i.e.,* an enzyme

binding site, an antigenic determinant or epitope) of the protein that is recognized by the binding molecule. For example, if a compound has specific binding for protein binding site "A", the presence of the compound in a reaction containing a protein including binding site A, and a labeled peptide that specifically binds to protein 5 binding site A will reduce the amount of labeled peptide bound to the protein. In contrast, nonspecific binding of a compound to the protein does not result in a concentration-dependent displacement of the labeled peptide from the protein.

The present disclosure is intended to include all isotopes of atoms occurring in the present compounds. Isotopes include those atoms having the same atomic 10 number but different mass numbers. By way of general example and without limitation, isotopes of hydrogen include deuterium and tritium. Isotopes of carbon include ^{13}C and ^{14}C . Isotopically-labeled compounds of the invention can generally be prepared by conventional techniques known to those skilled in the art or by processes analogous to those described herein, using an appropriate isotopically-15 labeled reagent in place of the non-labeled reagent otherwise employed. Such compounds may have a variety of potential uses, for example as standards and reagents in determining biological activity. In the case of stable isotopes, such compounds may have the potential to favorably modify biological, pharmacological, or pharmacokinetic properties.

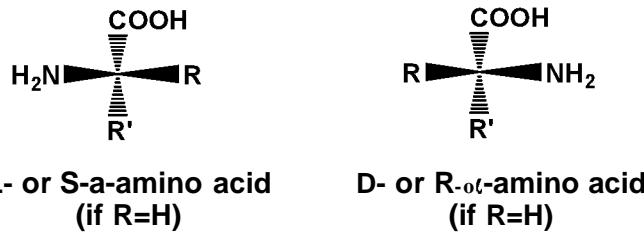
20 An additional aspect of the subject matter described herein is the use of the disclosed peptides as radiolabeled ligands for development of ligand binding assays or for monitoring of *in vivo* adsorption, metabolism, distribution, receptor binding or occupancy, or compound disposition. For example, a macrocyclic peptide described herein may be prepared using the radioactive isotope ^{125}I and the resulting 25 radiolabeled peptide may be used to develop a binding assay or for metabolism studies. Alternatively, and for the same purpose, a macrocyclic peptide described herein may be converted to a radiolabeled form by catalytic tritiation using methods known to those skilled in the art.

The macrocyclic peptides of the present disclosure can also be used as PET 30 imaging agents by adding a radioactive tracer using methods known to those skilled in the art.

Preferred peptides include at least one of the macrocyclic peptides provided herein and these peptides may be included in pharmaceutical compositions and combinations.

The definitions provided herein apply, without limitation, to the terms as used 5 throughout this specification, unless otherwise limited in specific instances.

Those of ordinary skill in the art of amino acid and peptide chemistry are aware that an amino acid includes a compound represented by the general structure:



where R and R' are as discussed herein.

Unless otherwise indicated, the term "amino acid" as employed herein, alone or as part of another group, includes, without limitation, an amino group and a carboxyl group linked to the same carbon, referred to as "a" carbon, where R and/or R' can be a natural or an un-natural side chain, including hydrogen. The absolute "S" configuration at the "a" carbon is commonly referred to as the "L" or "natural" 15 configuration. In the case where both the "R" and the "R"(prime) substituents equal hydrogen, the amino acid is glycine and is not chiral.

The terms "natural amino acid side chain" and "naturally occurring amino acid side chain," as used herein, refer to side chain of any of the naturally occurring amino acids (i.e., alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, 20 glutamic acid, glycine, -histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine) usually in the S-configuration (i.e., the L-amino acid).

The terms "unnatural amino acid side chain" and "non-naturally occurring amino acid side chain," as used herein, refer to a side chain of any naturally occurring 25 amino acid usually in the R-configuration (i.e., the D-amino acid) or to a group other than a naturally occurring amino acid side chain in R- or S-configuration (i.e., the D- or L-amino acid, respectively) selected from:

C₂-C₇alkenyl, Ci-C₃alkoxyCi-C₃alkyl, Ci-C₆alkoxycarbonylCi-C₃alkyl, Ci-C₇alkyl, Ci-C₃alkylsulfanylCi-C₃alkyl, amidoCi-C₃alkyl, aminoCi-C₃alkyl, azaindolylCi-C₃alkyl, benzothiazolylCi-C₃alkyl, benzothienylCi-C₃alkyl, benzyloxyCi-C₃alkyl, carboxyCi-C₃alkyl, C₃-C₄cycloalkylCi-C₃alkyl, 5 diphenylmethyl, furanylCi-C₃alkyl, imidazolylCi-C₃alkyl, naphthylCi-C₃alkyl, pyridinylCi-C₃alkyl, thiazolylCi-C₃alkyl, thienylCi-C₃alkyl;

biphenylCi-C₃alkyl wherein the biphenyl is optionally substituted with a methyl group;

heterocyclyl optionally substituted with one, two, three, four, or five groups 10 independently selected from Ci-C₄alkoxy, Ci-C₄alkyl, Ci-C₃alkylsulfonylamino, amido, amino, aminoCi-C₃alkyl, aminosulfonyl, carboxy, cyano, halo, haloCi-Csalkyl, hydroxy, -NC(NH₂)₂, nitro, and -OP(0)(OH)₂;

indolylCi-C₃alkyl, wherein the indolyl part is optionally substituted with one group selected from Ci-C₃alkyl, carboxyCi-C₃alkyl, halo, hydroxy, and phenyl, 15 wherein the phenyl is further optionally substituted by one, two, or three groups independently selected from Ci-C₃alkoxy, Ci-C₃alkyl, and halo;

NR^yR^{y'}(Ci-C₇alkyl), wherein R^y and R^{y'} are independently selected from 20 hydrogen, C₂-C₄alkenyloxycarbonyl, Ci-C₃alkyl, Ci-C₃alkylcarbonyl, C₃-Ci₄cycloalkylcarbonyl, furanylcarbonyl, and phenylcarbonyl. When the alkyl linker contains more than one carbon an additional NR^yR^{y'} group can be on the chain.

NR^qR^tcarbonylCi₁-C₃alkyl, wherein R^q and R^t are independently selected from hydrogen, Ci-C₃alkyl, and triphenylmethyl;

phenyl optionally substituted with one, two, three, four, or five groups 25 independently selected from Ci-C₄alkoxy, Ci-C₄alkyl, Ci-C₃alkylsulfonylamino, amido, amino, aminoCi-C₃alkyl, aminosulfonyl, carboxy, cyano, halo, haloCi-Csalkyl, hydroxy, -NC(NH₂)₂, nitro, and -OP(0)(OH)₂;

phenylCi-C₃alkyl wherein the phenyl part is optionally substituted with one, two, three, four, or five groups independently selected from Ci-C₄alkoxy, Ci-C₄alkyl, Ci-C₃alkylsulfonylamino, amido, amino, aminoCi-C₃alkyl, aminosulfonyl, carboxy, 30 cyano, halo, haloCi-C₃alkyl, hydroxy, -NC(NH₂)₂, nitro, and -OP(0)(OH)₂; and phenoxyCi-C₃alkyl wherein the phenyl is optionally substituted with a Ci-C₃alkyl group.

The term "C2-C4alkenyl," as used herein, refers to a straight or branched chain group of two to four carbon atoms containing at least one carbon-carbon double bond.

5 The term "C2-C7alkenyl," as used herein, refers to a straight or branched chain group of two to seven carbon atoms containing at least one carbon-carbon double bond.

The term "C2-C4alkenyloxy," as used herein, refers to a C₂-C4alkenyl group attached to the parent molecular moiety through an oxygen atom.

10 The term "Ci-C3alkoxy," as used herein, refers to a Ci-C3alkyl group attached to the parent molecular moiety through an oxygen atom.

The term "Ci-C4alkoxy," as used herein, refers to a Ci-C4alkyl group attached to the parent molecular moiety through an oxygen atom.

The term "Ci-C6alkoxy," as used herein, refers to a Ci-C₆alkyl group attached to the parent molecular moiety through an oxygen atom.

15 The term "Ci-C3alkoxyCi-C3alkyl," as used herein, refers to a Ci-C3alkoxy group attached to the parent molecular moiety through a Ci-C3alkyl group.

The term "Ci-C6alkoxyCi-C6alkyl," as used herein, refers to a Ci-C₆alkoxy group attached to the parent molecular moiety through a Ci-C₆alkyl group

20 The term "Ci-C6alkoxycarbonyl," as used herein, refers to a Ci-C₆alkoxy group attached to the parent molecular moiety through a carbonyl group.

The term "Ci-C6alkoxycarbonylCi-C3alkyl," as used herein, refers to a Ci-C₆alkoxycarbonyl group attached to the parent molecular moiety through a Ci-C3alkyl group.

25 The term "Ci-C3alkyl," as used herein, refers to a group derived from a straight or branched chain saturated hydrocarbon containing from one to three carbon atoms.

The term "Ci-C4alkyl," as used herein, refers to a group derived from a straight or branched chain saturated hydrocarbon containing from one to four carbon atoms.

30 The term "Ci-C6alkyl," as used herein, refers to a group derived from a straight or branched chain saturated hydrocarbon containing from one to six carbon atoms.

The term "Ci-C3alkylcarbonyl," as used herein, refers to a Ci-C3alkyl group attached to the parent molecular moiety through a carbonyl group.

The term "Ci-C3alkylsulfanyl," as used herein, refers to a Ci-C3alkyl group attached to the parent molecular moiety through a sulfur atom.

5 The term "Ci-C3alkylsulfanylCi-C3alkyl," as used herein, refers to a Ci-C3alkylsulfanyl group attached to the parent molecular moiety through a Ci-C3alkyl group.

The term "Ci-C3alkylsulfonyl," as used herein, refers to a Ci-C3alkyl group attached to the parent molecular moiety through a sulfonyl group.

10 The term "Ci-C3alkylsulfonylamino," as used herein, refers to a Ci-C3alkylsulfonyl group attached to the parent molecular moiety through an amino group.

The term "amido," as used herein, refers to -C(0)NH2.

15 The term "amidoCi-C3alkyl," as used herein, refers to an amido group attached to the parent molecular moiety through a Ci-C3alkyl group.

The term "amino," as used herein, refers to - NH2.

The term "aminoCi-C3alkyl," as used herein, refers to an amino group attached to the parent molecular moiety through a Ci-C3alkyl group.

20 The term "aminosulfonyl," as used herein, refers to an amino group attached to the parent molecular moiety through a sulfonyl group.

The term "azaindolylCi-C3alkyl," as used herein, refers to an azaindolyl group attached to the parent molecular through a Ci-C3alkyl group. The azaindolyl group can be attached to the alkyl moiety through any substitutable atom in the group.

25 The term "benzothiazolylCi-C3alkyl," as used herein, refers to an benzothiazolyl group attached to the parent molecular through a Ci-C3alkyl group. The benzothiazolyl group can be attached to the alkyl moiety through any substitutable atom in the group.

30 The term "benzothienylCi-C3alkyl," as used herein, refers to a benzothienyl group attached to the parent molecular through a Ci-C3alkyl group. The benzothienyl group can be attached to the alkyl moiety through any substitutable atom in the group.

The term "benzyloxy," as used herein, refers to a benzyl group attached to the parent molecular moiety through an oxygen atom.

The term "benzyloxyCi-C3alkyl," as used herein, refers to a benzyloxy group attached to the parent molecular moiety through a Ci-C3alkyl group.

5 The term "biphenylCi-C3alkyl," as used herein, refers to a biphenyl group attached to the parent molecular moiety through a Ci-C3alkyl group. The biphenyl group can be attached to the alkyl moiety through any substitutable atom in the group.

The term "carbonyl," as used herein, refers to -C(O)-.

10 The term "carboxy," as used herein, refers to -CO2H.

The term "carboxyCi-C3alkyl," as used herein, refers to a carboxy group attached to the parent molecular moiety through a Ci-C3alkyl group.

The term "carboxyCi-Cealkyl," as used herein, refers to a carboxy group attached to the parent molecular moiety through a Ci-C₆alkyl group.

15 The term "cyano," as used herein, refers to -CN.

The term "C3-Ci4cycloalkyl," as used herein, refers to a saturated monocyclic, bicyclic, or tricyclic hydrocarbon ring system having three to fourteen carbon atoms and zero heteroatoms. The bicyclic and tricyclic rings may be fused, spirocyclic, or bridged. Representative examples of cycloalkyl groups include, but are not limited 20 to, cyclopropyl, cyclopentyl, bicyclo[3.1.1]heptyl, and adamantly.

The term "C3-Ci4cycloalkylCi-C3alkyl," as used herein, refers to a C3-Ci4cycloalkyl group attached to the parent molecular moiety through a Ci-C3alkyl group.

25 The term "C3-Ci4cycloalkylcarbonyl," as used herein, refers to a C3-C14 cycloalkyl group attached to the parent molecular moiety through a carbonyl group.

The term "furanylCi-C3alkyl," as used herein, refers to a furanyl group attached to the parent molecular moiety through a Ci-C3alkyl group. The furanyl group can be attached to the alkyl moiety through any substitutable atom in the group.

30 The term "furanylcarbonyl," as used herein, refers to a furanyl group attached to the parent molecular moiety through a carbonyl group.

The terms "halo" and "halogen," as used herein, refer to F, Cl, Br, or I.

The term "haloCi-C3alkyl," as used herein, refers to a Ci-C3alkyl group substituted with one, two, or three halogen atoms.

The term "haloCi-Cealkyl," as used herein, refers to a Ci-C₆alkyl group substituted with one, two, or three halogen atoms.

5 The term "halomethyl," as used herein, refers to a methyl group substituted with one, two, or three halogen atoms.

The term "heterocyclyl," as used herein, refers to a five-, six-, or seven-membered ring containing one, two, or three heteroatoms independently selected from nitrogen, oxygen, and sulfur. The five-membered ring has zero to two double bonds and the six- and seven-membered rings have zero to three double bonds. The term "heterocyclyl" also includes bicyclic groups in which the heterocyclyl ring is fused to a four- to six-membered aromatic or non-aromatic carbocyclic ring or another monocyclic heterocyclyl group. The heterocyclyl groups of the present disclosure are attached to the parent molecular moiety through a carbon atom in the group. Examples of heterocyclyl groups include, but are not limited to, benzothienyl, furyl, imidazolyl, indolinyl, indolyl, isothiazolyl, isoxazolyl, morpholinyl, oxazolyl, piperazinyl, piperidinyl, pyrazolyl, pyridinyl, pyrrolidinyl, pyrrolopyridinyl, pyrrolyl, thiazolyl, thienyl, and thiomorpholinyl.

10 The term "hydroxy," as used herein, refers to -OH.

20 The term "hydroxyCi-Cealkyl," as used herein, refers to a Ci-C₆alkyl group substituted with one, two, or three hydroxy groups.

15 The term "imidazolylCi-C3alkyl," as used herein, refers to an imidazolyl group attached to the parent molecular moiety through a Ci-C3alkyl group. The imidazolyl group can be attached to the alkyl moiety through any substitutable atom in the group.

25 The term "indolylCi-C3alkyl," as used herein, refers to an indolyl group attached to the parent molecular moiety through a Ci-C3alkyl group. The indolyl group can be attached to the alkyl moiety through any substitutable atom in the group.

30 The term "naphthylCi-C3alkyl," as used herein, refers to a naphthyl group attached to the parent molecular moiety through a Ci-C3alkyl group. The naphthyl

group can be attached to the alkyl moiety through any substitutable atom in the group.

The term "nitro," as used herein, refers to -NO₂.

5 The term "NR^yR^{y'}," as used herein, refers to two groups, R^y and R^{y'}, which are attached to the parent molecular moiety through a nitrogen atom. R^y and R^{y'} are independently selected from hydrogen, C₂-C₄alkenyloxycarbonyl, Ci-C₃alkylcarbonyl, C₃-Ci₄cycloalkylcarbonyl, furanylcarbonyl, and phenylcarbonyl.

The term "NR^yR^{y'}(Ci-C₃)alkyl," as used herein, refers to an NR^yR^{y'} group attached to the parent molecular moiety through a Ci-C₃alkyl group.

10 The term "NR^qRV" as used herein, refers to two groups, R^q and R^t, which are attached to the parent molecular moiety through a nitrogen atom. R^q and R^t are independently selected from hydrogen, Ci-C₃alkyl, and triphenylmethyl.

The term "NR^qR^tarbonyl," as used herein, refers to an NR^qR^t group attached to the parent molecular moiety through a carbonyl group.

15 The term "NR^qR^tcarbonylC₁-C₃alkyl," as used herein, refers to an NR^qR^tcarbonyl group attached to the parent molecular moiety through a Ci-C₃alkyl group.

The term "phenoxy," as used herein, refers to a phenyl group attached to the parent molecular moiety through an oxygen atom.

20 The term "phenoxyCi-C₃alkyl," as used herein, refers to a phenoxy group attached to the parent molecular moiety through a Ci-C₃alkyl group.

The term "phenylCi-C₃alkyl," as used herein, refers to a phenyl group attached to the parent molecular moiety through a Ci-C₃alkyl group.

25 The term "phenylCi-C₆alkyl," as used herein, refers to a phenyl group attached to the parent molecular moiety through a Ci-C₆alkyl group.

The term "phenylcarbonyl," as used herein, refers to a phenyl group attached to the parent molecular moiety through a carbonyl group.

30 The term "pyridinylCi-C₃alkyl," as used herein, refers to a pyridinyl group attached to the parent molecular moiety through a Ci-C₃alkyl group. The pyridinyl group can be attached to the alkyl moiety through any substitutable atom in the group.

The term "sulfanyl," as used herein, refers to -S-.

The term "sulfonyl," as used herein, refers to -SO2-.

The term "thiazolylCi-C3alkyl," as used herein, refers to a thiazolyl group attached to the parent molecular moiety through a Ci-C3alkyl group. The thiazolyl group can be attached to the alkyl moiety through any substitutable atom in the 5 group.

The term "thienylCi-C3alkyl," as used herein, refers to a thienyl group attached to the parent molecular moiety through a Ci-C3alkyl group. The thienyl group can be attached to the alkyl moiety through any substitutable atom in the group.

10 The term "treating" refers to: (i) preventing a disease, disorder, or condition from occurring in a patient that may be predisposed to the disease, disorder, and/or condition but has not yet been diagnosed as having it; (ii) inhibiting the disease, disorder, or condition, i.e., arresting its development; and (iii) relieving the disease, disorder, or condition, i.e., causing regression of the disease, disorder, and/or condition and/or symptoms associated with the disease, disorder, and/or condition.

15 Binding of the macrocyclic peptides to PD-L1 can be measured, for example, by methods such as homogeneous time-resolved fluorescence (HTRF), Surface Plasmon Resonance (SPR), isothermal titration calorimetry (ITC), nuclear magnetic resonance spectroscopy (NMR), and the like. Further, binding of the macrocyclic peptides to PD-L1 expressed on the surface of cells can be measured as described 20 herein in cellular binding assays.

25 Administration of a therapeutic agent described herein includes, without limitation, administration of a therapeutically effective amount of therapeutic agent. The term "therapeutically effective amount" as used herein refers, without limitation, to an amount of a therapeutic agent to treat or prevent a condition treatable by administration of a composition of the PD-1/PD-L1 binding inhibitors described herein. That amount is the amount sufficient to exhibit a detectable therapeutic or preventative or ameliorative effect. The effect may include, for example and without limitation, treatment or prevention of the conditions listed herein. The precise 30 effective amount for a subject will depend upon the subject's size and health, the nature and extent of the condition being treated, recommendations of the treating

physician, and therapeutics or combination of therapeutics selected for administration. Thus, it is not useful to specify an exact effective amount in advance.

In another aspect, the disclosure pertains to methods of inhibiting growth of tumor cells in a subject using the macrocyclic peptides of the present disclosure. As 5 demonstrated herein, the macrocyclic peptides of the present disclosure are capable of binding to PD-L1, disrupting the interaction between PD-L1 and PD-1, competing with the binding of PD-L1 with anti-PD-1 monoclonal antibodies that are known to block the interaction with PD-1, enhancing CMV-specific T cell IFNy secretion, and enhancement of HIV-specific T cell IFNg secretion. As a result, the macrocyclic 10 peptides of the present disclosure are useful for modifying an immune response, treating diseases such as cancer or infectious disease, stimulating a protective autoimmune response or to stimulate antigen-specific immune responses (e.g., by coadministration of PD-L1 blocking peptides with an antigen of interest).

In order that the present disclosure may be more readily understood, certain 15 terms are first defined. Additional definitions are set forth throughout the detailed description.

The terms "Programmed Death Ligand 1", "Programmed Cell Death Ligand 1", "Protein PD-L1", "PD-L1", "PDL1", "PDCDL1", "hPD-L1", "hPD-L1", "CD274" and "B7-H1" are used interchangeably, and include variants, isoforms, species 20 homologs of human PD-L1, and analogs having at least one common epitope with PD-L1. The complete PD-L1 sequence can be found under GENBANK® Accession No. NP_054862.

The terms "Programmed Death 1", "Programmed Cell Death 1", "Protein PD-1", "PD-1", "PD1", "PDCD1", "hPD-1" and "hPD-I" are used interchangeably, and 25 include variants, isoforms, species homologs of human PD-1, and analogs having at least one common epitope with PD-1. The complete PD-1 sequence can be found under GENBANK® Accession No. U64863.

The terms "cytotoxic T lymphocyte-associated antigen-4", "CTLA-4", "CTLA4", "CTLA-4 antigen" and "CD152" (see, e.g., Murata, *Am. J. Pathol.*, 155:453-460 (1999)) are used interchangeably, and include variants, isoforms, 30 species homologs of human CTLA-4, and analogs having at least one common epitope with CTLA-4 (see, e.g., Balzano, *Int. J. Cancer Suppl.*, 7:28-32 (1992)).

The complete CTLA-4 nucleic acid sequence can be found under GENBANK® Accession No. L15006.

The term "immune response" refers to the action of, for example, lymphocytes, antigen presenting cells, phagocytic cells, granulocytes, and soluble 5 macromolecules produced by the above cells or the liver (including macrocyclic peptides, cytokines, and complement) that results in selective damage to, destruction of, or elimination from the human body of invading pathogens, cells or tissues infected with pathogens, cancerous cells, or, in cases of autoimmunity or pathological inflammation, normal human cells or tissues.

10 An "adverse event" (AE) as used herein is any unfavorable and generally unintended, even undesirable, sign (including an abnormal laboratory finding), symptom, or disease associated with the use of a medical treatment. For example, an adverse event may be associated with activation of the immune system or expansion of immune system cells (*e.g.*, T cells) in response to a treatment. A medical 15 treatment may have one or more associated AEs and each AE may have the same or different level of severity. Reference to methods capable of "altering adverse events" means a treatment regime that decreases the incidence and/or severity of one or more AEs associated with the use of a different treatment regime.

As used herein, "hyperproliferative disease" refers to conditions wherein cell 20 growth is increased over normal levels. For example, hyperproliferative diseases or disorders include malignant diseases (*e.g.*, esophageal cancer, colon cancer, biliary cancer) and non-malignant diseases (*e.g.*, atherosclerosis, benign hyperplasia, and benign prostatic hypertrophy).

As used herein, "about" or "comprising essentially of" mean within an 25 acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, *i.e.*, the limitations of the measurement system. For example, "about" or "comprising essentially of" can mean within one or more than one standard deviation per the practice in the art. Alternatively, "about" or "comprising essentially of" can mean a 30 range of up to 20%. Furthermore, particularly with respect to biological systems or processes, the terms can mean up to an order of magnitude or up to 5-fold of a value. When particular values are provided in the application and claims, unless otherwise

stated, the meaning of "about" or "comprising essentially of" should be assumed to be within an acceptable error range for that particular value.

As described herein, any concentration range, percentage range, ratio range or integer range is to be understood to include the value of any integer within the recited range and, when appropriate, fractions thereof (such as one tenth and one hundredth of an integer), unless otherwise indicated.

Competition Assays

The present disclosure is also directed to macrocyclic peptides that are capable of competing with the binding of a reference anti-PD-L1 antibody (MDX-1105) by at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, and at least about 100%. Such macrocyclic peptides may share structural homology with one or more macrocyclic peptides disclosed herein, including mutant, conservative substitution, functional substitution, and deletion forms, provided they specific bind to PD-L1. For example, if a macrocyclic peptide binds substantially to the same region of PD-L1 as a reference anti-PD-L1 antibody, the macrocyclic peptide should bind to an epitope of PD-L1 that at least overlaps with the PD-L1 epitope that the anti-PD-L1 monoclonal antibody binds to. The overlapping region can range from one amino acid residue to several hundred amino acid residues. The macrocyclic peptide should then compete with and/or block the binding of the anti-PD-L1 monoclonal antibody to PD-L1 and thereby decrease the binding of the anti-PD-L1 monoclonal antibody to PD-L1, preferably by at least about 50% in a competition assay.

Anti-PD-L1 antibodies that may be used as reference antibodies for competition assay purposes are known in the art. For example, the following representative anti-PD-L1 antibodies may be used: MDX-1 105 (BMS); L01X-C (Serono), L1X3 (Serono), MSB-0010718C (Serono), and PD-L1 Probody (CytomX), and the PD-L1 antibodies disclosed in co-owned WO 2007/005874.

Anti-PD-1 antibodies that may be used as reference antibodies for competition assay purposes are known in the art. For example, the following representative anti-PD-1 antibodies may be used: nivolumab (BMS); 17D8, 2D3,

4H1, 4A1 1, 7D3 and 5F4 each disclosed in co-owned U.S. Patent No. 8,008,449 (BMS), MK-3475 (Merck, disclosed in U.S. Patent No. 8,168,757), and the antibodies disclosed in U.S. Patent No. 7,488,802.

5 Pharmaceutical Compositions

In another aspect, the present disclosure provides a composition, *e.g.*, a pharmaceutical composition, containing one or a combination of macrocyclic peptides of the present disclosure, formulated together with a pharmaceutically acceptable carrier. Such compositions may include one or a combination of (*e.g.*, 10 two or more different) macrocyclic peptides, or immunoconjugates or bispecific molecules of the disclosure. For example, a pharmaceutical composition of the disclosure can comprise a combination of macrocyclic peptides (or immunoconjugates or bispecifics) that bind to different epitopes on the target antigen or that have complementary activities.

15 Pharmaceutical compositions of the disclosure also can be administered in combination therapy, *i.e.*, combined with other agents. For example, the combination therapy can include a macrocyclic peptide combined with at least one other anti-inflammatory or immunosuppressant agent. Examples of therapeutic agents that can be used in combination therapy are described in greater detail below in the section on 20 uses of the macrocyclic peptides of the disclosure.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Preferably, the carrier is suitable for intravenous, intramuscular, subcutaneous, 25 parenteral, spinal or epidermal administration (*e.g.*, by injection or infusion). Depending on the route of administration, the active compound, *i.e.*, a macrocyclic peptide, immunoconjugate, or bispecific molecule, may be coated in a material to protect the compound from the action of acids and other natural conditions that may inactivate the compound.

30 The pharmaceutical compounds of the disclosure may include one or more pharmaceutically acceptable salts. A "pharmaceutically acceptable salt" or "therapeutically acceptable salt" refers to a salt that retains the desired biological

activity of the parent compound and does not impart any undesired toxicological effects (see *e.g.*, Berge, S.M. et al., *J. Pharm. Sci.*, 66:1-19 (1977)). Examples of such salts include acid addition salts and base addition salts. Acid addition salts include those derived from nontoxic inorganic acids, such as hydrochloric, nitric, 5 phosphoric, sulfuric, hydrobromic, hydroiodic, phosphorous and the like, as well as from nontoxic organic acids such as aliphatic mono- and dicarboxylic acids, phenyl-substituted alkanoic acids, hydroxy alkanoic acids, aromatic acids, aliphatic and aromatic sulfonic acids and the like. Base addition salts include those derived from alkaline earth metals, such as sodium, potassium, magnesium, calcium and the like, 10 as well as from nontoxic organic amines, such as N,N'-dibenzylethylenediamine, N-methylglucamine, chloroprocaine, choline, diethanolamine, ethylenediamine, procaine and the like.

A pharmaceutical composition of the disclosure also may include a pharmaceutically acceptable anti-oxidant. Examples of pharmaceutically acceptable 15 antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabi sulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine 20 tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

Examples of suitable aqueous and nonaqueous carriers that may be employed in the pharmaceutical compositions of the disclosure include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such 25 as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of presence of 30 microorganisms may be ensured both by sterilization procedures, supra, and by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include

isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

5 Pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in
10 the pharmaceutical compositions of the disclosure is contemplated. Supplementary active compounds can also be incorporated into the compositions.

15 Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, liposome, or other ordered structure suitable to high drug concentration. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of
20 surfactants. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

25 Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by sterilization microfiltration. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the
30 required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying (lyophilization) that yield a powder

of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the subject being 5 treated, and the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the composition which produces a therapeutic effect. Generally, out of one hundred percent, this amount will range from about 0.01 percent to about ninety-nine percent of active ingredient, preferably from about 0.1 10 percent to about 70 percent, most preferably from about 1 percent to about 30 percent of active ingredient in combination with a pharmaceutically acceptable carrier.

Dosage regimens are adjusted to provide the optimum desired response (*e.g.*, a therapeutic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally 15 reduced or increased as indicated by the exigencies of therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each unit contains a predetermined quantity of active compound calculated to 20 produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the disclosure are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in 25 individuals.

For administration of the macrocyclic peptide, the dosage ranges from about 0.0001 to 100 mg/kg, and more usually 0.01 to 5 mg/kg, of the host body weight. For example dosages can be 0.3 mg/kg body weight, 1 mg/kg body weight, 3 mg/kg 30 body weight, 5 mg/kg body weight or 10 mg/kg body weight or within the range of 1-10 mg/kg. An exemplary treatment regime entails administration once per day, twice per day, bi-weekly, tri-weekly, weekly, once every two weeks, once every three weeks, once every four weeks, once a month, once every 3 months or once every

three to 6 months. Preferred dosage regimens for a macrocyclic peptide of the disclosure include 1 mg/kg body weight or 3 mg/kg body weight via intravenous administration, with the macrocycle being given using one of the following dosing schedules: (i) every four weeks for six dosages, then every three months; (ii) every 5 three weeks; (iii) 3 mg/kg body weight once followed by 1 mg/kg body weight every three weeks.

In some methods, two or more macrocyclic peptides with different binding specificities are administered simultaneously, in which case the dosage of each compound administered falls within the ranges indicated. The compounds are 10 usually administered on multiple occasions. Intervals between single dosages can be, for example, weekly, monthly, every three months or yearly. Intervals can also be irregular as indicated by measuring blood levels of macrocyclic peptide to the target antigen in the patient. In some methods, dosage is adjusted to achieve a plasma concentration of about 1-1000 .mu.g/ml and in some methods about 25-300 .mu.g/ml.

15 Alternatively, the macrocyclic peptide can be administered as a sustained release formulation, in which case less frequent administration is required. The dosage and frequency of administration can vary depending on whether the treatment is prophylactic or therapeutic. In prophylactic applications, a relatively low dosage is administered at relatively infrequent intervals over a long period of time. Some 20 patients continue to receive treatment for the rest of their lives. In therapeutic applications, a relatively high dosage at relatively short intervals is sometimes required until progression of the disease is reduced or terminated, and preferably until the patient shows partial or complete amelioration of symptoms of disease. Thereafter, the patient can be administered a prophylactic regime.

25 Actual dosage levels of the active ingredients in the pharmaceutical compositions of the present disclosure may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient. The selected dosage level will depend upon a variety of pharmacokinetic 30 factors including the activity of the particular compositions of the present disclosure employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the

duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

5 A "therapeutically effective dosage" of a macrocyclic peptide of the disclosure preferably results in a decrease in severity of disease symptoms, an increase in frequency and duration of disease symptom-free periods, or a prevention of impairment or disability due to the disease affliction. For example, for the treatment of tumors, a "therapeutically effective dosage" preferably inhibits cell
10 growth or tumor growth by at least about 20%, more preferably by at least about 40%, even more preferably by at least about 60%, and still more preferably by at least about 80% relative to untreated subjects. The ability of a compound to inhibit tumor growth and/or HIV can be evaluated in an animal model system predictive of efficacy in human tumors or viral efficacy. Alternatively, this property of a
15 composition can be evaluated by examining the ability of the compound to inhibit, such inhibition *in vitro* by assays known to the skilled practitioner. A therapeutically effective amount of a therapeutic compound can decrease tumor size, decrease viral load, or otherwise ameliorate symptoms in a subject. One of ordinary skill in the art would be able to determine such amounts based on such factors as the subject's size,
20 the severity of the subject's symptoms, and the particular composition or route of administration selected.

 In another aspect, the instant disclosure provides a pharmaceutical kit of parts comprising a macrocyclic peptide and an another immumodulator, as described herein. The kit may also further comprise instructions for use in the treatment of a
25 hyperproliferative disease (such as cancer as described herein) and/or anti-viral disease.

 A composition of the present disclosure can be administered via one or more routes of administration using one or more of a variety of methods known in the art. As will be appreciated by the skilled artisan, the route and/or mode of administration
30 will vary depending upon the desired results. Preferred routes of administration for macrocyclic peptides of the disclosure include intravenous, intramuscular, intradermal, intraperitoneal, subcutaneous, spinal or other parenteral routes of

administration, for example by injection or infusion. The phrase "parenteral administration" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, 5 intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion.

Alternatively, a macrocyclic peptide of the disclosure can be administered via a non-parenteral route, such as a topical, epidermal or mucosal route of 10 administration, for example, intranasally, orally, vaginally, rectally, sublingually or topically.

The active compounds can be prepared with carriers that will protect the compound against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems.

15 Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are patented or generally known to those skilled in the art. See, e.g., Robinson, J.R., ed., *Sustained and Controlled Release Drug Delivery Systems*, Marcel Dekker, Inc., New York (1978).

20 Therapeutic compositions can be administered with medical devices known in the art. For example, in a preferred embodiment, a therapeutic composition of the disclosure can be administered with a needleless hypodermic injection device, such as the devices disclosed in U.S. Patent Nos. 5,399,163, 5,383,851, 5,312,335, 5,064,413, 4,941,880, 4,790,824, or 4,596,556. Examples of well-known implants 25 and modules useful in the present disclosure include: U.S. Patent No. 4,487,603, which discloses an implantable micro-infusion pump for dispensing medication at a controlled rate; U.S. Patent No. 4,486,194, which discloses a therapeutic device for administering medication through the skin; U.S. Patent No. 4,447,233, which discloses a medication infusion pump for delivering medication at a precise infusion 30 rate; U.S. Patent No. 4,447,224, which discloses a variable flow implantable infusion apparatus for continuous drug delivery; U.S. Patent No. 4,439,196, which discloses an osmotic drug delivery system having multi-chamber compartments; and

U.S. Patent No. 4,475,196, which discloses an osmotic drug delivery system. These patents are incorporated herein by reference. Many other such implants, delivery systems, and modules are known to those skilled in the art.

In certain embodiments, the macrocyclic peptides of the disclosure can be 5 formulated to ensure proper distribution *in vivo*. For example, the blood-brain barrier (BBB) excludes many highly hydrophilic compounds. To ensure that therapeutic compounds of the disclosure cross the BBB (if desired), they can be formulated, for example, in liposomes. For methods of manufacturing liposomes, see, *e.g.*, U.S. Patent Nos. 4,522,811, 5,374,548, and 5,399,331. The liposomes may comprise one 10 or more moieties which are selectively transported into specific cells or organs, thus enhance targeted drug delivery (see, *e.g.*, Ranade, V.V., *J. Clin. Pharmacol.*, 29:685 (1989)). Exemplary targeting moieties include folate or biotin (see, *e.g.*, U.S. Patent No. 5,416,016 to Low et al.); mannosides (Umezawa et al., *Biochem. Biophys. Res. Commun.*, 153:1038 (1988)); macrocyclic peptides (Bloeman, P.G. et al., *FEBS Lett.*, 357:140 (1995); Owais, M. et al., *Antimicrob. Agents Chemother.*, 39:180 (1995)); surfactant protein A receptor (Briscoe et al., *Am. J. Physiol.*, 1233:134 (1995)); pl20 (Schreier et al., *J. Biol. Chem.*, 269:9090 (1994)); see also Keinanen, K. et al., *FEBS Lett.*, 346:123 (1994); Killion, J.J. et al., *Immunomethods* 4:273 (1994).
20

Uses and Methods of the Disclosure

The macrocyclic peptides, compositions and methods of the present disclosure have numerous *in vitro* and *in vivo* utilities involving, for example, detection of PD-L1 or enhancement of immune response by blockade of PD-L1. For 25 example, these molecules can be administered to cells in culture, *in vitro* or *ex vivo*, or to human subjects, *e.g.*, *in vivo*, to enhance immunity in a variety of situations. Accordingly, in one aspect, the disclosure provides a method of modifying an 30 immune response in a subject comprising administering to the subject the macrocyclic peptide of the disclosure such that the immune response in the subject is modified. Preferably, the response is enhanced, stimulated or up-regulated. In other respects, the macrocyclic peptide may have anti-cyno, anti-mouse, and/or anti-woodchuck binding and therapeutic activity.

As used herein, the term "subject" is intended to include human and non-human animals. Non-human animals includes all vertebrates, *e.g.*, mammals and non-mammals, such as non-human primates, sheep, dogs, cats, cows, horses, chickens, woodchuck, amphibians, and reptiles, although mammals are preferred, 5 such as non-human primates, sheep, dogs, cats, cows and horses. Preferred subjects include human patients in need of enhancement of an immune response. The methods are particularly suitable for treating human patients having a disorder that can be treated by augmenting the T-cell mediated immune response. In a particular embodiment, the methods are particularly suitable for treatment of cancer cells *in* 10 *vivo*. To achieve antigen-specific enhancement of immunity, the macrocyclic peptides can be administered together with an antigen of interest. When macrocyclic peptides to PD-L1 are administered together with another agent, the two can be administered in either order or simultaneously.

The disclosure further provides methods for detecting the presence of human, 15 woodchuck, cyno, and/or mouse PD-L1 antigen in a sample, or measuring the amount of human, woodchuck, cyno, and/or mouse PD-L1 antigen, comprising contacting the sample, and a control sample, with a reference macrocyclic peptide which specifically binds to human, woodchuck, cyno, and/or mouse PD-L1, under conditions that allow for formation of a complex between the macrocycle and human, 20 woodchuck, cyno, and/or mouse PD-L1. The formation of a complex is then detected, wherein a difference complex formation between the sample compared to the control sample is indicative the presence of human, woodchuck, cyno, and/or mouse PD-L1 antigen in the sample.

Given the specific binding of the macrocyclic peptides of the disclosure for 25 PD-L1, compared to CD28, ICOS and CTLA-4, the macrocyclic peptides of the disclosure can be used to specifically detect PD-L1 expression on the surface of cells and, moreover, can be used to purify PD-L1 via immunoaffinity purification.

Cancer

30 Blockade of PD-1 by macrocyclic peptides can enhance the immune response to cancerous cells in the patient. The ligand for PD-1, PD-L1, is not expressed in normal human cells, but is abundant in a variety of human cancers (Dong et al., *Nat.*

Med., 8:787-789 (2002)). The interaction between PD-1 and PD-L1 results in a decrease in tumor infiltrating lymphocytes, a decrease in T-cell receptor mediated proliferation, and immune evasion by the cancerous cells (Dong et al., *J. Mol. Med.*, 81:281-287 (2003); Blank et al., *Cancer Immunol. Immunother.*, 54:307-314 (2005); 5 Konishi et al., *Clin. Cancer Res.*, 10:5094-5100 (2004)). Immune suppression can be reversed by inhibiting the local interaction of PD-1 to PD-L1 and the effect is additive when the interaction of PD-1 to PD-L2 is blocked as well (Iwai et al., *Proc. Natl. Acad. Sci.*, 99:12293-12297 (2002); Brown et al., *J. Immunol.*, 170:1257-1266 (2003)). While previous studies have shown that T-cell proliferation can be restored 10 by inhibiting the interaction of PD-1 to PD-L1, there have been no reports of a direct effect on cancer tumor growth *in vivo* by blocking the PD-1/PD-L1 interaction. In one aspect, the present disclosure relates to treatment of a subject *in vivo* using a macrocyclic peptide such that growth of cancerous tumors is inhibited. A macrocyclic peptide may be used alone to inhibit the growth of cancerous tumors. 15 Alternatively, a macrocyclic peptide may be used in conjunction with other immunogenic agents, standard cancer treatments, or other macrocyclic peptides, as described below.

20 Accordingly, in one embodiment, the disclosure provides a method of inhibiting growth of tumor cells in a subject, comprising administering to the subject a therapeutically effective amount of a macrocyclic peptide.

Preferred cancers whose growth may be inhibited using the macrocyclic peptides of the disclosure include cancers typically responsive to immunotherapy. Non-limiting examples of preferred cancers for treatment include melanoma (*e.g.*, metastatic malignant melanoma), renal cell carcinoma (*e.g.*, clear cell carcinoma), 25 prostate cancer (*e.g.*, hormone refractory prostate adenocarcinoma and castration-resistant prostate cancer), breast cancer, colorectal cancer and lung cancer (*e.g.*, squamous and non-squamous non-small cell lung cancer). Additionally, the disclosure includes refractory or recurrent malignancies whose growth may be inhibited using the macrocyclic peptides of the disclosure.

30 Examples of other cancers that may be treated using the methods of the disclosure include bone cancer, pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular malignant melanoma, uterine cancer, ovarian cancer,

colon cancer, rectal cancer, cancer of the anal region, stomach/gastric cancer, testicular cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, Hodgkin's Disease, non-Hodgkin's lymphoma, cancer of the esophagus, cancer 5 of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, chronic or acute leukemias including acute myeloid leukemia, chronic myeloid leukemia, acute lymphoblastic leukemia, chronic lymphocytic leukemia, solid tumors of childhood, lymphocytic lymphoma, cancer of 10 the bladder, cancer of the kidney or ureter, carcinoma of the renal pelvis, neoplasm of the central nervous system (CNS), primary CNS lymphoma, tumor angiogenesis, spinal axis tumor, brain stem glioma, pituitary adenoma, Kaposi's sarcoma, epidermoid cancer, squamous cell cancer, T-cell lymphoma, environmentally induced cancers including those induced by asbestos, and combinations of said cancers. The 15 present disclosure is also useful for treatment of metastatic cancers, especially metastatic cancers that express PD-L1 (Iwai et al., *Int. Immunol.*, 17:133-144 (2005)).

Optionally, macrocyclic peptides to PD-L1 can be combined with an 20 immunogenic agent, such as cancerous cells, purified tumor antigens (including recombinant proteins, peptides, and carbohydrate molecules), cells, and cells transfected with genes encoding immune stimulating cytokines (He et al., *J. Immunol.*, 173:4919-4928 (2004)). Non-limiting examples of tumor vaccines that can be used include peptides of melanoma antigens, such as peptides of gp100, 25 MAGE antigens, Trp-2, MART1 and/or tyrosinase, or tumor cells transfected to express the cytokine GM-CSF (discussed further below).

In humans, some tumors have been shown to be immunogenic such as 30 melanomas. It is anticipated that by raising the threshold of T cell activation by PD-L1 blockade, we may expect to activate tumor responses in the host.

PD-L1 blockade is likely to be most effective when combined with a vaccination protocol. Many experimental strategies for vaccination against tumors have been devised (see Rosenberg, S., Development of Cancer Vaccines, ASCO Educational Book Spring: 60-62 (2000); Logothetis, C., ASCO Educational Book

Spring: 300-302 (2000); Khayat, D., ASCO Educational Book Spring: 414-428 (2000); Foon, K., ASCO Educational Book Spring: 730-738 (2000); see also Restifo, N. et al., Cancer Vaccines, Chapter 61, pp. 3023-3043, in DeVita, V. et al., eds., *Cancer: Principles and Practice of Oncology*, Fifth Edition (1997)). In one of these 5 strategies, a vaccine is prepared using autologous or allogeneic tumor cells. These cellular vaccines have been shown to be most effective when the tumor cells are transduced to express GM-CSF. GM-CSF has been shown to be a potent activator of antigen presentation for tumor vaccination (Dranoff et al., *Proc. Natl. Acad. Sci. USA*, 90: 3539-3543 (1993)).

10 The study of gene expression and large scale gene expression patterns in various tumors has led to the definition of so called tumor specific antigens (Rosenberg, S.A., *Immunity*, 10:281-287 (1999)). In many cases, these tumor specific antigens are differentiated antigens expressed in the tumors and in the cell from which the tumor arose, for example melanocyte antigens gp100, MAGE 15 antigens, and Trp-2. More importantly, many of these antigens can be shown to be the targets of tumor specific T cells found in the host. PD-L1 blockade may be used in conjunction with a collection of recombinant proteins and/or peptides expressed in a tumor in order to generate an immune response to these proteins. These proteins are normally viewed by the immune system as self antigens and are therefore tolerant 20 to them. The tumor antigen may also include the protein telomerase, which is required for the synthesis of telomeres of chromosomes and which is expressed in more than 85% of human cancers and in only a limited number of somatic tissues (Kim, N et al., *Science*, 266:201 1-2013 (1994)). (These somatic tissues may be protected from immune attack by various means). Tumor antigen may also be "neo- 25 antigens" expressed in cancer cells because of somatic mutations that alter protein sequence or create fusion proteins between two unrelated sequences (*i.e.*, bcr-abl in the Philadelphia chromosome), or idiotype from B cell tumors.

Other tumor vaccines may include the proteins from viruses implicated in 30 human cancers such a Human Papilloma Viruses (HPV), Hepatitis Viruses (HBV and HCV) and Kaposi's Herpes Sarcoma Virus (KHSV). Another form of tumor specific antigen which may be used in conjunction with PD-L1 blockade is purified heat shock proteins (HSP) isolated from the tumor tissue itself. These heat shock proteins

contain fragments of proteins from the tumor cells and these HSPs are highly efficient at delivery to antigen presenting cells for eliciting tumor immunity (Suot, R. et al., *Science*, 269:1585-1588 (1995); Tamura, Y. et al., *Science*, 278:1 17-120 (1997)).

5 Dendritic cells (DC) are potent antigen presenting cells that can be used to prime antigen-specific responses. DCs can be produced *ex vivo* and loaded with various protein and peptide antigens as well as tumor cell extracts (Nestle, F. et al., *Nat. Med.*, 4:328-332 (1998)). DCs may also be transduced by genetic means to express these tumor antigens as well. DCs have also been fused directly to tumor 10 cells for the purposes of immunization (Kugler, A. et al., *Nat. Med.*, 6:332-336 (2000)). As a method of vaccination, DC immunization may be effectively combined with PD-L1 blockade to activate more potent anti-tumor responses.

PD-L1 blockade may also be combined with standard cancer treatments. PD-L1 blockade may be effectively combined with chemotherapeutic regimes. In these 15 instances, it may be possible to reduce the dose of chemotherapeutic reagent administered (Mokyr, M. et al., *Cancer Res.*, 58:5301-5304 (1998)). An example of such a combination is a macrocyclic peptide in combination with decarbazine for the treatment of melanoma. Another example of such a combination is a macrocyclic peptide in combination with interleukin-2 (IL-2) for the treatment of melanoma. The 20 scientific rationale behind the combined use of PD-L1 blockade and chemotherapy is that cell death, that is a consequence of the cytotoxic action of most chemotherapeutic compounds, should result in increased levels of tumor antigen in the antigen presentation pathway. Other combination therapies that may result in synergy with PD-L1 blockade through cell death are radiation, surgery, and hormone 25 deprivation. Each of these protocols creates a source of tumor antigen in the host. Angiogenesis inhibitors may also be combined with PD-L1 blockade. Inhibition of angiogenesis leads to tumor cell death which may feed tumor antigen into host antigen presentation pathways.

PD-L1 blocking macrocyclic peptides can also be used in combination with 30 bispecific macrocyclic peptides that target Fc alpha or Fc gamma receptor-expressing effectors cells to tumor cells (see, e.g., U.S. Patent Nos. 5,922,845 and 5,837,243). Bispecific macrocyclic peptides can be used to target two separate antigens. For

example anti-Fc receptor/anti tumor antigen (*e.g.*, Her-2/neu) bispecific macrocyclic peptides have been used to target macrophages to sites of tumor. This targeting may more effectively activate tumor specific responses. The T cell arm of these responses would be augmented by the use of PD-L1 blockade. Alternatively, antigen may be 5 delivered directly to DCs by the use of bispecific macrocyclic peptides which bind to tumor antigen and a dendritic cell specific cell surface marker.

Tumors evade host immune surveillance by a large variety of mechanisms. Many of these mechanisms may be overcome by the inactivation of proteins which are expressed by the tumors and which are immunosuppressive. These include 10 among others TGF-beta (Kehrl, J. et al., *J. Exp. Med.*, 163:1037-1050 (1986)), IL-10 (Howard, M. et al., *Immunology Today*, 13:198-200 (1992)), and Fas ligand (Hahne, M. et al., *Science*, 274:1363-1365 (1996)). Macrocylic peptides to each of these entities may be used in combination with anti-PD-L1 to counteract the effects of the immunosuppressive agent and favor tumor immune responses by the host.

15 Other macrocyclic peptides which may be used to activate host immune responsiveness can be used in combination with anti-PD-L1. These include molecules on the surface of dendritic cells which activate DC function and antigen presentation. Anti-CD40 macrocyclic peptides are able to substitute effectively for T cell helper activity (Ridge, J. et al., *Nature*, 393:474-478 (1998)) and can be used in 20 conjunction with PD-1 antibodies (Ito, N. et al., *Immunobiology*, 201(5):527-540 (2000)). Activating macrocyclic peptides to T cell costimulatory molecules such as CTLA-4 (*e.g.*, U.S. Patent No. 5,811,097), OX-40 (Weinberg, A. et al., *Immunol.*, 164:2160-2169 (2000)), 4-1BB (Melero, I. et al., *Nat. Med.*, 3:682-685 (1997), and ICOS (Hutloff, A. et al., *Nature*, 397:262-266 (1999)) may also provide for 25 increased levels of T cell activation.

30 Bone marrow transplantation is currently being used to treat a variety of tumors of hematopoietic origin. While graft versus host disease is a consequence of this treatment, therapeutic benefit may be obtained from graft vs. tumor responses. PD-L1 blockade can be used to increase the effectiveness of the donor engrafted tumor specific T cells.

There are also several experimental treatment protocols that involve *ex vivo* activation and expansion of antigen specific T cells and adoptive transfer of these

cells into recipients in order to antigen-specific T cells against tumor (Greenberg, R. et al., *Science*, 285:546-551 (1999)). These methods may also be used to activate T cell responses to infectious agents such as CMV. *Ex vivo* activation in the presence of macrocyclic peptides may be expected to increase the frequency and activity of the 5 adoptively transferred T cells.

Infectious Diseases

Other methods of the disclosure are used to treat patients that have been exposed to particular toxins or pathogens. Accordingly, another aspect of the 10 disclosure provides a method of treating an infectious disease in a subject comprising administering to the subject a macrocyclic peptide of the present disclosure such that the subject is treated for the infectious disease.

Similar to its application to tumors as discussed above, PD-L1 blockade can be used alone, or as an adjuvant, in combination with vaccines, to stimulate the 15 immune response to pathogens, toxins, and self-antigens. Examples of pathogens for which this therapeutic approach may be particularly useful, include pathogens for which there is currently no effective vaccine, or pathogens for which conventional vaccines are less than completely effective. These include, but are not limited to HIV, Hepatitis (A, B, and C), Influenza, Herpes, Giardia, Malaria (Butler, N.S. et al., 20 *Nature Immunology* 13, 188-195 (2012); Hafalla, J.C.R., et al. *PLOS Pathogens*; February 2, 2012)), Leishmania, *Staphylococcus aureus*, *Pseudomonas Aeruginosa*. PD-L1 blockade is particularly useful against established infections by agents such as HIV that present altered antigens over the course of the infections. These novel epitopes are recognized as foreign at the time of anti-human PD-L1 administration, 25 thus provoking a strong T cell response that is not damped by negative signals through PD-L1.

Some examples of pathogenic viruses causing infections treatable by methods of the disclosure include HIV, hepatitis (A, B, or C), herpes virus (e.g., VZV, HSV-1, HAV-6, HSV-II, and CMV, Epstein Barr virus), adenovirus, influenza virus, 30 flaviviruses, echovirus, rhinovirus, coxsackie virus, cornovirus, respiratory syncytial virus, mumps virus, rotavirus, measles virus, rubella virus, parvovirus, vaccinia virus,

HTLV vims, dengue vims, papillomavims, molluscum vims, poliovims, rabies vims, JC vims and arboviral encephalitis vims.

Some examples of pathogenic bacteria causing infections treatable by methods of the disclosure include chlamydia, rickettsial bacteria, mycobacteria, 5 staphylococci, streptococci, pneumonococci, meningococci and conoococci, Klebsiella, proteus, serratia, pseudomonas, legionella, diphtheria, salmonella, bacilli, cholera, tetanus, botulism, anthrax, plague, leptospirosis, and Lyme disease bacteria.

Some examples of pathogenic fungi causing infections treatable by methods of the disclosure include *Candida* (*albicans*, *kmsei*, *glabrata*, *tropicalis*, etc.), 10 *Cryptococcus neoformans*, *Aspergillus* (*fumigatus*, *niger*, etc.), *Genus Mucorales* (*mucor*, *absidia*, *rhizophus*), *Sporothrix schenkii*, *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, *Coccidioides immitis* and *Histoplasma capsulatum*.

Some examples of pathogenic parasites causing infections treatable by methods of the disclosure include *Entamoeba histolytica*, *Balantidium coli*, 15 *Naegleria fowleri*, *Acanthamoeba* sp., *Giardia lamblia*, *Cryptosporidium* sp., *Pneumocystis carinii*, *Plasmodium vivax*, *Babesia microti*, *Trypanosoma brucei*, *Trypanosoma cruzi*, *Leishmania donovani*, *Toxoplasma gondii*, and *Nippostrongylus brasiliensis*.

In all of the above methods, PD-L1 blockade can be combined with other 20 forms of immunotherapy such as cytokine treatment (e.g., interferons, agents targeting VEGF activity or VEGF-receptors, GM-CSF, G-CSF, IL-2), or bispecific antibody therapy, which provides for enhanced presentation of tumor antigens (see, e.g., Holliger, *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993); Poljak, *Structure*, 2:1 121-1 123 (1994)).

25

Autoimmune Reactions

The macrocyclic peptides may provoke and amplify autoimmune responses. Indeed, induction of anti-tumor responses using tumor cell and peptide vaccines 30 reveals that many anti-tumor responses involve anti-self reactivities (depigmentation observed in anti-CTLA-4+GM-CSF-modified B 16 melanoma in van Elsas et al. supra; depigmentation in Trp-2 vaccinated mice (Overwijk, W. et al., *Proc. Natl. Acad. Sci. USA*, 96:2982-2987 (1999)); autoimmune prostatitis evoked by TRAMP

tumor cell vaccines (Hurwitz, A., *supra* (2000)), melanoma peptide antigen vaccination and vitiligo observed in human clinical trials (Rosenberg, S.A. et al., *J. Immunother. Emphasis Tumor Immunol.*, 19(1):81-84 (1996)).

Therefore, it is possible to consider using anti-PD-L1 blockade in conjunction 5 with various self proteins in order to devise vaccination protocols to efficiently generate immune responses against these self proteins for disease treatment. For example, Alzheimer's disease involves inappropriate accumulation of A.beta. peptide in amyloid deposits in the brain; antibody responses against amyloid are able to clear these amyloid deposits (Schenk et al., *Nature*, 400:173-177 (1999)).

10 Other self proteins may also be used as targets such as IgE for the treatment of allergy and asthma, and TNF.alpha for rheumatoid arthritis. Finally, antibody responses to various hormones may be induced by the use of the macrocycles disclosed herein. Neutralizing antibody responses to reproductive hormones may be used for contraception. Neutralizing antibody response to hormones and other 15 soluble factors that are required for the growth of particular tumors may also be considered as possible vaccination targets.

Analogous methods as described above for the use of anti-PD-L1 macrocycles can be used for induction of therapeutic autoimmune responses to treat 20 patients having an inappropriate accumulation of other self-antigens, such as amyloid deposits, including A.beta. in Alzheimer's disease, cytokines such as TNF.alpha., and IgE.

Vaccines

The macrocyclic peptides may be used to stimulate antigen-specific immune 25 responses by coadministration of an anti-PD-1 macrocycle with an antigen of interest (e.g., a vaccine). Accordingly, in another aspect the disclosure provides a method of enhancing an immune response to an antigen in a subject, comprising administering to the subject: (i) the antigen; and (ii) an anti-PD-1 macrocycle such that an immune response to the antigen in the subject is enhanced. The antigen can be, for example, a 30 tumor antigen, a viral antigen, a bacterial antigen or an antigen from a pathogen. Non-limiting examples of such antigens include those discussed in the sections

above, such as the tumor antigens (or tumor vaccines) discussed above, or antigens from the viruses, bacteria or other pathogens described above.

Suitable routes of administering the compositions (*e.g.*, macrocyclic peptides, multispecific and bispecific molecules and immunoconjugates) of the disclosure *in vivo* and *in vitro* are well known in the art and can be selected by those of ordinary skill. For example, the compositions can be administered by injection (*e.g.*, intravenous or subcutaneous). Suitable dosages of the molecules used will depend on the age and weight of the subject and the concentration and/or formulation of the composition.

As previously described the macrocyclic peptides of the disclosure can be co-administered with one or other more therapeutic agents, *e.g.*, a cytotoxic agent, a radiotoxic agent or an immunosuppressive agent. The peptide can be linked to the agent (as an immunocomplex) or can be administered separate from the agent. In the latter case (separate administration), the peptide can be administered before, after or concurrently with the agent or can be co-administered with other known therapies, *e.g.*, an anti-cancer therapy, *e.g.*, radiation. Such therapeutic agents include, among others, anti-neoplastic agents such as doxorubicin (adriamycin), cisplatin bleomycin sulfate, carmustine, chlorambucil, decarbazine and cyclophosphamide hydroxyurea which, by themselves, are only effective at levels which are toxic or subtoxic to a patient. Cisplatin is intravenously administered as a 100 mg/dose once every four weeks and adriamycin is intravenously administered as a 60-75 mg/ml dose once every 21 days. Co-administration of the macrocyclic peptides of the present disclosure with chemotherapeutic agents provides two anti-cancer agents which operate via different mechanisms which yield a cytotoxic effect to human tumor cells. Such co-administration can solve problems due to development of resistance to drugs or a change in the antigenicity of the tumor cells which would render them unreactive with the peptides.

Also within the scope of the present disclosure are kits comprising the compositions of the disclosure (*e.g.*, macrocyclic peptides, bispecific or multispecific molecules, or immunoconjugates) and instructions for use. The kit can further contain at least one additional reagent, or one or more additional macrocyclic peptides of the disclosure (*e.g.*, a human antibody having a complementary activity

which binds to an epitope in PD-L1 antigen distinct from the macrocycle). Kits typically include a label indicating the intended use of the contents of the kit. The term label includes any writing, or recorded material supplied on or with the kit, or which otherwise accompanies the kit.

5

Combination Therapy

The combination of the macrocyclic peptides of the present disclosure with another PD-L1 antagonist and/or other immunomodulator is useful for enhancement of an immune response against a hyperproliferative disease. For example, these 10 molecules can be administered to cells in culture, *in vitro* or *ex vivo*, or to human subjects, *e.g.*, *in vivo*, to enhance immunity in a variety of situations. Accordingly, in one aspect, the disclosure provides a method of modifying an immune response in a subject comprising administering to the subject a macrocyclic peptide of the disclosure such that the immune response in the subject is modified. Preferably, the 15 response is enhanced, stimulated or up-regulated. In another embodiment, the instant disclosure provides a method of altering adverse events associated with treatment of a hyperproliferative disease with an immunostimulatory therapeutic agent, comprising administering a macrocyclic peptide of the present disclosure and a subtherapeutic dose of another immunomodulator to a subject.

20 Blockade of PD-L1 by macrocyclic peptides can enhance the immune response to cancerous cells in the patient. Cancers whose growth may be inhibited using the macrocyclic peptides of the instant disclosure include cancers typically responsive to immunotherapy. Representative examples of cancers for treatment with the combination therapy of the instant disclosure include melanoma (*e.g.*, metastatic malignant melanoma), renal cancer, prostate cancer, breast cancer, colon cancer and lung cancer. Examples of other cancers that may be treated using the 25 methods of the instant disclosure include bone cancer, pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular malignant melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, testicular cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the 30 endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, Hodgkin's Disease, non-Hodgkin's lymphoma, cancer of the esophagus, cancer

of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, chronic or acute leukemias including acute myeloid leukemia, chronic myeloid leukemia, acute lymphoblastic leukemia, chronic 5 lymphocytic leukemia, solid tumors of childhood, lymphocytic lymphoma, cancer of the bladder, cancer of the kidney or ureter, carcinoma of the renal pelvis, neoplasm of the central nervous system (CNS), primary CNS lymphoma, tumor angiogenesis, spinal axis tumor, brain stem glioma, pituitary adenoma, Kaposi's sarcoma, epidermoid cancer, squamous cell cancer, T-cell lymphoma, environmentally induced 10 cancers including those induced by asbestos, and combinations of said cancers. The present disclosure is also useful for treatment of metastatic cancers.

In certain embodiments, the combination of therapeutic agents containing at least one macrocyclic peptide discussed herein may be administered concurrently as a single composition in a pharmaceutically acceptable carrier, or concurrently as 15 separate compositions wherein each agent can be administered sequentially. For example, a second immunomodulator and a macrocyclic peptide of the present disclosure can be administered sequentially, such as the second immunomodulator administered first and the macrocyclic peptide second, or the macrocyclic peptide being administered first and the second immunomodulator second. Furthermore, if 20 more than one dose of the combination therapy is administered sequentially, the order of the sequential administration can be reversed or kept in the same order at each time point of administration, sequential administrations may be combined with concurrent administrations, or any combination thereof. For example, the first administration of a second immunomodulator and the macrocyclic peptide may be 25 concurrent, the second administration may be sequential with the second immunomodulator first and the macrocyclic peptide second, and the third administration may be sequential with the macrocyclic peptide first and second immunomodulator second, etc. Another representative dosing scheme may involve a first administration that is sequential with the macrocyclic peptide first and the 30 second immunomodulator second, and subsequent administrations may be concurrent.

Optionally, the combination of the macrocyclic peptide and a second immunomodulator can be further combined with an immunogenic agent, such as cancerous cells, purified tumor antigens (including recombinant proteins, peptides, and carbohydrate molecules), cells, and cells transfected with genes encoding 5 immune stimulating cytokines (He et al., *J. Immunol.*, 173:4919-4928 (2004)). Non-limiting examples of tumor vaccines that can be used include peptides of melanoma antigens, such as peptides of gp100, MAGE antigens, Trp-2, MART-1 and/or tyrosinase, or tumor cells transfected to express the cytokine GM-CSF (discussed further below).

10 A combined PD-L1 macrocyclic peptide and a second immunomodulator can be further combined with a vaccination protocol. Many experimental strategies for vaccination against tumors have been devised (see Rosenberg, S., Development of Cancer Vaccines, ASCO Educational Book Spring: 60-62 (2000); Logothetis, C., ASCO Educational Book Spring: 300-302 (2000); Khayat, D., ASCO Educational Book Spring: 414-428 (2000); Foon, K., ASCO Educational Book Spring: 730-738 (2000); see also Restifo et al., Cancer Vaccines, Chapter 61, pp. 3023-3043 in 15 DeVita et al., eds., *Cancer: Principles and Practice of Oncology*, Fifth Edition (1997)). In one of these strategies, a vaccine is prepared using autologous or allogeneic tumor cells. These cellular vaccines have been shown to be most effective 20 when the tumor cells are transduced to express GM-CSF. GM-CSF has been shown to be a potent activator of antigen presentation for tumor vaccination (Dranoff et al., *Proc. Natl. Acad. Sci. USA*, 90:3539-3543 (1993)).

The study of gene expression and large scale gene expression patterns in various tumors has led to the definition of so called tumor specific antigens 25 (Rosenberg, *Immunity*, 10:281-287 (1999)). In many cases, these tumor specific antigens are differentiation antigens expressed in the tumors and in the cell from which the tumor arose, for example melanocyte antigens gp100, MAGE antigens, and Trp-2. More importantly, many of these antigens can be shown to be the targets 30 of tumor specific T cells found in the host. In certain embodiments, a combined PD-L1 macrocyclic peptide and a second immunomodulator may be used in conjunction with a collection of recombinant proteins and/or peptides expressed in a tumor in order to generate an immune response to these proteins. These proteins are normally

viewed by the immune system as self-antigens and are, therefore, tolerant to them. The tumor antigen may also include the protein telomerase, which is required for the synthesis of telomeres of chromosomes and which is expressed in more than 85% of human cancers and in only a limited number of somatic tissues (Kim et al., *Science*, 266:201 1-2013 (1994)). (These somatic tissues may be protected from immune attack by various means). Tumor antigen may also be "neo-antigens" expressed in cancer cells because of somatic mutations that alter protein sequence or create fusion proteins between two unrelated sequences (*i.e.*, bcr-abl in the Philadelphia chromosome), or idiotype from B cell tumors.

10 Other tumor vaccines may include the proteins from viruses implicated in human cancers such a Human Papilloma Viruses (HPV), Hepatitis Viruses (HBV and HCV) and Kaposi's Herpes Sarcoma Virus (KHSV). Another form of tumor specific antigen which may be used in conjunction with PD-L1 macrocyclic peptide blockade is purified heat shock proteins (HSP) isolated from the tumor tissue itself. These heat 15 shock proteins contain fragments of proteins from the tumor cells and these HSPs are highly efficient at delivery to antigen presenting cells for eliciting tumor immunity (Suot et al., *Science*, 269:1585-1588 (1995); Tamura et al., *Science*, 278:1 17-120 (1997)).

20 Dendritic cells (DC) are potent antigen presenting cells that can be used to prime antigen-specific responses. DCs can be produced *ex vivo* and loaded with various protein and peptide antigens as well as tumor cell extracts (Nestle et al., *Nat. Med.*, 4:328-332 (1998)). DCs may also be transduced by genetic means to express these tumor antigens as well. DCs have also been fused directly to tumor cells for the purposes of immunization (Kugler et al., *Nat. Med.*, 6:332-336 (2000)). As a 25 method of vaccination, DC immunization may be effectively further combined with a combined anti-PD-L1 macrocyclic peptide and a second immunomodulator to activate more potent anti-tumor responses.

30 A combined anti-PD-L1 macrocyclic peptide and additional immunomodulator may also be further combined with standard cancer treatments. For example, a combination of a macrocyclic peptide and a second immunomodulator may be effectively combined with chemotherapeutic regimes. In these instances, as is observed with the combination of a macrocyclic peptide and a

second immunomodulator, it may be possible to reduce the dose of other chemotherapeutic reagent administered with the combination of the instant disclosure (Mokyr et al., *Cancer Res.*, 58:5301-5304 (1998)). An example of such a combination is a combination of a macrocyclic peptide and a second immunomodulator further in combination with decarbazine for the treatment of melanoma. Another example is a combination of a macrocyclic peptide and a second immunomodulatory agent further in combination with interleukin-2 (IL-2) for the treatment of melanoma. The scientific rationale behind the combined use of PD-L1 macrocyclic peptide and another immunomodulator with chemotherapy is that cell death, which is a consequence of the cytotoxic action of most chemotherapeutic compounds, should result in increased levels of tumor antigen in the antigen presentation pathway. Other combination therapies that may result in synergy with a combined anti-PD-L1 macrocyclic peptide and additional immunomodulator through cell death include radiation, surgery, or hormone deprivation. Each of these protocols creates a source of tumor antigen in the host. Angiogenesis inhibitors may also be combined with a combined PD-L1 and second immunomodulator. Inhibition of angiogenesis leads to tumor cell death, which may also be a source of tumor antigen to be fed into host antigen presentation pathways.

A combination of PD-L1 and another immunomodulator can also be used in combination with bispecific macrocyclic peptides that target Fc.alpha. or Fc.gamma. receptor-expressing effector cells to tumor cells (see, e.g., U.S. Patent Nos. 5,922,845 and 5,837,243). Bispecific macrocyclic peptides can be used to target two separate antigens. For example anti-Fc receptor/anti tumor antigen (e.g., Her-2/neu) bispecific macrocyclic peptides have been used to target macrophages to sites of tumor. This targeting may more effectively activate tumor specific responses. The T cell arm of these responses would be augmented by the use of a combined PD-L1 and a second immunomodulator. Alternatively, antigen may be delivered directly to DCs by the use of bispecific macrocyclic peptides which bind to tumor antigen and a dendritic cell specific cell surface marker.

In another example, a combination of a macrocyclic peptide and a second immunomodulator can be used in conjunction with anti-neoplastic macrocyclic agents, such as RITUXAN® (rituximab), HERCEPTIN® (trastuzumab), BEXXAR®

(tositumomab), ZEVALIN® (ibritumomab), CAMPATH® (alemtuzumab), Lymphocide (eprtuzumab), AVASTEST® (bevacizumab), and TARCEVA® (erlotinib), and the like. By way of example and not wishing to be bound by theory, treatment with an anti-cancer antibody or an anti-cancer antibody conjugated to a 5 toxin can lead to cancer cell death (e.g., tumor cells) which would potentiate an immune response mediated by the second immunomodulator target or PD-L1. In an exemplary embodiment, a treatment of a hyperproliferative disease (e.g., a cancer tumor) may include an anti-cancer antibody in combination with a macrocyclic peptide and a second immunomodulator concurrently or sequentially or any 10 combination thereof, which may potentiate an anti-tumor immune responses by the host.

Tumors evade host immune surveillance by a large variety of mechanisms. Many of these mechanisms may be overcome by the inactivation of proteins, which are expressed by the tumors and which are immunosuppressive. These include, 15 among others, TGF-.beta. (Kehrl, J. et al., *J. Exp. Med.*, 163:1037-1050 (1986)), IL-10 (Howard, M. et al., *Immunology Today*, 13:198-200 (1992)), and Fas ligand (Hahne, M. et al., *Science*, 274:1363-1365 (1996)). In another example, antibodies to each of these entities may be further combined with a macrocyclic peptide and another immunomodulator to counteract the effects of immunosuppressive agents and 20 favor anti-tumor immune responses by the host.

Other agents that may be used to activate host immune responsiveness can be further used in combination with a macrocyclic peptide of the present disclosure. These include molecules on the surface of dendritic cells that activate DC function and antigen presentation. Anti-CD40 macrocyclic peptides are able to substitute 25 effectively for T cell helper activity (Ridge, J. et al., *Nature*, 393:474-478 (1998)) and can be used in conjunction with the macrocyclic peptides of the present disclosure, either alone or in combination with an anti-CTLA-4 combination (Ito, N. et al., *Immunobiology*, 201(5):527-540 (2000)). Activating macrocyclic peptides to T cell costimulatory molecules, such as OX-40 (Weinberg, A. et al., *Immunol.*, 30 164:2160-2169 (2000)), 4-IBB (Melero, I. et al., *Nat. Med.*, 3:682-685 (1997), and ICOS (Hutloff, A. et al., *Nature*, 397:262-266 (1999)) may also provide for increased levels of T cell activation.

Bone marrow transplantation is currently being used to treat a variety of tumors of hematopoietic origin. While graft versus host disease is a consequence of this treatment, therapeutic benefit may be obtained from graft vs. tumor responses. A macrocyclic peptide of the present disclosure, either alone or in combination with 5 another immunomodulator, can be used to increase the effectiveness of the donor engrafted tumor specific T cells.

There are also several experimental treatment protocols that involve *ex vivo* activation and expansion of antigen specific T cells and adoptive transfer of these 10 cells into recipients in order to antigen-specific T cells against tumor (Greenberg, R. et al., *Science*, 285:546-551 (1999)). These methods may also be used to activate T cell responses to infectious agents such as CMV. *Ex vivo* activation in the presence a macrocyclic peptide of the present disclosure, either alone or in combination with another immunomodulator, may be expected to increase the frequency and activity of the adoptively transferred T cells.

15 In certain embodiments, the present disclosure provides a method for altering an adverse event associated with treatment of a hyperproliferative disease with an immunostimulatory agent, comprising administering a macrocyclic peptide of the present disclosure in combination with a subtherapeutic dose of another immunomodulator to a subject. For example, the methods of the present disclosure 20 provide for a method of reducing the incidence of immunostimulatory therapeutic antibody-induced colitis or diarrhea by administering a non-absorbable steroid to the patient. Because any patient who will receive an immunostimulatory therapeutic antibody is at risk for developing colitis or diarrhea induced by such treatment, this entire patient population is suitable for therapy according to the methods of the 25 present disclosure. Although steroids have been administered to treat inflammatory bowel disease (IBD) and prevent exacerbations of IBD, they have not been used to prevent (decrease the incidence of) IBD in patients who have not been diagnosed with IBD. The significant side effects associated with steroids, even non-absorbable steroids, have discouraged prophylactic use.

30 In further embodiments, a macrocyclic peptide of the present disclosure, either alone or in combination with another immunomodulator, can be further combined with the use of any non-absorbable steroid. As used herein, a "non-

absorbable steroid" is a glucocorticoid that exhibits extensive first pass metabolism such that, following metabolism in the liver, the bioavailability of the steroid is low, *i.e.*, less than about 20%. In one embodiment of the disclosure, the non-absorbable steroid is budesonide. Budesonide is a locally-acting glucocorticosteroid, which is 5 extensively metabolized, primarily by the liver, following oral administration.

ENTOCORT® EC (Astra-Zeneca) is a pH- and time-dependent oral formulation of budesonide developed to optimize drug delivery to the ileum and throughout the colon. ENTOCORT® EC is approved in the U.S. for the treatment of mild to moderate Crohn's disease involving the ileum and/or ascending colon. The usual oral dosage of ENTOCORT® EC for the treatment of Crohn's disease is 6 to 9 mg/day. ENTOCORT® EC is released in the intestines before being absorbed and retained in the gut mucosa. Once it passes through the gut mucosa target tissue, ENTOCORT® EC is extensively metabolized by the cytochrome P450 system in the liver to metabolites with negligible glucocorticoid activity. Therefore, the bioavailability is low (about 10%). The low bioavailability of budesonide results in an improved therapeutic ratio compared to other glucocorticoids with less extensive first-pass metabolism. Budesonide results in fewer adverse effects, including less hypothalamic-pituitary suppression, than systemically-acting corticosteroids. However, chronic administration of ENTOCORT® EC can result in systemic glucocorticoid effects such as hypercorticism and adrenal suppression. See *Physicians' Desk Reference Supplement*, 58th Edition, 608-610 (2004).

In still further embodiments, a combination PD-L1 and another immunomodulator in conjunction with a non-absorbable steroid can be further combined with a salicylate. Salicylates include 5-ASA agents such as, for example:

30 Dosage and Formulation

A suitable peptide of Formula I, or more specifically a macrocyclic peptide described herein, can be administered to patients to treat diabetes and other related

diseases as the compound alone and or mixed with an acceptable carrier in the form of pharmaceutical formulations. Those skilled in the art of treating diabetes can easily determine the dosage and route of administration of the compound to mammals, including humans, in need of such treatment. The route of administration 5 may include but is not limited to oral, intraoral, rectal, transdermal, buccal, intranasal, pulmonary, subcutaneous, intramuscular, intradermal, sublingual, intracolonic, intraocular, intravenous, or intestinal administration. The compound is formulated according to the route of administration based on acceptable pharmacy practice (Fingl et al., in *The Pharmacological Basis of Therapeutics*, Chapter 1, p. 1 (1975); 10 *Remington's Pharmaceutical Sciences*, 18th Edition, Mack Publishing Co., Easton, PA (1990)).

The pharmaceutically acceptable peptide compositions described herein can be administered in multiple dosage forms such as tablets, capsules (each of which includes sustained release or timed release formulations), pills, powders, granules, 15 elixirs, in situ gels, microspheres, crystalline complexes, liposomes, micro-emulsions, tinctures, suspensions, syrups, aerosol sprays and emulsions. The compositions described herein can also be administered in oral, intravenous (bolus or infusion), intraperitoneal, subcutaneous, transdermally or intramuscular form, all using dosage forms well known to those of ordinary skill in the pharmaceutical arts. 20 The compositions may be administered alone, but generally will be administered with a pharmaceutical carrier selected on the basis of the chosen route of administration and standard pharmaceutical practice.

The dosage regimen for the compositions described herein will, of course, vary depending upon known factors, such as the pharmacodynamic characteristics of 25 the particular agent and its mode and route of administration; the species, age, sex, health, medical condition, and weight of the recipient; the nature and extent of the symptoms; the kind of concurrent treatment; the frequency of treatment; the route of administration, the renal and hepatic function of the patient, and the effect desired. A physician or veterinarian can determine and prescribe the effective amount of the 30 drug required to prevent, counter, or arrest the progress of the disease state.

By way of general guidance, the daily oral dosage of the active ingredient, when used for the indicated effects, will range between about 0.001 to 1000 mg/kg of

body weight, preferably between about 0.01 to 100 mg/kg of body weight per day, and most preferably between about 0.6 to 20 mg/kg/day. Intravenously, the daily dosage of the active ingredient when used for the indicated effects will range between 0.001ng to 100.0 ng per min/per Kg of body weight during a constant rate infusion.

5 Such constant intravenous infusion can be preferably administered at a rate of 0.01 ng to 50 ng per min per Kg body weight and most preferably at 0.01 ng to 10.0 mg per min per Kg body weight. The compositions described herein may be administered in a single daily dose, or the total daily dosage may be administered in divided doses of two, three, or four times daily. The compositions described herein may also be
10 administered by a depot formulation that will allow sustained release of the drug over a period of days/weeks/months as desired.

The compositions described herein can be administered in intranasal form via topical use of suitable intranasal vehicles, or via transdermal routes, using transdermal skin patches. When administered in the form of a transdermal delivery
15 system, the dosage administration will, of course, be continuous rather than intermittent throughout the dosage regimen.

The compositions are typically administered in a mixture with suitable pharmaceutical diluents, excipients, or carriers (collectively referred to herein as pharmaceutical carriers) suitably selected with respect to the intended form of
20 administration, that is, oral tablets, capsules, elixirs, aerosol sprays generated with or without propellant and syrups, and consistent with conventional pharmaceutical practices.

For instance, for oral administration in the form of a tablet or capsule, the active drug component can be combined with an oral, non-toxic, pharmaceutically
25 acceptable, inert carrier such as but not limited to, lactose, starch, sucrose, glucose, methyl cellulose, magnesium stearate, dicalcium phosphate, calcium sulfate, mannitol, and sorbitol; for oral administration in liquid form, the oral drug components can be combined with any oral, non-toxic, pharmaceutically acceptable inert carrier such as, but not limited to, ethanol, glycerol, and water. Moreover, when
30 desired or necessary, suitable binders, lubricants, disintegrating agents, and coloring agents can also be incorporated into the mixture. Suitable binders include, but not limited to, starch, gelatin, natural sugars such as, but not limited to, glucose or beta-

lactose, corn sweeteners, natural and synthetic gums such as acacia, tragacanth, or sodium alginate, carboxymethylcellulose, polyethylene glycol, and waxes.

Lubricants used in these dosage forms include sodium oleate, sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, and sodium chloride.

5 Disintegrants include, but are not limited to, starch, methyl cellulose, agar, bentonite, and xanthan gum.

The compositions described herein may also be administered in the form of mixed micellar or liposome delivery systems, such as small unilamellar vesicles, large unilamellar vesicles, and multilamellar vesicles. Liposomes can be formed 10 from a variety of phospholipids, such as cholesterol, stearylamine, or phosphatidylcholines. Permeation enhancers may be added to enhance drug absorption.

Since prodrugs are known to enhance numerous desirable qualities of pharmaceuticals (*i.e.*, solubility, bioavailability, manufacturing, etc.) the compounds 15 described herein may be delivered in prodrug form. Thus, the subject matter described herein is intended to cover prodrugs of the presently claimed compounds, methods of delivering the same, and compositions containing the same.

The compositions described herein may also be coupled with soluble polymers as targetable drug carriers. Such polymers can include polyvinyl-pyrrolidone, pyran copolymer, polyhydroxypropyl- methacrylamide-phenol, polyhydroxyethylaspartamidephenol, or polyethyleneoxide-polylysine substituted with palmitoyl residues. Furthermore, the compositions described herein may be combined with a class of biodegradable polymers useful in achieving controlled release of a drug, for example, polylactic acid, polyglycolic acid, copolymers of 20 polylactic and polyglycolic acid, polyepsilon caprolactone, polyhydroxy butyric acid, polyorthoesters, polyacetals, polydihydropyrans, polycyanoacylates, and crosslinked or amphipathic block copolymers of hydrogels.

Dosage forms (pharmaceutical compositions) suitable for administration may contain from about 0.01 milligram to about 500 milligrams of active ingredient per 30 dosage unit. In these pharmaceutical compositions the active ingredient will ordinarily be present in an amount of about 0.5-95% by weight based on the total weight of the composition.

Gelatin capsules may contain the active ingredient and powdered carriers, such as lactose, starch, cellulose derivative, magnesium stearate, and stearic acid. Similar diluents can be used to make compressed tablets. Both tablets and capsules can be manufactured as sustained release products to provide for continuous release 5 of medication over a period of hours. Compressed tablets can be sugar coated or film coated to mask any unpleasant taste and protect the tablet from the atmosphere, or enteric coated for selective disintegration in the gastrointestinal tract.

Liquid dosage forms for oral administration can contain coloring and flavoring to increase patient acceptance.

10 In general, water, a suitable oil, saline, aqueous dextrose (glucose), and related sugar solutions and glycols such as propylene glycol or polyethylene glycols are suitable carriers for parenteral solutions. Solution for parenteral administration preferably contains a water-soluble salt of the active ingredient, suitable stabilizing agents, and if necessary, buffer substances. Antioxidizing agents such as sodium 15 bisulfite, sodium sulfite, or ascorbic acid, either alone or combined, are suitable stabilizing agents. Also used are citric acid and its salts and sodium EDTA. In addition, parenteral solutions can contain preservatives, such as benzalkonium chloride, methyl- or propyl-paraben, and chlorobutanol.

20 Suitable pharmaceutical carriers are described in *Remington: The Science and Practice of Pharmacy*, Nineteenth Edition, Mack Publishing Company (1995), a standard reference text in this field.

Representative useful pharmaceutical dosage forms for administration of the compounds described herein can be illustrated as follows:

25 **Capsules**

A large number of unit capsules can be prepared by filling standard two-piece hard gelatin capsules with 100 milligrams of powdered active ingredient, 150 milligrams of lactose, 50 milligrams of cellulose, and 6 milligrams magnesium stearate.

30

Soft Gelatin Capsules

A mixture of active ingredient in a digestible oil such as soybean oil, cottonseed oil or olive oil may be prepared and injected by means of a positive displacement pump into gelatin to form soft gelatin capsules containing 100 milligrams of the active ingredient. The capsules should be washed and dried.

Tablets

Tablets may be prepared by conventional procedures so that the dosage unit, for example is 100 milligrams of active ingredient, 0.2 milligrams of colloidal silicon dioxide, 5 milligrams of magnesium stearate, 275 milligrams of microcrystalline cellulose, 11 milligrams of starch and 98.8 milligrams of lactose. Appropriate coatings may be applied to increase palatability or delay absorption.

Injectable

An injectable formulation of a peptide composition described herein may or may not require the use of excipients such as those that have been approved by regulatory bodies. These excipients include, but are not limited to, solvents and co-solvents, solubilizing, emulsifying or thickening agents, chelating agents, anti-oxidants and reducing agents, antimicrobial preservatives, buffers and pH adjusting agents, bulking agents, protectants and tonicity adjustors and special additives. An injectable formulation has to be sterile, pyrogen free and, in the case of solutions, free of particulate matter.

A parenteral composition suitable for administration by injection may be prepared by stirring for example, 1.5% by weight of active ingredient in a pharmaceutically acceptable buffer that may or may not contain a co-solvent or other excipient. The solution should be made isotonic with sodium chloride and sterilized.

Suspension

An aqueous suspension can be prepared for oral and/or parenteral administration so that, for example, each 5 mL contains 100 mg of finely divided active ingredient, 20 mg of sodium carboxymethyl cellulose, 5 mg of sodium

benzoate, 1.0 g of sorbitol solution, U.S.P., and 0.025 mL of vanillin or other palatable flavoring.

Biodegradable Microparticles

5 A sustained-release parenteral composition suitable for administration by injection may be prepared, for example, by dissolving a suitable biodegradable polymer in a solvent, adding to the polymer solution the active agent to be incorporated, and removing the solvent from the matrix thereby forming the matrix of the polymer with the active agent distributed throughout the matrix.

10

Peptide Synthesis

The description of the present disclosure herein should be construed in congruity with the laws and principals of chemical bonding. It should be understood that the compounds encompassed by the present disclosure are those that are suitably 15 stable for use as pharmaceutical agent. One of skill in the art will know what compounds would and would not be stable based on the general principles of chemical bonding and stability.

Chemical synthesis of a macrocyclic peptide of the present disclosure can be carried out using a variety of art recognized methods, including stepwise solid phase 20 synthesis, semi-synthesis through the conformationally-assisted re-ligation of peptide fragments, enzymatic ligation of cloned or synthetic peptide segments, and chemical ligation. A preferred method to synthesize the macrocyclic peptides and analogs thereof described herein is chemical synthesis using various solid-phase techniques such as those described in Chan, W.C. et al., eds., *Fmoc Solid Phase Synthesis*, 25 Oxford University Press, Oxford (2000); Barany, G. et al., *The Peptides: Analysis, Synthesis, Biology*, Vol. 2: "Special Methods in Peptide Synthesis, Part A", pp. 3-284, Gross, E. et al., eds., Academic Press, New York (1980); and in Stewart, J.M. et al., *Solid-Phase Peptide Synthesis*, 2nd Edition, Pierce Chemical Co., Rockford, IL (1984). The preferred strategy is based on the Fmoc (9-Fluorenylmethyl methyl-30 oxy carbonyl) group for temporary protection of the α-amino group, in combination with the *tert*-butyl group for temporary protection of the amino acid side chains (see for example Atherton, E. et al., "The Fluorenylmethoxycarbonyl Amino Protecting

Group", in *The Peptides: Analysis, Synthesis, Biology*, Vol. 9: "Special Methods in Peptide Synthesis, Part C", pp. 1-38, Udenfriend, S. et al., eds., Academic Press, San Diego (1987).

5 The peptides can be synthesized in a stepwise manner on an insoluble polymer support (also referred to as "resin") starting from the C-terminus of the peptide. A synthesis is begun by appending the C-terminal amino acid of the peptide to the resin through formation of an amide or ester linkage. This allows the eventual release of the resulting peptide as a C-terminal amide or carboxylic acid, respectively.

10 The C-terminal amino acid and all other amino acids used in the synthesis are required to have their a-amino groups and side chain functionalities (if present) differentially protected such that the a-amino protecting group may be selectively removed during the synthesis. The coupling of an amino acid is performed by activation of its carboxyl group as an active ester and reaction thereof with the unblocked a-amino group of the N-terminal amino acid appended to the resin. The 15 sequence of a-amino group deprotection and coupling is repeated until the entire peptide sequence is assembled. The peptide is then released from the resin with concomitant deprotection of the side chain functionalities, usually in the presence of appropriate scavengers to limit side reactions. The resulting peptide is finally purified by reverse phase HPLC.

20 The synthesis of the peptidyl-resins required as precursors to the final peptides utilizes commercially available cross-linked polystyrene polymer resins (Novabiochem, San Diego, CA; Applied Biosystems, Foster City, CA). Preferred solid supports are: 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetyl-p-methyl benzhydrylamine resin (Rink amide MBHA resin); 9-Fmoc-amino-xanthen-3-yloxy-Merrifield resin (Sieber amide resin); 4-(9-Fmoc)aminomethyl-3,5-dimethoxyphenoxy)valeryl-aminomethyl-Merrifield resin (PAL resin), for C-terminal carboxamides. Coupling of first and subsequent amino acids can be accomplished using HOEt, 6-Cl-HOEt or HOAt active esters produced from DIC/HOEt, HBTU/HOEt, BOP, PyBOP, or from DIC/6-Cl-HOEt, HCTU, DIC/HOAt or HATU, 25 respectively. Preferred solid supports are: 2-Chlorotriyl chloride resin and 9-Fmoc-amino-xanthen-3 -yloxy-Merrifield resin (Sieber amide resin) for protected peptide fragments. Loading of the first amino acid onto the 2-chlorotriyl chloride resin is

best achieved by reacting the Fmoc-protected amino acid with the resin in dichloromethane and DIEA. If necessary, a small amount of DMF may be added to facilitate dissolution of the amino acid.

5 The syntheses of the peptide analogs described herein can be carried out by using a single or multi-channel peptide synthesizer, such as an CEM Liberty Microwave synthesizer, or a Protein Technologies, Inc. Prelude (6 channels) or Symphony (12 channels) synthesizer.

10 The peptidyl-resin precursors for their respective peptides may be cleaved and deprotected using any standard procedure (see, for example, King, D.S. et al., *Int. J. Peptide Protein Res.*, 36:255-266 (1990)). A desired method is the use of TFA in the presence of water and TIS as scavengers. Typically, the peptidyl-resin is stirred in TFA/water/TIS (94:3:3, v:v:v; 1 mL/100 mg of peptidyl resin) for 2-6 hrs at room temperature. The spent resin is then filtered off and the TFA solution is concentrated or dried under reduced pressure. The resulting crude peptide is either precipitated 15 and washed with Et₂O or is redissolved directly into DMSO or 50% aqueous acetic acid for purification by preparative UPLC.

20 Peptides with the desired purity can be obtained by purification using preparative UPLC, for example, on a Waters Model 4000 or a Shimadzu Model LC-8A liquid chromatograph. The solution of crude peptide is injected into a YMC S5 ODS (20X 100 mm) column and eluted with a linear gradient of MeCN in water, both buffered with 0.1% TFA, using a flow rate of 14-20 mL/min with effluent monitoring by UV absorbance at 220 nm. The structures of the purified peptides can be confirmed by electro-spray MS analysis.

25

Experimental Procedures

30 The abbreviations used in the present application, including particularly in the illustrative schemes and examples which follow, are well-known to those skilled in the art. Some of the abbreviations used are as follows: DMF for N,N-dimethylformamide; HATU for 0-(7-azabenzotriazole-1-yl)-1,3,3-tetramethyluronium hexafluorophosphate; HCTU for 0-(6-Cl-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate; TFA for trifluoroacetic acid; DBU for 1,8-diazobicyclo[5.4.0]undec-7-ene; DIAD for diisopropyl

azodicarboxylate; TIS for triisopropylsilane; DMSO for dimethylsulfoxide; MeCN or ACN for acetonitrile; DCM for 1,1-dichloromethane; DIEA or DIPEA for diisopropylethylamine; Fmoc for 9-fluorenylmethyloxycarbonyl; NMM for N-methylmorpholine; DMAP for 4-N,N-dimethylaminopyridine; NMP for N-methylpyrrolidone; Ac for acetyl; and Et for ethyl.

5 *Analytical Data:*

Mass Spectrometry: "ESI-MS(+)" signifies electrospray ionization mass spectrometry performed in positive ion mode; "ESI-MS(-)" signifies electrospray ionization mass spectrometry performed in negative ion mode; "ESI-HRMS(+)" signifies high-resolution electrospray ionization mass spectrometry performed in positive ion mode; "ESI-HRMS(-)" signifies high-resolution electrospray ionization mass spectrometry performed in negative ion mode. The detected masses are reported following the "w/z" unit designation. Compounds with exact masses greater than 10 1000 were often detected as double-charged or triple-charged ions.

15 *Analysis LCMS Condition A:*

Column: Waters BEH C18, 2.1 x 50 mm, 1.7- μ m particles; Mobile Phase A : water with 0.05% TFA; Mobile Phase B :Acetonitrile with 0.05% TFA; Temperature: 50 °C; Gradient: 2% B to 98% B over 2 min., then a 0.5 min. hold at 98% B ; Flow: 0.8 mL/min; Detection: UV at 220 nm.

20 *Analysis LCMS Condition C:*

Column: Waters BEH C18, 2.1 x 50 mm, 1.7- μ m particles; Mobile Phase A : water with 0.2% Formic Acid and 0.01% TFA; Mobile Phase B : Acetonitrile with 0.2% Formic acid an 0.01% TFA; Temperature: 50 °C; Gradient: 2% B to 80% B over 2 min., 80% B to 98% B over 0.1 minute then a 0.5 min. hold at 98% B ; Flow: 0.8 mL/min; Detection: UV at 220 nm.

30 *Analysis LCMS Condition D:*

Column: Waters BEH C18, 2.1 x 50 mm, 1.7- μ m particles; Mobile Phase A : 5:95 acetonitrile:water with 10 mM ammonium acetate; Mobile Phase B : 95:5

acetonitrile:water with 10 mM ammonium acetate; Temperature: 70 °C; Gradient: 0-100% B over 3 min., then a 2.0-minute hold at 100% B; Flow: 0.75 mL/min; Detection: UV at 220 nm.

5 *Analysis LCMS Condition E:*

Column: Waters BEH C18, 2.1 x 50 mm, 1.7- μ m particles; Mobile Phase A : 5:95 acetonitrile:water with 0.1% trifluoroacetic acid; Mobile Phase B : 95:5 acetonitrile:water with 0.1% trifluoroacetic acid; temperature: 50 °C or 70 °C; Gradient: 0-100% B over 3 min., then a 0.75 or 2.0-minute hold at 100% B; Flow: 10 0.75 or 1.11 mL/min; Detection: UV at 220 nm.

Analysis LCMS Condition F:

Column: Waters XBridge C18, 2.1 x 50 mm; Mobile Phase A : 5:95 acetonitrile:water with 10 mM ammonium acetate; Mobile Phase B : 95:5 acetonitrile:water with 10 mM ammonium acetate; Temperature: 35 °C; Gradient: 0-100% B over 4 min., then a 15 1-minute hold at 100% B; Flow: 4 mL/min; Detection: UV at 220 nm.

Analysis LCMS Condition G:

Column: Waters BEH C18, 2.0 x 50 mm, 1.7- μ m particles; Mobile Phase A : 5:95 20 methanol:water with 10 mM ammonium acetate; Mobile Phase B : 95:5 methanol:water with 10 mM ammonium acetate; Temperature: 50 °C; Gradient: 0-100% B over 3 min., then a 0.5-minute hold at 100% B; Flow: 0.5 mL/min; Detection: UV at 220 nm.

25 *Analysis LCMS Condition H:*

Column: Waters BEH C18, 2.0 x 50 mm, 1.7- μ m particles; Mobile Phase A : 5:95 acetonitrile:water with 10 mM ammonium acetate; Mobile Phase B : 95:5 acetonitrile:water with 10 mM ammonium acetate; Temperature: 50 °C; Gradient: 0-100% B over 3 minutes, then a 0.5-minute hold at 100% B; Flow: 30 1.0 mL/min; Detection: UV at 220 nm.

Analysis LCMS Condition I:

Column: Waters BEH C18, 2.0 x 50 mm, 1.7- μ m particles; Mobile Phase A : 5:95 methanol:water with 10 mM ammonium acetate; Mobile Phase B : 95:5 methanol:water with 10 mM ammonium acetate; Temperature: 50 °C; Gradient: 0-5 100% B over 3 minutes, then a 0.5-minute hold at 100% B ; Flow: 0.5 mL/min; Detection: UV at 220 nm.

Analysis LCMS Condition J:

Waters CSH C18, 2.1 x 50 mm, 1.7- μ m particles; Mobile Phase A : 5:95 10 acetonitrile:water with trifluoroacetic acid; Mobile Phase B : 95:5 acetonitrile:water with trifluoroacetic acid; Temperature: 70 °C; Gradient: 0% B, 0-100% B over 3 minutes, then a 2.0-minute hold at 100% B ; Flow: 0.75 mL/min; Detection: UV at 220 nm.

15 *General Procedures:**Symphony Method A:*

All manipulations were performed under automation on a Symphony peptide synthesizer (Protein Technologies). All procedures unless noted were performed in a 20 Symphony polypropylene vessel fitted with a bottom frit. The vessel connects to the Symphony peptide synthesizer through both the bottom and the top of the tube. All solvents, DMF, DCM, amino acids and reagents are added through the bottom of the vessel and pass up through the frit to contact the resin. All solutions are removed through the bottom of the vessel. "Periodic agitation" describes a brief pulse of N₂ 25 gas through the bottom frit; the pulse lasts approximately 5 seconds and occurs every 15 seconds. Amino acid solutions were generally not used beyond three weeks from preparation. HATU solution was used within 5 days of preparation. DMF = dimethylformamide; DCM = dichloromethane; THF = tetrahydrofuran; HCTU = 2-(6-Chloro-1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium; HATU = 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate; NMM= n-Methyl morpholine; DIPEA = diisopropylethylamine; DMAP = N,N-dimethylpyridin-4-amine; Rink resin=4-(2',4'-

dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-aminomethyl resin;
Sieber resin= Fmoc-amino-xanthen-3-yloxy, where "3-yloxy" describes the position
and type of connectivity to the polystyrene resin. The resins used are Merrifield
polymer (polystyrene) with either a Rink or a Sieber linker (Fmoc-protected at
5 nitrogen); 100-200 mesh, 1% DVB, 0.35 mmol/g or 0.71 mmol/g loading,
respectively. Other common acid sensitive resins can also be used in the synthesis
such as functionalized Chlorotriyl Resin. Common amino acids used are listed
below with side-chain protecting groups indicated inside parenthesis. Fmoc-Ala-OH;
Fmoc-Arg(Pbf)-OH; Fmoc-Asn(Trt)-OH; Fmoc-Asp(OtBu)-OH; Fmoc-Cys(Trt)-
10 OH; Fmoc-Dab(Boc)-OH (Dab = 2,4-diaminobutyric acid); Fmoc-Dap(Boc)-OH
(Dap = 2,3-diaminopropionic acid); Fmoc-Gln(Trt)-OH; Fmoc-Gly-OH; Fmoc-
His(Trt)-OH; Fmoc-*t*-Hyp(tBu)-OH (*t*-Hyp = trans-4-hydroxyproline); Fmoc-Ile-OH;
Fmoc-Leu-OH; Fmoc-Lys(Boc)-OH; Fmoc-Nle-OH; Fmoc-Met-OH; Fmoc-[N-
15 Me]Ala-OH; Fmoc-[N-Me]Nle-OH; Fmoc-Phe-OH; Fmoc-Pro-OH; Fmoc-Sar-OH
(Sar = Sarcosine or [N-Me]Gly); Fmoc-Ser(tBu)-OH; Fmoc-Thr(tBu)-OH; Fmoc-
Trp(Boc)-OH; Fmoc-Tyr(tBu)-OH; Fmoc-Val-OH.

The procedures of "Symphony Method A" describe an experiment performed
on a 0.050-0.100 mmol scale, where the scale is determined by the resin substitution.
This scale corresponds to approximately 143-286 mg or 70-140 mg of the Rink or
20 Sieber-Merrifield resins, respectively, described above. All procedures can be scaled
beyond the 0.050-0.100 mmol scale by adjusting the described volumes by the
multiple of the scale. Prior to amino acid coupling, all peptide synthesis sequences
began with a resin-swelling procedure, described below as "Swelling procedure".
Coupling of amino acids to a primary amine N-terminus used the "Standard-coupling
25 procedure" described below. Coupling of amino acids to a secondary amine N-
terminus used the "Secondary-amine coupling" procedure described below.

Swelling procedure:

To a Symphony polypropylene reaction vessel was added Merrifield Rink or
30 Sieber resin (70 mg, 0.050 mmol or 140 mg, 0.100 mmol). The resin was washed
(swelled) three times as follows: to the reaction vessel was added DMF (2.5-5.0 mL)

upon which the mixture was periodically agitated with N2 bubbling from the bottom of the reaction vessel for 10 minutes before the solvent was drained through the frit.

Standard-coupling procedure:

5 The resin was washed three times as follows: to the reaction vessel was added DMF (2.5-5.0 mL) upon which the mixture was periodically agitated with N2 bubbling from the bottom of the reaction vessel for 30 seconds before the solvent was drained through the frit. To the reaction vessel was added piperidine:DMF (20:80 v/v, 2.5-5.0 mL). The mixture was periodically agitated for 5 minutes and then the 10 solution was drained through the frit. The procedure was repeated one more time. The resin was washed 6 times as follows: for each wash, DMF (2.5-5.0 mL) was added through the bottom of the vessel and the resulting mixture was periodically agitated for 30 seconds before the solution was drained through the frit. To the reaction vessel was added the amino acid (0.2M in DMF, 1.25-2.5 mL, 5 eq), then 15 HATU (0.2M in DMF, 1.25-2.5 mL, 5 eq), and finally NMM (0.8M in DMF, 1.25-2.5 mL, 10 eq). The mixture was periodically agitated for one hour, then the reaction solution was drained through the frit. The resin was washed with DMF (2.5-5.0 mL) five times, stirring it for 30 seconds each time. To the reaction vessel was added a solution of acetic anhydride:DIEA:DMF (10:5:85 v/v/v, 2.5-5.0 mL). The mixture 20 was periodically agitated for 20 min., then the solution was drained through the frit. The resin was washed successively five times as follows: for each wash, DMF (2.5-5.0 mL) was added through the top of the vessel and the resulting mixture was periodically agitated for 90 seconds before the solution was drained through the frit. The resulting resin was used directly in the next step.

25

Secondary amine-coupling procedure:

 The resin was washed three times as follows: to the reaction vessel was added DMF (2.5-5.0 mL) upon which the mixture was periodically agitated with N2 bubbling from the bottom of the reaction vessel for 30 seconds before the solvent was 30 drained through the frit. To the reaction vessel was added piperidine:DMF (20:80 v/v, 2.5-5.0 mL). The mixture was periodically agitated for 5 minutes and then the solution was drained through the frit. The procedure was repeated one more time.

The resin was washed 6 times as follows: for each wash, DMF (2.5-5.0 mL) was added through the bottom of the vessel and the resulting mixture was periodically agitated for 30 seconds before the solution was drained through the frit. To the reaction vessel was added the amino acid (0.2M in DMF, 2.5-5.0 mL, 10 eq), then 5 HATU (0.2M in DMF, 2.5-5.0 mL, 10 eq), and finally NMM (0.8M in DMF, 2.5-5.0 mL, 20 eq). The mixture was periodically agitated for two hours, then the reaction solution was drained through the frit. The resin was washed with DMF (2.5-5.0 mL) five times, stirring it for 30 seconds each time. To the reaction vessel was added a solution of acetic anhydride:DIEA:DMF (10:5:85 v/v/v, 2.5-5.0 mL). The mixture 10 was periodically agitated for 20 min., then the solution was drained through the frit. The resin was washed successively five times as follows: for each wash, DMF (2.5-5.0 mL) was added through the top of the vessel and the resulting mixture was periodically agitated for 90 seconds before the solution was drained through the frit. The resulting resin was used directly in the next step.

15

Secondary amine-coupling without Fmoc deprotection procedure:

The resin was washed three times as follows: to the reaction vessel was added DMF (2.5-5.0 mL) upon which the mixture was periodically agitated with N_2 bubbling from the bottom of the reaction vessel for 30 seconds before the solvent was 20 drained through the frit. To the reaction vessel was added the amino acid (0.2M in DMF, 2.5-5.0 mL, 10 eq), then HATU (0.2M in DMF, 2.5-5.0 mL, 10 eq), and finally NMM (0.8M in DMF, 2.5-5.0 mL, 20 eq). The mixture was periodically agitated for six hours, then the reaction solution was drained through the frit. The resin was washed with DMF (2.5-5.0 mL) five times, stirring it for 30 seconds each 25 time. To the reaction vessel was added a solution of acetic anhydride:DIEA:DMF (10:5:85 v/v/v, 2.5-5.0 mL). The mixture was periodically agitated for 20 min., then the solution was drained through the frit. The resin was washed successively five times as follows: for each wash, DMF (2.5-5.0 mL) was added through the top of the vessel and the resulting mixture was periodically agitated for 90 seconds before the 30 solution was drained through the frit. The resulting resin was used directly in the next step.

Chloroacetic Anhydride capping procedure:

The resin was washed three times as follows: to the reaction vessel was added DMF (2.5-5.0 mL) upon which the mixture was periodically agitated with N_2 bubbling from the bottom of the reaction vessel for 30 seconds before the solvent was 5 drained through the frit. To the reaction vessel was added piperidine:DMF (20:80 v/v, 2.5-5.0 mL). The mixture was periodically agitated for 5 minutes and then the solution was drained through the frit. The resin was washed once with DMF (2.5-5.0 mL). To the reaction vessel was added piperidine:DMF (20:80 v/v, 2.5-5.0 mL). The mixture was periodically agitated for 5 minutes and then the solution was 10 drained through the frit. The resin was washed 6 times as follows: for each wash, DMF (2.5-5.0 mL) was added through the bottom of the vessel and the resulting mixture was periodically agitated for 30 seconds before the solution was drained through the frit. To the reaction vessel was added NMM (0.8M in DMF, 3.0 mL, 48 eq) followed by Chloroacetic anhydride (0.4M in DMF, 3.0 mL, 24 eq). The mixture 15 was periodically agitated for 30 minutes, then the reaction solution was drained through the frit. The resin was washed once with DMF (4.0 mL). To the reaction vessel was added NMM (0.8M in DMF, 3.0 mL, 48 eq) followed by Chloroacetic anhydride (0.4M in DMF, 3.0 mL, 24 eq). The mixture was periodically agitated for 30 minutes, then the reaction solution was drained through the frit. The resin was 20 washed six times with DMF (3.0 mL) with periodically agitation of the mixture for 30 seconds before the solution was drained through the frit. The resin was then washed five times with DCM (3.0 mL) with periodic agitation of the resulting mixture for 30 seconds before the solution was drained through the frit. The resulting resin was then dried with a stream of Nitrogen for 10 mins.

25

Symphony Method B:

All manipulations were performed under automation on a Symphony peptide synthesizer (Protein Technologies). All procedures unless noted were performed in a Symphony 20 mL polypropylene tube fitted with a bottom frit. The tube connects to 30 the Symphony peptide synthesizer through both the bottom and the top of the tube. All Solvents, DMF, DCM, amino acids and reagents are added through the bottom of the tube and pass up through the frit to contact the resin. All solutions are removed

through the bottom of the tube. "Periodic agitation" describes a brief pulse of N2 gas through the bottom frit; the pulse lasts approximately 5 seconds and occurs every 15 seconds. Amino acid solutions were generally not used beyond three weeks from preparation. HATU solution was used within 5 days of preparation. DMF = 5 dimethylformamide; HCTU = 2-(6-Chloro-1-H-benzotriazol-1-yl)-1, 1,3,3-tetramethyluronium; HATU = 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate; NMM= N-methyl morpholine; DIPEA = diisopropylethylamine; Sieber = Fmoc-amino-xanthen-3-yloxy, where "3-yloxy" describes the position and type of connectivity to the 10 polystyrene resin. The resin used is Merrifield polymer (polystyrene) with a Sieber linker (Fmoc-protected at nitrogen); 100-200 mesh, 1% DVB, 0.71 mmol/g loading. Other common acid sensitive resins can also be used in the synthesis such as Rink or functionalized Chloro trityl Resin. Common amino acids used are listed below with side-chain protecting groups indicated inside parenthesis.

15 Fmoc-Ala-OH; Fmoc-Arg(Pbf)-OH; Fmoc-Asn(Trt)-OH; Fmoc-Asp(OtBu)-OH; Fmoc-Bzt-OH; Fmoc-Cys(Trt)-OH; Fmoc-Dab(Boc)-OH; Fmoc-Dap(Boc)-OH; Fmoc-Gln(Trt)-OH; Fmoc-Gly-OH; Fmoc-His(Trt)-OH; Fmoc-*t*-Hyp(tBu)-OH (*t*-Hyp = *t*-az *s*-4-hydroxyproline; Fmoc-Ile-OH; Fmoc-Leu-OH; Fmoc-Lys(Boc)-OH; Fmoc-Nle-OH; Fmoc-Met-OH; Fmoc-[N-Me]Ala-OH; Fmoc-[N-Me]Nle-OH; Fmoc- 20 Phe-OH; Fmoc-Pro-OH; Fmoc-Sar-OH; Fmoc-Ser(tBu)-OH; Fmoc-Thr(tBu)-OH; Fmoc-Trp(Boc)-OH; Fmoc-Tyr(tBu)-OH; Fmoc-Val-OH.

The procedures of "Symphony Method B" describes an experiment performed on a 0.10 mmol scale, where the scale is determined by the amount of Sieber linker bound to the resin. This scale corresponds to approximately 140 mg of the Sieber- 25 Merrifield resin described above. All procedures can be scaled beyond 0.10 mmol scale by adjusting the described volumes by the multiple of the scale. Prior to amino acid coupling, all peptide synthesis sequences began with a resin-swelling procedure, described below as "Swelling procedure". Coupling of amino acids to a primary amine N-terminus used the "Standard-coupling procedure" described below.

30 Coupling of amino acids to a secondary amine N-terminus used the "*Secondary amine-coupling procedure B*", Custom amino acids are coupled via a manual blank addition of the amino acid "*Custom amino acids-coupling procedure*" described

below, and Chloroacetyl Anhydride is added to the final position of the sequence using the "Final capping procedure" described below.

Swelling procedure:

5 To a Symphony polypropylene reaction vessel was added Merrifield Sieber resin (140 mg, 0.100 mmol). The resin was washed (swelled) three times as follows: to the reaction vessel was added DMF (2.5-5.0 mL) upon which the mixture was periodically agitated with N₂ bubbling from the bottom of the reaction vessel for 10 minutes before the solvent was drained through the frit.

10

Standard-coupling procedure:

The resin was washed three times as follows: to the reaction vessel was added DMF (2.5 mL) upon which the mixture was periodically agitated with N₂ bubbling from the bottom of the reaction vessel for 30 seconds before the solvent was drained through the frit. To the reaction vessel was added piperidine:DMF (20:80 v/v, 5 mL). The mixture was periodically agitated for 5 minutes and then the solution was drained through the frit. To the reaction vessel was added piperidine:DMF (20:80 v/v, 5 mL). The mixture was periodically agitated for 5 minutes and then the solution was drained through the frit. The resin was washed 6 times as follows: for each wash, DMF (2.5 mL) was added through the bottom of the vessel and the resulting mixture was periodically agitated for 30 seconds before the solution was drained through the frit. To the reaction vessel was added the amino acid (0.2M in DMF, 2.5 mL, 10 eq), then HATU (0.2M in DMF, 2.5 mL, 10 eq), and finally NMM (0.8M in DMF, 2.5 mL, 20 eq). The mixture was periodically agitated for 30 minutes, then the reaction solution was drained through the frit. The resin was washed 6 times as follows: DMF (2.5 mL) was added through the bottom of the vessel and the resulting mixture was periodically agitated for 30 seconds before the solution was drained through the frit. To the reaction vessel was added AC2O/DIPEA/DMF (v/v/v 1:1:3 2.5 mL) the mixture was periodically agitated for 10 minutes, then the reaction solution was drained through the frit. The resin was washed successively six times as follows: for each wash, DMF (2.5 mL) was added through the bottom of the vessel

and the resulting mixture was periodically agitated for 90 seconds before the solution was drained through the frit. The resulting resin was used directly in the next step.

Secondary amine-coupling procedure:

5 The resin was washed three times as follows: to the reaction vessel was added DMF (2.5 mL) upon which the mixture was periodically agitated with N2 bubbling from the bottom of the reaction vessel for 30 seconds before the solvent was drained through the frit. To the reaction vessel was added piperidine:DMF (20:80 v/v, 5 mL). The mixture was periodically agitated for 5 minutes and then the solution was
10 drained through the frit. To the reaction vessel was added piperidine:DMF (20:80 v/v, 5 mL). The mixture was periodically agitated for 5 minutes and then the solution was drained through the frit. The resin was washed 6 times as follows: for each wash, DMF (2.5 mL) was added through the bottom of the vessel and the resulting mixture was periodically agitated for 30 seconds before the solution was drained
15 through the frit. To the reaction vessel was added the amino acid (0.2M in DMF, 2.5 mL, 10 eq), then HATU (0.2M in DMF, 2.5 mL, 10 eq), and finally NMM (0.8M in DMF, 2.5 mL, 20 eq). The mixture was periodically agitated for 60 minutes, then the reaction solution was drained through the frit. The resin was washed with DMF (6.25 mL) was added through the bottom of the vessel and the resulting mixture was
20 periodically agitated for 30 seconds before the solution was drained through the frit. To the reaction vessel was added the amino acid (0.2M in DMF, 2.5 mL, 10 eq.), then HATU (0.2M in DMF, 2.5 mL, 10 eq.), and finally NMM (0.8M in DMF, 2.5 mL, 20 eq.). The mixture was periodically agitated for 60 minutes, then the reaction solution was drained through the frit. The resin was washed successively three times
25 as follows: for each wash, DMF (2.5 mL) was added through the bottom of the vessel and the resulting mixture was periodically agitated for 30 seconds before the solution was drained through the frit. To the reaction vessel was added AC2O/DIPEA/DMF (v/v/v 1:1:3 2.5 mL) the mixture was periodically agitated for 10 minutes, then the reaction solution was drained through the frit. The resin was washed successively six
30 times as follows: for each wash, DMF (2.5 mL) was added through the bottom of the vessel and the resulting mixture was periodically agitated for 90 seconds before the

solution was drained through the frit. The resulting resin was used directly in the next step.

Custom amino acids-coupling procedure:

5 The resin was washed three times as follows: to the reaction vessel was added DMF (2.5 mL) upon which the mixture was periodically agitated with N2 bubbling from the bottom of the reaction vessel for 30 seconds before the solvent was drained through the frit. To the reaction vessel was added the amino acid (0.2M in DMF, 2.5 mL, 10 eq), then HATU (0.2M in DMF, 2.5 mL, 10 eq), and finally NMM (0.8M in 10 DMF, 2.5 mL, 20 eq). The mixture was periodically agitated for six hours, then the reaction solution was drained through the frit. The resin was washed 6 times as follows: DMF (2.5 mL) was added through the bottom of the vessel and the resulting mixture was periodically agitated for 30 seconds before the solution was drained through the frit. To the reaction vessel was added AC2O/DIPEA/DMF (v/v/v 1:1:3 15 2.5 mL) the mixture was periodically agitated for 10 minutes, then the reaction solution was drained through the frit. The resin was washed successively six times as follows: for each wash, DMF (2.5 mL) was added through the bottom of the vessel and the resulting mixture was periodically agitated for 90 seconds before the solution was drained through the frit. The resulting resin was used directly in the next step.

20

Final capping procedure:

25 The resin was washed three times as follows: to the reaction vessel was added DMF (2.5 mL) upon which the mixture was periodically agitated with N2 bubbling from the bottom of the reaction vessel for 30 seconds before the solvent was drained through the frit. To the reaction vessel was added piperidine:DMF (20:80 v/v, 2.5-5.0 mL). The mixture was periodically agitated for 5 minutes and then the solution was drained through the frit. The resin was washed once with DMF (2.5-5.0 mL). To the reaction vessel was added piperidine:DMF (20:80 v/v, 2.5-5.0 mL). The mixture was periodically agitated for 5 minutes and then the solution was drained 30 through the frit. The resin was washed six times as follows: for each wash, DMF (2.5 mL) was added through the bottom of the vessel and the resulting mixture was periodically agitated for 30 seconds before the solution was drained through the frit.

To the reaction vessel was added NMM (0.8M in DMF, 2.5 mL, 20 eq) followed by the addition of the Chloroacetic anhydride (0.4M in DMF, 2.5 mL, 10 eq). The mixture was periodically agitated for 15 minutes, then the reaction solution was drained through the frit. The resin was washed with DMF (6.25 mL) was added 5 through the bottom of the vessel and the resulting mixture was periodically agitated for 30 seconds before the solution was drained through the frit. To the reaction vessel was added NMM (0.8M in DMF, 2.5 mL, 10 eq) followed by the addition of the Chloroacetic anhydride (0.4M in DMF, 2.5 mL, 10 eq). The mixture was periodically agitated for 15 minutes, then the reaction solution was drained through 10 the frit. The resin was washed 6 times as follows: DMF (2.5 mL) was added through the bottom of the vessel and the resulting mixture was periodically agitated for 30 seconds before the solution was drained through the frit. To the reaction vessel was added AC2O/DIPEA/DMF (v/v/v 1:1:3 2.5 mL) the mixture was periodically agitated for 10 minutes, then the reaction solution was drained through the frit. The resin was 15 washed successively six times as follows: for each wash, DMF (2.5 mL) was added through the bottom of the vessel and the resulting mixture was periodically agitated for 30 seconds before the solution was drained through the frit. The resin was washed successively four times as follows: for each wash, DCM (2.5 mL) was added through the bottom of the vessel and the resulting mixture was periodically agitated 20 for 30 seconds before the solution was drained through the frit. The resulting resin was then dried with a stream of Nitrogen for 10 mins.

Manual Procedures

Bromoacetic Acid Coupling:

25 All manipulations were performed manually at room temperature, unless noted otherwise. The resin was washed three times as follows: the resins from four 0.100 mmol scale Symphony syntheses used to prepare Resin Intermediates A and B were combined into a 50 mL fritted glass reactor equipped with a three-way stopcock and washed with DMF (10 mL) three times with agitation by N₂ bubbling from the 30 bottom of the reaction vessel. To the reaction vessel was added piperidine:DMF (20:80 v/v, 10 mL). The mixture was periodically agitated for 5 minutes and then the solution was drained through the frit. The procedure was repeated once more. The

resin was washed six times with DMF (10 mL). To the reaction vessel was added a solution of bromoacetic acid (10.0 eq) in DMF (5.0 mL) and DIC (10.3 eq). The mixture was agitated by N_2 bubbling for one hour, then the reaction solution was drained through the frit. The resin was washed with DMF (5 mL) five times. The 5 resulting resin was suspended in DCM/DMF (3:2) and divided, by volume, into seven equal parts (0.057 mmol), which were transferred into 25 mL fritted syringes. Each resin aliquot was used directly in the next on-resin N-alkylation step.

N-Gly-Alkylation On-resin Method A:

10 All manipulations were performed manually at room temperature, unless noted otherwise. To the resin from the bromoacetylation step (0.057 mmol) was added a solution of the required amine (10 eq.) and DIEA (11 eq.) in DMF (mL) and the resulting mixture was stirred for one hour. The resin was washed five times with DMF (10 mL). The procedure was repeated once more. When the hydrochloride salt 15 of the amine was used, an additional 10 eq. of DIEA was used. Reaction progress was monitored by TFA micro-cleavage of small resin samples. Upon reaction completion, the N-alkylated resin was washed five times with DMF (10 mL) and placed back into a Symphony reaction vessel for completion of sequence assembly on the Symphony peptide synthesizer.

20

N-Gly-Alkylation On-resin Method B:

25 All manipulations were performed manually at room temperature, unless noted otherwise. To the resin from the bromoacetylation step (0.057 mmol) was added a solution of the required amine (10 eq.) and DIEA (11 eq.) in DMF (mL) and the resulting mixture was stirred for one hour. The resin was washed five times with DMF (10 mL). The procedure was repeated once more. When the hydrochloride salt of the amine was used, an additional 10 eq. of DIEA was used. The resin was then 30 treated with a solution of amine (10-20 eq.) and DMAP (21 eq.) for one to sixteen hours. Reaction progress was monitored by TFA micro-cleavage of small resin samples. Upon reaction completion, the N-alkylated resin was washed five times with DMF (10 mL) and placed back into a Symphony reaction vessel for completion of sequence assembly on the Symphony peptide synthesizer.

N-Gly-Alkylation On-resin Method C:

All manipulations were performed manually at room temperature, unless noted otherwise. This procedure was used when the required amine was ethylamine. To the resin from the bromoacetylation step (0.057 mmol) was added a solution of 5 the ethylamine hydrochloride (10 eq.) and DIEA (21 eq.) in DMF (mL) and the resulting mixture was stirred for one hour. The resin was washed five times with DMF (10 mL). The procedure was repeated once more. The resin was then treated with a 2 M solution of ethylamine in THF (10 mL) for sixteen hours. Reaction 10 progress was monitored by TFA micro-cleavage of small resin samples. Upon reaction completion, the N-alkylated resin was washed five times with DMF (10 mL) and placed back into a Symphony reaction vessel for completion of sequence assembly on the Symphony peptide synthesizer.

N-Gly-Alkylation On-resin Method D

15 All manipulations were performed manually at room temperature, unless noted otherwise. To the bromoacetylated resin (0.100 mmol) was added a solution of the required amine (10 eq., 1.0 mmol) and DBU (5 eq., 0.5 mmol) in DMF (3 mL) and the resulting mixture was stirred for three hours. The resin was washed once with DMF (5 mL). The procedure was repeated once more, but the reaction was 20 allowed to proceed for 16 hrs. The resin was washed four times with DMF (4 mL) and DCM (4 mL), and was then placed back into a Symphony reaction vessel for completion of sequence assembly on the Symphony peptide synthesizer.

Alkylation Method A:

25 A solution of the alcohol corresponding to the alkylating group (0.046 g, 1.000 mmol), triphenylphosphine (0.131 g, 0.500 mmol), and DIAD (0.097 mL, 0.500 mmol) in 3 mL of THF was added to nosylated resin (0.186 g, 0.100 mmol), and the reaction mixture was stirred for 16 hours at room temperature. The resin was washed three times with TFIF (5 mL) Tetrahydrofuran, and the above procedure was 30 repeated 1-3 times. Reaction progress was monitored by TFA micro-cleavage of small resin samples treated with a solution of 50 μ L of TIS in 1 mL of TFA for 1.5 hours.

Alkylation Method B:

The nosylated resin (0.100 mmol) was washed three times with N-methylpyrrolidone (NMP) (3 mL). A solution of NMP (3 mL), Alkyl Bromide (20 eq, 2.000 mmol) and DBU (20 eq, 0.301 mL, 2.000 mmol) was added to the resin, 5 and the reaction mixture was stirred for 16 hours at room temperature. The resin was washed with NMP (3 mL) and the above procedure was repeated once more. Reaction progress was monitored by TFA micro-cleavage of small resin samples treated with a solution of 50 μ L of TIS in 1 mL of TFA for 1.5 hours.

10 *Nosylate formation:*

A solution of collidine (10 eq.) in DCM (2 mL) was added to the resin, followed by a solution of Nos-Cl (8 eq.) in DCM (1 mL). The reaction mixture was stirred for 16 hours at room temperature. The resin was washed three times with DCM (4 mL) and three times with DMF (4 mL). The alternating DCM and DMF 15 washes were repeated three times, followed by one final set of four DCM washes (4 mL).

Nosylate removal:

The resin (0.100 mmol) was swelled using three washes with DMF (3 mL) 20 and three washes with NMP (3 mL). A solution of NMP (3 mL), DBU (0.075 mL, 0.500 mmol) and 2-mercaptoethanol (0.071 mL, 1.000 mmol) was added to the resin and the reaction mixture was stirred for 5 minutes at room temperature. After filtering and washing with NMP (3 mL), the resin was re-treated with a solution of NMP (3 mL), DBU (0.075 mL, 0.500 mmol) and 2-mercaptoethanol (0.071 mL, 25 1.000 mmol) for 5 minutes at room temperature. The resin was washed three times with NMP (3 mL), four times with DMF (4 mL) and four times with DCM (4 mL), and was placed back into a Symphony reaction vessel for completion of sequence assembly on the Symphony peptide synthesizer.

30 *Chloroacetyl chloride coupling procedure:*

To the reaction vessel containing the resin from the previous step was added piperidine:DMF (20:80 v/v, 5.0 mL). The mixture was periodically agitated for 5

minutes and then the solution was drained through the frit. To the reaction vessel was added piperidine:DMF (20:80 v/v, 5.0 mL). The mixture was periodically agitated for 5 minutes and then the solution was drained through the frit. The resin was washed successively six times as follows: for each wash, DMF (5.0 mL) was 5 added through the top of the vessel and the resulting mixture was periodically agitated for 30 seconds before the solution was drained through the frit. To the reaction vessel was added 3.5 mL of a solution of DIPEA (40 eq), and chloroacetyl chloride (20 eq) in DMF. The mixture was periodically agitated for three hours, then the solution was drained through the frit. The resin was washed successively five 10 times as follows: for each wash, DMF (5.0 mL) was added to top of the vessel and the resulting mixture was periodically agitated for 60 seconds before the solution was drained through the frit. The resin was washed successively three times as follows: for each wash, CH₂Cl₂ (5.0 mL) was added to top of the vessel and the resulting mixture was periodically agitated for 60 seconds before the solution was drained 15 through the frit. The resin was then dried under high vacuum.

CEMMethodA:

All manipulations were performed under automation on a CEM Liberty microwave peptide synthesizer (CEM Corporation). All procedures unless noted 20 were performed in a 30 or 125 mL polypropylene tube fitted with a bottom frit to a CEM Discovery microwave unit. The tube connects to the CEM Liberty synthesizer through both the bottom and the top of the tube. DMF and DCM can be added through the top and bottom of the tube, which washes down the sides of the tube equally. All solutions are removed through the bottom of the tube except while 25 transferring resin from the top. "Periodic bubbling" describes a brief bubbling of N₂ gas through the bottom frit. Amino acid solutions were generally not used beyond three weeks from preparation. HATU solution was used within 5 days of preparation. DMF = dimethylformamide; HCTU = 2-(6-Chloro-1-H-benzotriazol-1-yl)-1, 1,3,3-tetramethyluronium; HATU = 1-[Bis(dimethylamino)methylene]-1H-1,2,3-30 triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate; DIPEA = diisopropylethylamine; Sieber = Fmoc-amino-xanthen-3-yloxy, where "3-yloxy" describes the position and type of connectivity to the polystyrene resin. The resin

used is Merrifield polymer (polystyrene) with a Sieber linker (Fmoc-protected at nitrogen); 100-200 mesh, 1% DVB, 0.71 mmol/g loading. Common amino acids used are listed below with side-chain protecting groups indicated inside parenthesis.

Fmoc-Ala-OH; Fmoc-Arg(Pbf)-OH; Fmoc-Asn(Trt)-OH; Fmoc-Asp(OtBu)-OH;

5 Fmoc-Bzt-OH; Fmoc-Cys(Trt)-OH; Fmoc-Dab(Boc)-OH; Fmoc-Dap(Boc)-OH;
Fmoc-Gln(Trt)-OH; Fmoc-Gly-OH; Fmoc-His(Trt)-OH; Fmoc-Hyp(tBu)-OH; Fmoc-
Ile-OH; Fmoc-Leu-OH; Fmoc-Lys(Boc)-OH; Fmoc-Nle-OH; Fmoc-Met-OH; Fmoc-
[N-Me]Ala-OH; Fmoc-[N-Me]Nle-OH; Fmoc-Phe-OH; Fmoc-Pro-OH; Fmoc-Sar-
OH; Fmoc-Ser(tBu)-OH; Fmoc-Thr(tBu)-OH; Fmoc-Trp(Boc)-OH; Fmoc-Tyr(tBu)-

10 OH; Fmoc-Val-OH

The procedures of "CEM Method A" describe an experiment performed on a 0.100 mmol scale, where the scale is determined by the amount of Sieber linker bound to the resin. This scale corresponds to approximately 140 mg of the Sieber-Merrifield resin described above. All procedures can be scaled beyond 0.100 mmol scale by adjusting the described volumes by the multiple of the scale. Prior to amino acid coupling, all peptide synthesis sequences began with a resin-swelling procedure, described below as "Resin-swelling procedure". Coupling of amino acids to a primary amine N-terminus used the "Single-coupling procedure" described below. Coupling of amino acids to a secondary amine N-terminus used the "Secondary 15 amine-coupling procedure" described below. Coupling of chloroacetyl group to the N-terminus of the peptide is described by the "Chloroacetyl chloride coupling procedure" or "Chloroacetic acid coupling procedure" detailed above.

Resin-swelling procedure:

25 To 50 mL polypropylene conical tube was added Merrifield: Sieber resin (140 mg, 0.100 mmol). Then DMF (7 mL) was added to the tube followed by DCM (7 mL). The resin was then transferred to the reaction vessel from top of the vessel. The procedure is repeated additionally two times. DMF (7 mL) was added followed by DCM (7 mL). The resin was allowed to swell with N2 bubbling from the bottom 30 of the reaction vessel for 15 minutes before the solvent was drained through the frit.

Standard Couplingprocedure:

To the reaction vessel containing resin from the previous step was added a solution of piped dine:DMF (20:80 v/v, 5.0 mL). The mixture was periodically agitated for 3 minutes and then the solution was drained through the frit. To the 5 reaction vessel was added a solution of piperidine:DMF (20:80 v/v, 5.0 mL). The mixture was periodically agitated for 3 minutes and then the solution was drained through the frit. The resin was washed successively three times as follows: DMF (7 mL) wash from top, followed by DMF (7 mL) wash from bottom and finally with DMF (7 mL) wash from top. To the reaction vessel was added the amino acid (0.2M 10 in DMF, 2.5 mL, 5 eq), HATU (0.5M in DMF, 1.0 mL, 5 eq), and DIPEA (2M in NMP, 0.5 mL, 10 eq). The mixture was mixed by N2 bubbling for 5 minutes at 75 °C for all amino acids, except Fmoc-Cys(Trt)-OH and Fmoc-His(Trt)-OH which are coupled at 50 °C, the reaction solution was drained through the frit. The resin was washed successively three times as follows: DMF (7 mL) wash from top, followed by 15 DMF (7 mL) wash from bottom and finally with DMF (7 mL) wash from top. To the reaction vessel was added a solution of acetic anhydride:DIEA:DMF (10:1:89 v/v/v, 5.0 mL). The mixture was periodically bubbled for 2 minutes at 65 °C, then the solution was drained through the frit. The resin was washed successively three times as follows: DMF (7 mL) wash from top, followed by DMF (7 mL) wash from bottom 20 and finally with DMF (7 mL) wash from top. The resulting resin was used directly in the next step.

Double-couple Couplingprocedure:

To the reaction vessel containing resin from the previous step was added a solution of piperidine:DMF (20:80 v/v, 5.0 mL). The mixture was periodically agitated for 3 minutes and then the solution was drained through the frit. To the 25 reaction vessel was added a solution of piperidine:DMF (20:80 v/v, 5.0 mL). The mixture was periodically agitated for 3 minutes and then the solution was drained through the frit. The resin was washed successively three times as follows: DMF (7 mL) wash from top, followed by DMF (7 mL) wash from bottom and finally with DMF (7 mL) wash from top. To the reaction vessel was added the amino acid (0.2M 30 in DMF, 2.5 mL, 5 eq), HATU (0.5M in DMF, 1.0 mL, 5 eq), and DIPEA (2M in

NMP, 0.5 mL, 10 eq). The mixture was mixed by N2 bubbling for 5 minutes at 75 °C for all amino acids, except Fmoc-Cys(Trt)-OH and Fmoc-His(Trt)-OH which are coupled at 50 °C, the reaction solution was drained through the frit. The resin was washed successively three times as follows: DMF (7 mL) wash from top, followed by 5 DMF (7 mL) wash from bottom and finally with DMF (7 mL) wash from top. To the reaction vessel was added the amino acid (0.2M in DMF, 2.5 mL, 5 eq), HATU (0.5M in DMF, 1.0 mL, 5 eq), and DIPEA (2M in NMP, 0.5 mL, 10 eq). The mixture was mixed by N2 bubbling for 5 minutes at 75 °C for all amino acids, except Fmoc-Cys(Trt)-OH and Fmoc-His(Trt)-OH which are coupled at 50 °C, the reaction 10 solution was drained through the frit. The resin was washed successively three times as follows: DMF (7 mL) wash from top, followed by DMF (7 mL) wash from bottom and finally with DMF (7 mL) wash from top. To the reaction vessel was added a solution of acetic anhydride:DIEA:DMF (10: 1:89 v/v/v, 5.0 mL). The mixture was periodically bubbled for 2 minutes at 65 °C, then the solution was drained through 15 the frit. The resin was washed successively three times as follows: DMF (7 mL) wash from top, followed by DMF (7 mL) wash from bottom and finally with DMF (7 mL) wash from top. The resulting resin was used directly in the next step.

Custom amino acids-coupling procedure:

20 To the reaction vessel containing resin from the previous step was added a solution of piperidine:DMF (20:80 v/v, 5.0 mL). The mixture was periodically agitated for 3 minutes and then the solution was drained through the frit. To the reaction vessel was added a solution of piperidine:DMF (20:80 v/v, 5.0 mL). The mixture was periodically agitated for 3 minutes and then the solution was drained 25 through the frit. The resin was washed successively three times as follows: DMF (7 mL) wash from top, followed by DMF (7 mL) wash from bottom and finally with DMF (7 mL) wash from top. To the reaction vessel was added the amino acid solution (1.25 mL to 5 mL, 2.5 eq to 10 eq) containing HATU (2.5 eq to 10 eq), and finally DIPEA (2M in NMP, 0.5 mL to 1 mL, 20 eq). The mixture was mixed by N2 30 bubbling for 5 minutes to 2 hours at 25 °C to 75 °C, then the reaction solution was drained through the frit. The resin was washed successively three times as follows: DMF (7 mL) wash from top, followed by DMF (7 mL) wash from bottom and finally

with DMF (7 mL) wash from top. To the reaction vessel was added a solution of acetic anhydride:DIEA:DMF (10: 1:89 v/v/v, 5.0 mL). The mixture was periodically bubbled for 2 minutes at 65 °C, then the solution was drained through the frit. The resin was washed successively three times as follows: DMF (7 mL) wash from top, 5 followed by DMF (7 mL) wash from bottom and finally with DMF (7 mL) wash from top. The resulting resin was used directly in the next step.

Chloroacetyl chloride coupling procedure:

To the reaction vessel containing the resin from the previous step was added 10 piperidine:DMF (20:80 v/v, 5.0 mL). The mixture was periodically agitated for 3 minutes and then the solution was drained through the frit. To the reaction vessel was added piperidine:DMF (20:80 v/v, 5.0 mL). The mixture was periodically agitated for 3 minutes and then the solution was drained through the frit. The resin was washed successively five times as follows: for each wash, DMF (4.0 mL) was 15 added through the top of the vessel and the resulting mixture was periodically agitated for 30 seconds before the solution was drained through the frit. To the reaction vessel was added 3.0 mL of a solution of DIPEA (4.0 mmol, 0.699 mL, 40 eq), and chloroacetyl chloride (2.0 mmol, 0.160 mL, 20 eq) in DMF. The mixture was periodically agitated for 12 to 18 hours, then the solution was drained through 20 the frit. The resin was washed successively three times as follows: for each wash, DMF (4.0 mL) was added to top of the vessel and the resulting mixture was periodically agitated for 90 seconds before the solution was drained through the frit. The resin was washed successively four times as follows: for each wash, CH₂Cl₂ (2.0 mL) was added to top of the vessel and the resulting mixture was periodically 25 agitated for 90 seconds before the solution was drained through the frit.

Global Deprotection Method A:

All manipulations were performed manually at room temperature unless noted otherwise. The procedure of "Global Deprotection Method A" describes an 30 experiment performed on a 0.057-0.100 mmol scale, where the scale is determined by the amount of Rink or Sieber linker bound to the resin. The procedure can be scaled beyond 0.057-0.100 mmol scale by adjusting the described volumes by the multiple

of the scale. A "deprotection solution" was prepared using trifluoroacetic acid:triisopropylsilane:dithiothreitol (95:2.5:2.5 v:v:w) or trifluoroacetic acid:triisopropylsilane:dithiothreitol (96.5:2.5:1.0 v:v:w). The solution was pre-cooled in ice prior to adding it to the resin. To the resin in a 25 mL fritted syringe 5 was added the "deprotection solution" (2.5-4.0 mL). The mixture was mixed in a shaker for 60 min. The solution was filtered through the frit into cold diethyl ether (30 mL). The precipitated solid was centrifuged for 3 min. The supernatant solution was decanted and the solid was re-suspended in diethyl ether (15 mL). This procedure was repeated two more times. The supernatant was decanted and the 10 remaining solid was dried under high vacuum. The crude peptide was obtained as a white to off-white solid.

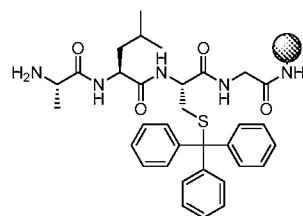
Cyclization Method A :

All manipulations were performed manually unless noted otherwise. The procedure 15 of "Cyclization Method A" describes an experiment performed on a 0.057-0.100 mmol scale. The crude peptide solid was dissolved in a solution of acetonitrile:aqueous 0.1M ammonium bicarbonate buffer (1:3 or 1:2, v:v; 30-40 mL), and the pH of the solution was carefully adjusted to 8.5-9.0 using aqueous NaOH (1.0 M). The solution was then mixed using a shaker for 12 to 18 hours. The 20 reaction solution was concentrated and the residue was then dissolved in acetonitrile:water. This solution was subjected to reverse-phase HPLC purification to afford the desired cyclic peptide.

Preparation of Intermediate Resins for Examples 3003-3050

25

Preparation of Intermediate Resin A :



To a Symphony 20 mL reaction vessels was added Sieber resin (0.100 mmol) and the vessel was placed on the Symphony peptide synthesizer. The following procedures were then performed sequentially:

Fmoc-Gly-OH "Symphony Method B : *Resin-swelling procedure*" was followed;

5 Fmoc-Cys(Trt)-OH "Symphony Method B : *Standard-coupling procedure*" was followed;

Fmoc-Leu-OH "Symphony Method B : *Standard-coupling procedure*" was followed;

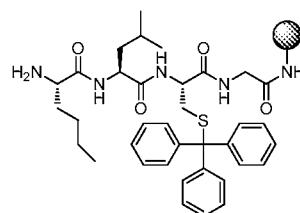
Fmoc-Ala-OH "Symphony Method B : *Standard-coupling procedure*" was followed.

The Fmoc group was removed using 20% Piperidine/DMF (5 mL) for 5 mins with

10 periodic Nitrogen stirring. The resin was washed with DMF (2.5 mL) and then 20% Piperidine/DMF (5 mL) was added to the resin, and periodic Nitrogen stirring was continued for 5 mins. The resulting resin was washed six times with DMF (2.5 mL), then five times with DCM (2.5 mL), and was used as an intermediate for the N-alkylation procedures.

15

Preparation of Intermediate Resin B :



To a Symphony 20 mL reaction vessels was added Sieber resin (0.100 mmol) and the vessel was placed on the Symphony peptide synthesizer. The following

20 procedures were then performed sequentially:

Fmoc-Gly-OH "Symphony Method B : *Resin-swelling procedure*" was followed;

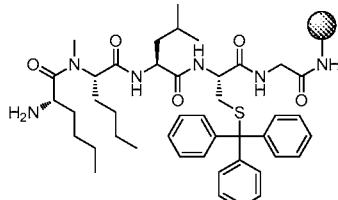
Fmoc-Cys(Trt)-OH "Symphony Method B : *Standard-coupling procedure*" was followed;

Fmoc-Leu-OH "Symphony Method B : *Standard-coupling procedure*" was followed;

25 Fmoc-Nle-OH "Symphony Method B : *Standard-coupling procedure*" was followed. The Fmoc group was removed using 20% Piperidine/DMF (5 mL) for 5 mins with periodic Nitrogen stirring. The resin was washed with DMF (2.5 mL) and then 20% Piperidine/DMF (5 mL) was added to the resin, and periodic Nitrogen stirring was continued for 5 mins. The resulting resin was washed six times with DMF (2.5 mL),

then five times with DCM (2.5 mL), and was used as an intermediate for the N-alkylation procedures.

Preparation of Intermediate Resin C:



5

To a Symphony 20 mL reaction vessels was added Sieber resin (0.100 mmol) and the vessel was placed on the Symphony peptide synthesizer. The following procedures were then performed sequentially:

Fmoc-Gly-OH "Symphony Method B : *Resin-swelling procedure*" was followed;

10 Fmoc-Cys(Trt)-OH "Symphony Method B : *Standard-coupling procedure*" was followed;

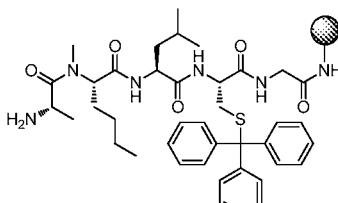
Fmoc-Leu-OH "Symphony Method B : *Standard-coupling procedure*" was followed;

Fmoc-nMethyl-Nle-OH "Symphony Method B : *Standard-coupling procedure*" was followed;

15 Fmoc-Nle-OH "Symphony Method B: *Secondary amine-coupling procedure*" was followed.

The Fmoc group was removed using 20% Piperidine/DMF (5 mL) for 5 mins with periodic Nitrogen stirring. The resin was washed with DMF (2.5 mL) and then 20% Piperidine/DMF (5 mL) was added to the resin, and periodic Nitrogen stirring 20 was continued for 5 mins. The resulting resin was washed six times with DMF (2.5 mL), then five times with DCM (2.5 mL), and was used as an intermediate for the N-alkylation procedures.

Preparation of Intermediate Resin D:



25

To a Symphony 20 mL reaction vessels was added Sieber resin (0.100 mmol) and the vessel was placed on the Symphony peptide synthesizer. The following procedures were then performed sequentially:

Fmoc-Gly-OH "Symphony Method B : *Resin-swelling procedure*" was followed;

5 Fmoc-Cys(Trt)-OH "Symphony Method B : *Standard-coupling procedure*" was followed;

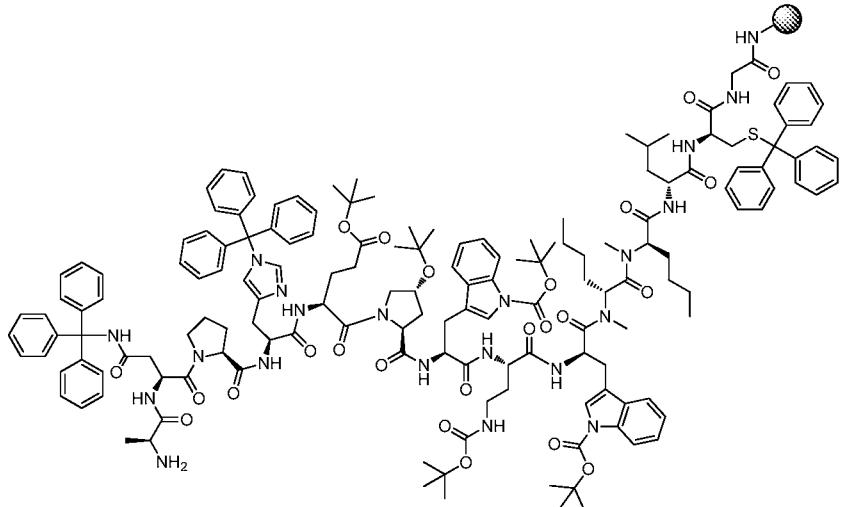
Fmoc-Leu-OH "Symphony Method B : *Standard-coupling procedure*" was followed;

Fmoc-nMethyl-Nle-OH "Symphony Method B : *Standard-coupling procedure*" was followed;

10 Fmoc-Ala-OH "Symphony Method B: *Secondary amine-coupling procedure*" was followed.

The Fmoc group was removed using 20% Piperidine/DMF (5 mL) for 5 mins with periodic Nitrogen stirring. The resin was washed with DMF (2.5 mL) and then 20% Piperidine/DMF (5 mL) was added to the resin, and periodic Nitrogen stirring was continued for 5 mins. The resulting resin was washed six times with DMF (2.5 mL), then five times with DCM (2.5 mL), and was used as an intermediate for the N-alkylation procedures.

Preparation of Intermediate Resin E:



20

To a Symphony 20 mL reaction vessels was added Sieber resin (0.100 mmol) and the vessel was placed on the Symphony peptide synthesizer. The following procedures were then performed sequentially:

Fmoc-Gly-OH "Symphony Method B : *Resin-swellingprocedure*" was followed;

Fmoc-Cys(Trt)-OH "Symphony Method B : *Standard-couplingprocedure*" was followed;

5 Fmoc-Leu-OH "Symphony Method B : *Standard-couplingprocedure*" was followed;

5 Fmoc-nMethyl-Nle-OH "Symphony Method B : *Standard-couplingprocedure*" was followed;

10 Fmoc-nMethyl-Nle-OH "Symphony Method B : *Secondary amine-coupling procedure*", was followed;

10 Fmoc-Trp(Boc)-OH "Symphony Method B : *Secondary amine-couplingprocedure*", was followed;

15 Fmoc-Dab(Boc)-OH "Symphony Method B : *Standard-couplingprocedure*" was followed;

15 Fmoc-Trp(Boc)-OH "Symphony Method B : *Standard-couplingprocedure*" was followed;

20 Fmoc-Hyp(tBu)-OH "Symphony Method B : *Standard-couplingprocedure*" was followed;

20 Fmoc-Glu(tBu)-OH "Symphony Method B : *Secondary amine-couplingprocedure*" was followed;

25 Fmoc-His(Trt)-OH "Symphony Method B : *Standard-couplingprocedure*" was followed;

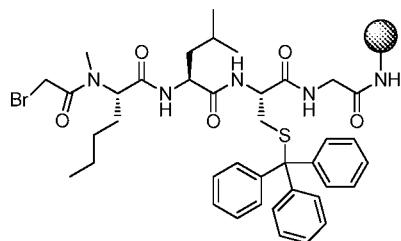
25 Fmoc-Pro-OH "Symphony Method B : *Standard-couplingprocedure*" was followed;

30 Fmoc-Asn(Trt)-OH "Symphony Method B : *Secondary amine-couplingprocedure*" was followed;

30 Fmoc-Ala-OH "Symphony Method B : *Standard-couplingprocedure*" was followed.

30 The Fmoc group was removed using 20% Piperidine/DMF (5 mL) for 5 mins with periodic Nitrogen stirring. The resin was washed with DMF (2.5 mL) and then 20% Piperidine/DMF (5 mL) was added to the resin, and periodic Nitrogen stirring was continued for 5 mins. The resulting resin was washed six times with DMF (2.5 mL), then five times with DCM (2.5 mL), and was used as an intermediate for the N-alkylation procedures.

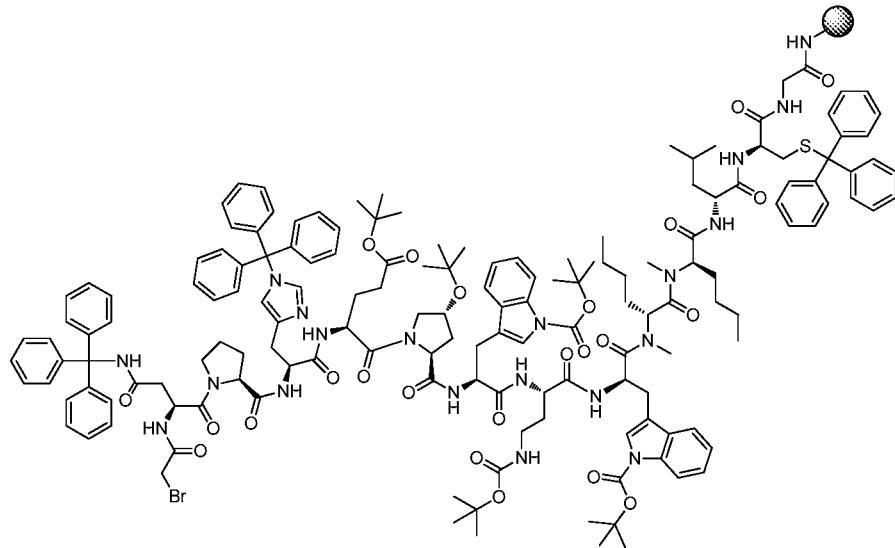
Preparation of Intermediate Resin F:



To a Symphony 20 mL reaction vessels was added Sieber resin (0.100 mmol) and the vessel was placed on the Symphony peptide synthesizer. The following 5 procedures were then performed sequentially:
Fmoc-Gly-OH "Symphony Method B : *Resin-swelling procedure*" was followed;
Fmoc-Cys(Trt)-OH "Symphony Method B : *Standard-coupling procedure*" was followed;
Fmoc-Leu-OH "Symphony Method B : *Standard-coupling procedure*" was followed;
10 Fmoc-nMethyl-Nle-OH "Symphony Method B : *Standard-coupling procedure*" was followed.

The Fmoc group was removed using 20% Piperidine/DMF (5 mL) for 5 mins with periodic Nitrogen stirring. The resin was washed with DMF (2.5 mL) and then 20% Piperidine/DMF (5 mL) was added to the resin, and periodic Nitrogen stirring was continued for 5 mins. The resulting resin was washed six times with DMF (2.5 mL), and was then transferred to a BioRad tube, and treated with the following solution: Bromoacetic acid (10 eq., 1 mmol) and DIC (11 eq., 1.1 mmol) in DMF (3 mL). The reaction mixture was stirred for 3 hours. After filtration and a DMF wash (3 mL), the resin was re-treated with the following solution: Bromoacetic acid (10 eq., 1 mmol) and DIC (11 eq., 1.1 mmol) in DMF (3 mL). The reaction mixture was stirred for 16 hours. After filtration, the resin was washed five times with DMF (3.0 mL), then five times with DCM (2.5 mL), and was used as an intermediate for the N-alkylation procedures.

Preparation of Intermediate Resin G:



To a Symphony 20 mL reaction vessels was added Sieber resin (0.100 mmol) and the vessel was placed on the Symphony peptide synthesizer. The following 5 procedures were then performed sequentially:

Fmoc-Gly-OH "Symphony Method B : *Resin-swelling procedure*" was followed;

Fmoc-Cys(Trt)-OH "Symphony Method B : *Standard-coupling procedure*" was followed;

Fmoc-Leu-OH "Symphony Method B : *Standard-coupling procedure*" was followed;

10 Fmoc-nMethyl-Nle-OH "Symphony Method B : *Standard-coupling procedure*" was followed;

Fmoc-nMethyl-Nle-OH "Symphony Method B: *Secondary amine-coupling procedure*" was followed;

15 Fmoc-Trp(Boc)-OH "Symphony Method B: *Secondary amine-coupling procedure*" was followed;

Fmoc-Dab(Boc)-OH "Symphony Method B : *Standard-coupling procedure*" was followed;

Fmoc-Trp(Boc)-OH "Symphony Method B : *Standard-coupling procedure*" was followed;

20 Fmoc-Hyp(tBu)-OH "Symphony Method B : *Standard-coupling procedure*" was followed;

Fmoc-Glu(tBu)-OH "Symphony Method B : *Secondary amine-coupling procedure*" was followed;

Fmoc-His(Trt)-OH "Symphony Method B : *Standard-couplingprocedure*" was followed;

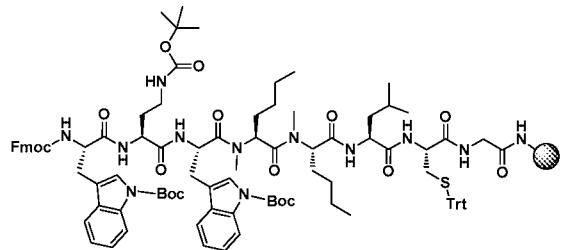
Fmoc-Pro-OH "Symphony Method B : *Standard-couplingprocedure*" was followed;

Fmoc-Asn(Trt)-OH "Symphony Method B : *Secondary amine-couplingprocedure*"

5 was followed.

The Fmoc group was removed using 20% Piperidine/DMF (5 mL) for 5 mins with periodic Nitrogen stirring. The resin was washed with DMF (2.5 mL) and then 20% Piperidine/DMF (5 mL) was added to the resin, and periodic Nitrogen stirring was continued for 5 mins. The resulting resin was washed six times with DMF (2.5 mL), and was then transferred to a BioRad tube, and treated with the following solution: Bromoacetic acid (10 eq., 1 mmol) and DIC (11 eq., 1.1 mmol) in DMF (3 mL). The reaction mixture was stirred for 3 hours. After filtration and a DMF wash (3 mL), the resin was re-treated with the following solution: Bromoacetic acid (10 eq., 1 mmol) and DIC (11 eq., 1.1 mmol) in DMF (3 mL). The reaction mixture was stirred for 16 hours. After filtration, the resin was washed five times with DMF (3.0 mL), then five times with DCM (2.5 mL), and was used as an intermediate for the N-alkylation procedures.

Preparation of Intermediate Resin H.



20

To four Symphony reaction vessels was added Rink resin (mg, 0.100 mmol) and the vessels were placed on the Symphony peptide synthesizer. The following procedures were then performed sequentially:

"Symphony Method A : *Resin-swellingprocedure*" was followed;

25 "Symphony Method A : *Standard couplingprocedure*" was followed with Fmoc-Gly-OH;

"Symphony Method A : *Standard couplingprocedure*" was followed with Fmoc-Cys(Trt)-OH;

"Symphony Method A: *Standard couplingprocedure*" was followed with Fmoc-Leu-OH;

"Symphony Method A: *Secondary-amine Couplingprocedure*" was followed with Fmoc-[N-Me]Nle-OH;

5 "Symphony Method A: *Secondary-amine Couplingprocedure*" was followed with Fmoc-[N-Me]Nle-OH

"Symphony Method A: *Secondary-amine Couplingprocedure*" was followed with Fmoc-Trp(Boc)-OH;

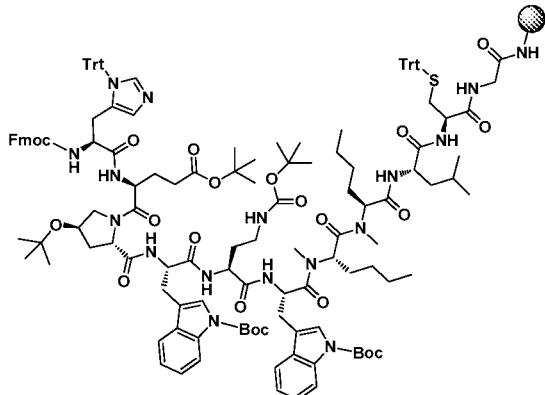
10 "Symphony Method A: *Standard couplingprocedure*" was followed with Fmoc-Dab(Boc)-OH;

"Symphony Method A: *Standard couplingprocedure*" was followed with Fmoc-Trp(Boc)-OH.

The resulting resins were combined into a 50 mL fritted glass reactor and washed

15 with DMF as described in the above bromoacetic acid coupling procedure.

Preparation of Intermediate Resin I.



20 To four Symphony reaction vessels was added Rink resin (mg, 0.100 mmol) and the vessels were placed on the Symphony peptide synthesizer. The following procedures were then performed sequentially:

"Symphony Method A: *Resin-swellingprocedure*" was followed;

"Symphony Method A: *Standard couplingprocedure*" was followed with Fmoc-Gly-

25 OH;

"Symphony Method A : *Standard couplingprocedure*" was followed with Fmoc-Cys(Trt)-OH;

"Symphony Method A : *Standard couplingprocedure*" was followed with Fmoc-Leu-OH;

5 "Symphony Method A : *Secondary-amine couplingprocedure*" was followed with Fmoc-[N-Me]Nle-OH;

"Symphony Method A : *Secondary-amine couplingprocedure*" was followed with Fmoc-[N-Me]Nle-OH

"Symphony Method A : *Secondary-amine couplingprocedure*" was followed with 10 Fmoc-Trp(Boc)-OH;

"Symphony Method A : *Standard couplingprocedure*" was followed with Fmoc-Dab(Boc)-OH;

"Symphony Method A : *Standard couplingprocedure*" was followed with Fmoc-Trp(Boc)-OH.

15 "Symphony Method A : *Standard couplingprocedure*" was followed with Fmoc-*t*-Hyp(tBu)-OH;

"Symphony Method A : *Secondary-amine Couplingprocedure*" was followed with Fmoc-Glu(OtBu)-OH;

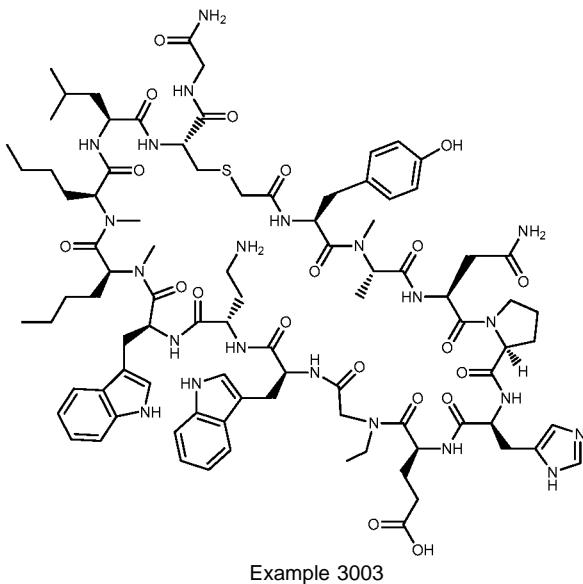
"Symphony Method A : *Standard couplingprocedure*" was followed with Fmoc- 20 His(Trt)-OH.

The resulting resins were combined into a 50 mL fritted glass reactor and washed with DMF as described in the above bromoacetic acid coupling procedure.

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30

Preparation of Example 3003



Example 3003 was prepared following the general synthetic sequence described below, starting from Intermediate Resin H. Intermediate Resin A (0.400 mmol) was placed into a 50 mL fritted glass reactor. The following procedures were then performed sequentially:

5 "Bromoacetic Acid Coupling" procedure was followed;

"N-Gly-Alkylation On-resin Method C" procedure was followed;

"Symphony Method A: Secondary-amine coupling without Fmoc deprotection" procedure was followed with Fmoc-Glu(OtBu)-OH;

10 "Symphony Method A: Standard coupling procedure" was followed with Fmoc-His(Trt)-OH;

"Symphony Method A: Standard coupling procedure" was followed with Fmoc-Pro-OH;

15 "Symphony Method A: Secondary-amine coupling procedure" was followed with Fmoc-Asn(Trt)-OH;

Symphony Method A: Standard coupling procedure" was followed with Fmoc-[N-Me]Ala-OH;

"Symphony Method A: Secondary-amine coupling procedure" was followed with

20 Fmoc-Tyr(tBu)-OH;

"Chloroacetic Anhydride capping procedure" was followed;

"Global Deprotection Method A" was followed;

"Cyclization Method A" was followed.

The crude material was purified via preparative LC/MS with the following 5 conditions: Column: XBridge CI8, 19 x 200 mm, 5- μ m particles; Mobile Phase A : 5:95 methanol: water with 10-mM ammonium acetate; Mobile Phase B : 95:5 methanol: water with 10-mM ammonium acetate; Gradient: 45-85% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing 10 the desired product were combined and dried via centrifugal evaporation. The yield of product was 6.3 mg, and its estimated purity by LCMS analysis was 99% using "Analysis LCMS condition D".

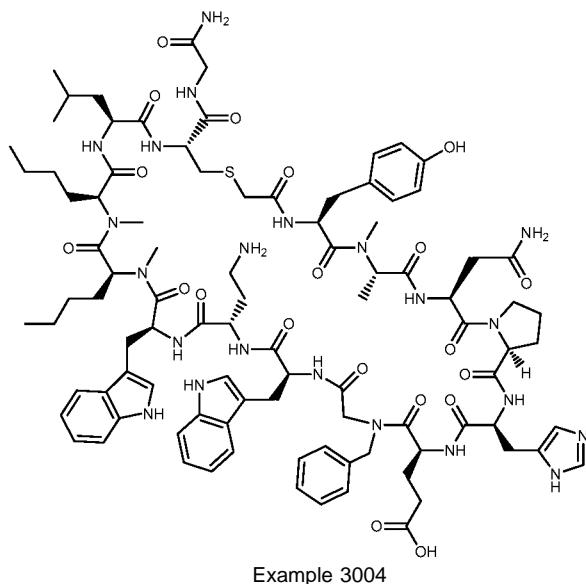
Analysis LCMS condition D : Retention time = 1.88 min; ESI-MS(+) *m/z* 935.1 (M+2H).

ESI-HRMS(+) m/z:

15 Calculated: 934.4692 (M+2H)

Found: 934.4674 (M+2H)

Preparation of Example 3004



20 Example 3004 was prepared following the general synthetic sequence described for the preparation of Example 3003, starting from Intermediate Resin H, using the following general procedures: "Bromoacetic Acid Coupling" procedure;

"N-Gly-Alkylation On-resin Method A" procedure; "Symphony Method A : Secondary-amine coupling without Fmoc deprotection" procedure; "Symphony Method A : Standard coupling procedure"; "Symphony Method A : Secondary-amine coupling procedure"; "Chloroacetic Anhydride capping" procedure; "Global Deprotection Method A"; "Cyclization Method A".

The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A : 5:95 methanol: water with 10-mM ammonium acetate; Mobile Phase B : 95:5 methanol: water with 10-mM ammonium acetate; Gradient: 50-90% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of product was 6.6 mg, and its estimated purity by LCMS analysis was 95% using "Analysis LCMS condition D".

Analysis LCMS condition D : Retention time = 1.87 min; ESI-MS(+) *m/z* 961.9 (M+2H).

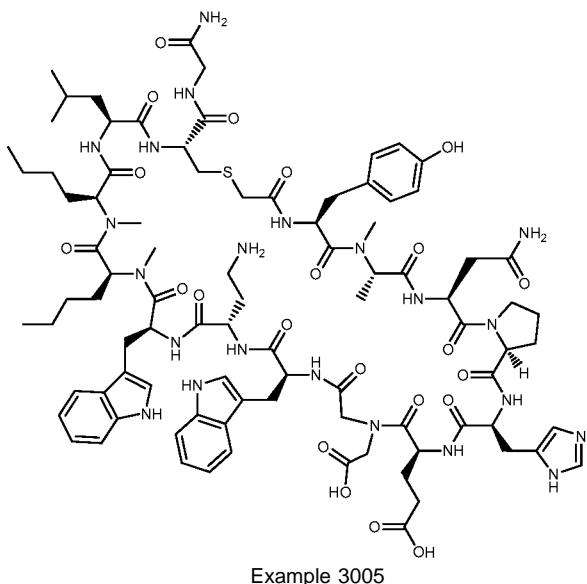
ESI-HRMS(+) *m/z*:

Calculated: 965.4771 (M+2H)

Found: 965.4754 (M+2H)

20

Preparation of Example 3005



Example 3005 was prepared following the general synthetic sequence described for the preparation of Example 3003, starting from Intermediate Resin H, using the following general procedures: "*Bromoacetic Acid Coupling*" procedure; "*N-Gly-Alkylation On-resin Method B*" procedure; "*Symphony Method A*": 5 *Secondary-amine coupling without Fmoc deprotection*" procedure; "*Symphony Method A : Standard coupling procedure*"; "*Symphony Method A : Secondary-amine coupling procedure*"; "*Chloroacetic Anhydride capping*" procedure; "*Global Deprotection Method A*"; "*Cyclization Method A*".

The crude material was purified via preparative LC/MS with the following 10 conditions: Column: XBridge CI8, 19 x 200 mm, 5- μ m particles; Mobile Phase A : 5:95 methanol: water with 10-mM ammonium acetate; Mobile Phase B : 95:5 methanol: water with 10-mM ammonium acetate; Gradient: 45-85% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield 15 of product was 3.9 mg, and its estimated purity by LCMS analysis was 100% using "*Analysis LCMS condition D*".

Analysis LCMS condition D : Retention time = 1.72 min; ESI-MS(+) *m/z* 950.4 (M+2H).

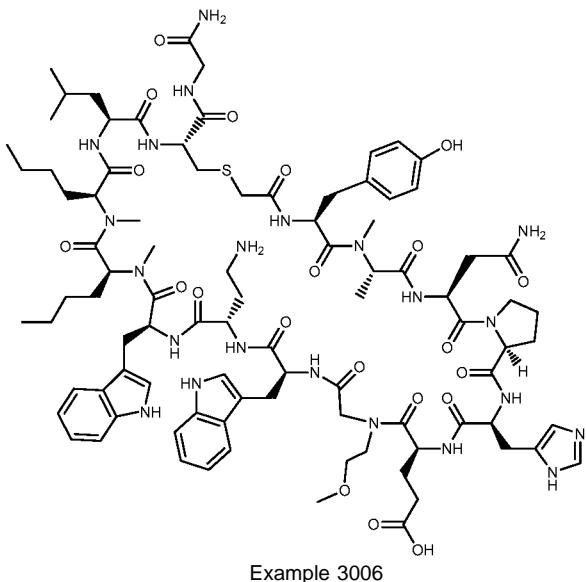
ESI-HRMS(+) m/z:

20 Calculated: 949.4563 (M+2H)
Found: 949.4547 (M+2H)

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Preparation of Example 3006



Example 3006 was prepared following the general synthetic sequence described for the preparation of Example 3003, starting from Intermediate Resin H, 5 using the following general procedures: "*Bromoacetic Acid Coupling*" procedure; "*N-Gly-Alkylation On-resin Method A*" procedure; "*Symphony Method A: Secondary-amine coupling without Fmoc deprotection*" procedure; "*Symphony Method A: Standard coupling procedure*"; "*Symphony Method A: Secondary-amine coupling procedure*"; "*Chloroacetic Anhydride capping*" procedure; "*Global Deprotection Method A*"; "*Cyclization Method A*".

The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A: 5:95 methanol: water with 10-mM ammonium acetate; Mobile Phase B: 95:5 methanol: water with 10-mM ammonium acetate; Gradient: 50-90% B over 30 15 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of product was 13.8 mg, and its estimated purity by LCMS analysis was 100%, using "*Analysis LCMS conditions D and E*".

Analysis LCMS condition D: Retention time = 1.90 min; ESI-MS(+) m/z 950.5 20 (M+2H).

Analysis LCMS condition E: Retention time = 1.58 min; ESI-MS(+) m/z 950.1 (M+2H).

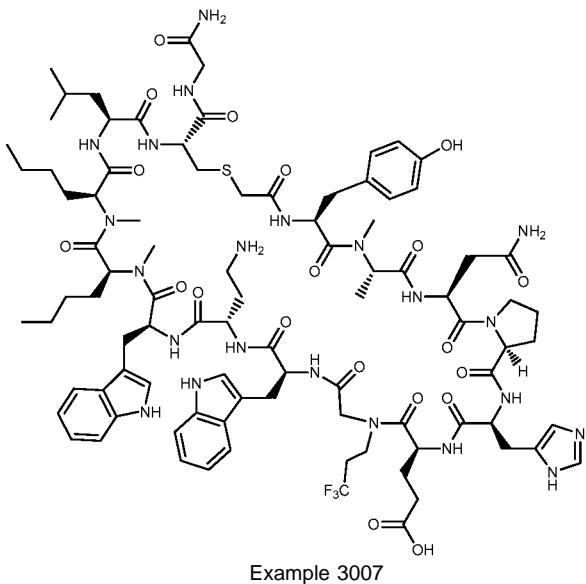
ESI-HRMS(+) *m/z*:

Calculated: 949.4745 (M+2H)

Found: 949.4725 (M+2H)

5

Preparation of Example 3007



Example 3007 was prepared following the general synthetic sequence described for the preparation of Example 3003, starting from Intermediate Resin H, using the following general procedures: "*Bromoacetic Acid Coupling*" procedure; "N-Gly-Alkylation On-resin Method B" procedure; "Symphony Method A: Secondary-amine coupling without Fmoc deprotection" procedure; "Symphony Method A: Standard coupling procedure"; "Symphony Method A: Secondary-amine coupling" procedure; "*Chloroacetic Anhydride capping*" procedure; "*Global Deprotection Method A*"; "*Cyclization Method A*".

15 The crude material was purified via preparative LC/MS with the following
conditions: Column: XBridge CI8, 19 x 200 mm, 5- μ m particles; Mobile Phase A :
5:95 methanol: water with 10-mM ammonium acetate; Mobile Phase B : 95:5
methanol: water with 10-mM ammonium acetate; Gradient: 50-90% B over 30
minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the
20 desired product were combined and dried via centrifugal evaporation. The yield of
product was 3.9 mg, and its estimated purity by LCMS analysis was 100% using
"Analysis LCMS condition D".

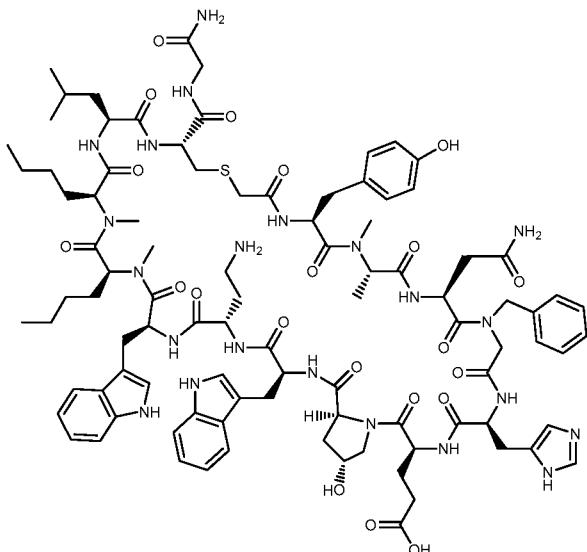
Analysis LCMS condition D : Retention time = 1.96 min; ESI-MS(+) *m/z* 969.3 (M+2H).

ESI-HRMS(+) m/z:

Calculated: 968.4629 (M+2H)

5 Found: 968.4617 (M+2H).

Preparation of Example 3008



Example 3008

Example 3008 was prepared following the general synthetic sequence described below, starting from Intermediate Resin I. Intermediate Resin B (0.400 mmol) was placed into a 50 mL fritted glass reactor. The following procedures were then performed sequentially:

"Bromoacetic Acid Coupling" procedure was followed;

"N-Gly-Alkylation On-resin Method A" procedure was followed;

15 "Symphony Method A: Secondary-amine coupling without Fmoc deprotection" procedure was followed with Fmoc-Asn(Trt)-OH;

Symphony Method A: Standard coupling procedure" was followed with Fmoc-[N-Me]Ala-OH;

"Symphony Method A: Secondary-amine coupling procedure" was followed with

20 Fmoc-Tyr(tBu)-OH;

"Chloroacetic Anhydride capping procedure" was followed;

"Global Deprotection Method A" was followed;

"Cyclization Method A" was followed.

The crude material was purified via preparative LC/MS with the following

5 conditions: Column: XBridge CI8, 19 x 200 mm, 5- μ m particles; Mobile Phase A : 5:95 methanol: water with 10-mM ammonium acetate; Mobile Phase B : 95:5 methanol: water with 10-mM ammonium acetate; Gradient: 50-90% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation.

10 The yield of product was 10.6 mg, and its estimated purity by LCMS analysis was 99% using "Analysis LCMS condition D".

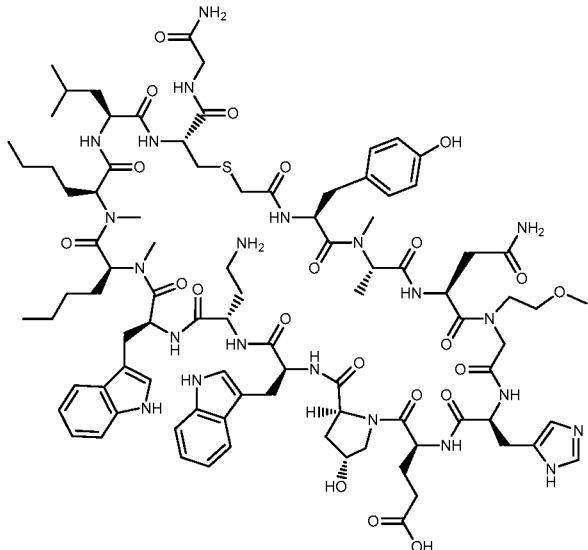
Analysis LCMS condition D: Retention time = 1.87 min; ESI-MS(+) *m/z* 914.3 (M+2H).

ESI-HRMS(+) m/z:

15 Calculated: 973.4745 (M+2H)

Found: 973.4729 (M+2H).

Preparation of Example 3009



Example 3009

20 Example 3009 was prepared following the general synthetic sequence described for the preparation of Example 3008, starting from Intermediate Resin I, using the following general procedures: "Bromoacetic Acid Coupling" procedure;

"*N*-Gly-Alkylation On-resin Method A" procedure; "Symphony Method A : Secondary-amine coupling without Fmoc deprotection" procedure; Symphony Method A : Standard coupling" procedure; "Symphony Method A : Secondary-amine coupling" procedure; "Chloroacetic Anhydride capping" procedure ; "Global Deprotection Method A"; "Cyclization Method A".

The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A : 5:95 methanol: water with 10-mM ammonium acetate; Mobile Phase B : 95:5 methanol: water with 10-mM ammonium acetate; Gradient: 45-85% B over 35 minutes, then a 5-minute hold at 100% B ; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of product was 9.1 mg, and its estimated purity by LCMS analysis was 100% using "Analysis LCMS condition E".

Analysis LCMS condition E: Retention time = 1.56 min; ESI-MS(+) *m/z* 958.0 (M+2H).

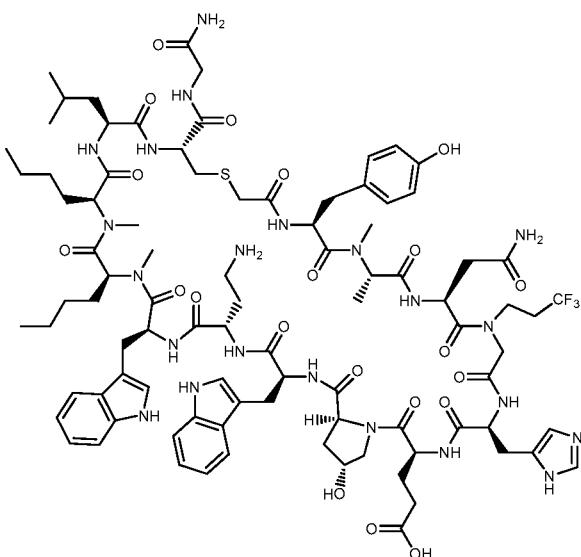
ESI-HRMS(+) *m/z*:

Calculated: 957.4720 (M+2H)

Found: 957.4693 (M+2H).

20

Preparation of Example 3010



Example 3010

Example 3010 was prepared following the general synthetic sequence described for the preparation of Example 3008, starting from Intermediate Resin I, using the following general procedures: "*Bromoacetic Acid Coupling*" procedure; "*N-Gly-Alkylation On-resin Method B*" procedure; "*Symphony Method A*": 5 *Secondary-amine coupling without Fmoc deprotection*" procedure; *Symphony Method A : Standard coupling*" procedure; "*Symphony Method A : Secondary-amine coupling*" procedure; "*Chloroacetic Anhydride capping*" procedure ; "*Global Deprotection Method A*"; "*Cyclization Method A*".

The crude material was purified via preparative LC/MS with the following 10 conditions: Column: XBridge CI8, 19 x 200 mm, 5- μ m particles; Mobile Phase A : 5:95 methanol: water with 10-mM ammonium acetate; Mobile Phase B : 95:5 methanol: water with 10-mM ammonium acetate; Gradient: 50-90% B over 30 minutes, then a 5-minute hold at 100% B ; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield 15 of product was 4.7 mg, and its estimated purity by LCMS analysis was 97% using "*Analysis LCMS condition D*".

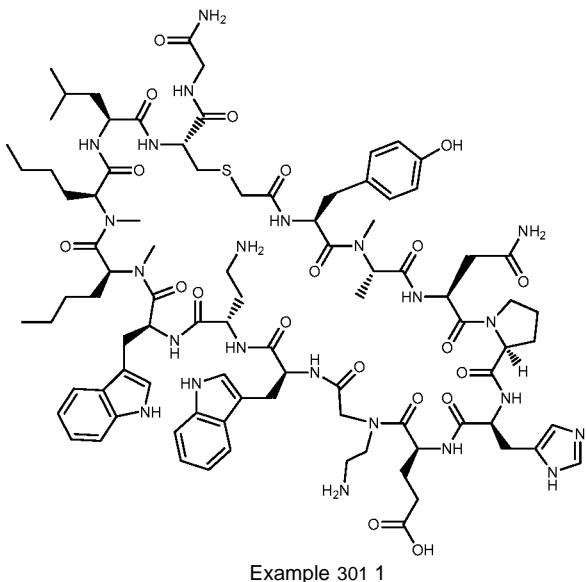
Analysis LCMS condition D : Retention time = 1.83 min; ESI-MS(+) *m/z* 977.7 (M+2H).

ESI-HRMS(+) *m/z*:
20 Calculated: 976.4604 (M+2H)
Found: 976.4592 (M+2H).

25

30

Preparation of Example 3011



Example 301 1 was prepared following the general synthetic sequence described for the preparation of Example 3003, starting from Intermediate Resin H, 5 using the following general procedures: "*Bromoacetic Acid Coupling*" procedure; "*N-Gly-Alkylation On-resin Method A*" procedure; "*Symphony Method A: Secondary-amine coupling without Fmoc deprotection*" procedure; "*Symphony Method A: Standard coupling procedure*"; "*Symphony Method A: Secondary-amine coupling procedure*"; "*Chloroacetic Anhydride capping*" procedure; "*Global Deprotection Method A*"; "*Cyclization Method A*".

The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge CI8, 19 x 200 mm, 5- μ m particles; Mobile Phase A : 5:95 methanol: water with 10-mM ammonium acetate; Mobile Phase B : 95:5 methanol: water with 10-mM ammonium acetate; Gradient: 50-90% B over 30 15 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of product was 10.6 mg, and its estimated purity by LCMS analysis was 89% using "*Analysis LCMS conditions D and E*".

Analysis LCMS condition D: Retention time = 1.85 min; ESI-MS(+) *m/z* 942.9 20 (M+2H).

Analysis LCMS condition E: Retention time = 1.47 min; ESI-MS(+) *m/z* 942.6 (M+2H).

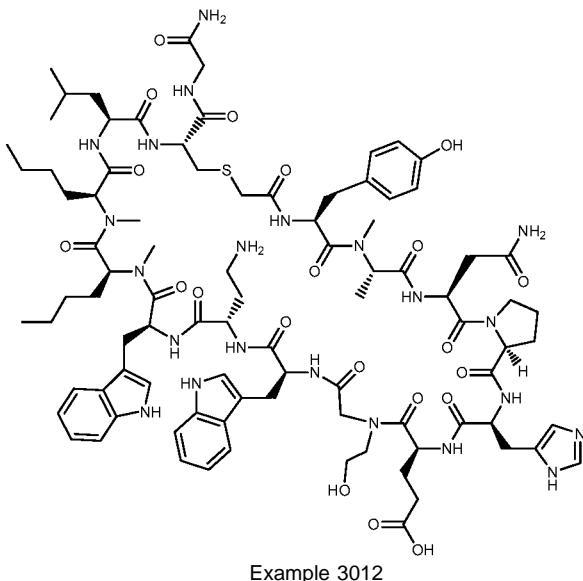
ESI-HRMS(+) *m/z*:

Calculated: 941.9723 (M+2H)

Found: 941.9747 (M+2H).

5

Preparation of Example 3012



Example 3012 was prepared following the general synthetic sequence described for the preparation of Example 3003, starting from Intermediate Resin H, using the following general procedures: "*Bromoacetic Acid Coupling*" procedure; 10 "*N-Gly-Alkylation On-resin Method A*" procedure; "*Symphony Method A : Secondary-amine coupling without Fmoc deprotection*" procedure; "*Symphony Method A : Standard coupling procedure*"; "*Symphony Method A : Secondary-amine coupling procedure*"; "*Chloroacetic Anhydride capping*" procedure; "*Global Deprotection Method A*"; "*Cyclization Method A*".

15 The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge CI8, 19 x 200 mm, 5- μ m particles; Mobile Phase A : 5:95 methanol: water with 10-mM ammonium acetate; Mobile Phase B : 95:5 methanol: water with 10-mM ammonium acetate; Gradient: 45-85% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the 20 desired product were combined and dried via centrifugal evaporation. The yield of product was 3.4 mg, and its estimated purity by LCMS analysis was 95% using "*Analysis LCMS conditions D and E*".

Analysis LCMS condition D: Retention time = 1.95 min; ESI-MS(+) *m/z* (M+2H), not detected.

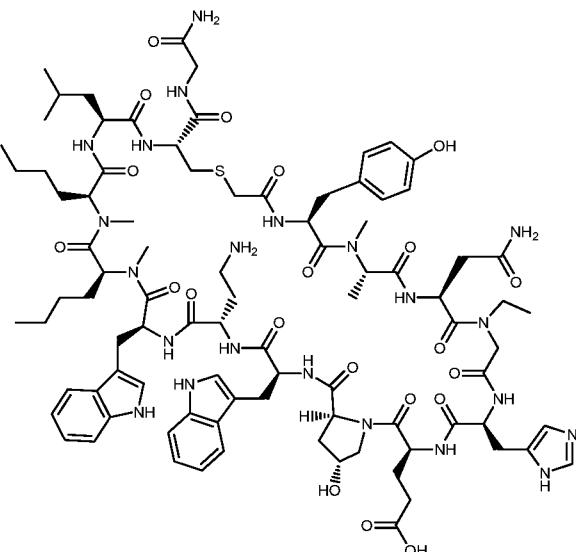
Analysis LCMS condition E: Retention time = 1.60 min; ESI-MS(+) *m/z* (M+2H), not detected.

5 *ESI-HRMS(+) m/z*:

Calculated: 942.4667 (M+2H)

Found: 942.4659 (M+2H).

Preparation of Example 3013



10

Example 3013

Example 3013 was prepared following the general synthetic sequence described for the preparation of Example 3008, starting from Intermediate Resin I, using the following general procedures: "Bromoacetic Acid Coupling" procedure; "N-Gly-Alkylation On-resin Method C" procedure; "Symphony Method A :

15 *Secondary-amine coupling without Fmoc deprotection*" procedure; Symphony Method A : *Standard coupling*" procedure; "Symphony Method A : *Secondary-amine coupling*" procedure; "Chloroacetic Anhydride capping" procedure ; "Global Deprotection Method A "; "Cyclization Method A".

The crude material was purified via preparative LC/MS with the following 20 conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A : 5:95 methanol: water with 10-mM ammonium acetate; Mobile Phase B : 95:5 methanol: water with 10-mM ammonium acetate; Gradient: 45-85% B over 40

minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of product was 5.8 mg, and its estimated purity by LCMS analysis was 97% using "Analysis LCMS conditions D and E".

5 *Analysis LCMS condition D*: Retention time = 1.80 min; ESI-MS(+) *m/z* 942.9 (M+2H);

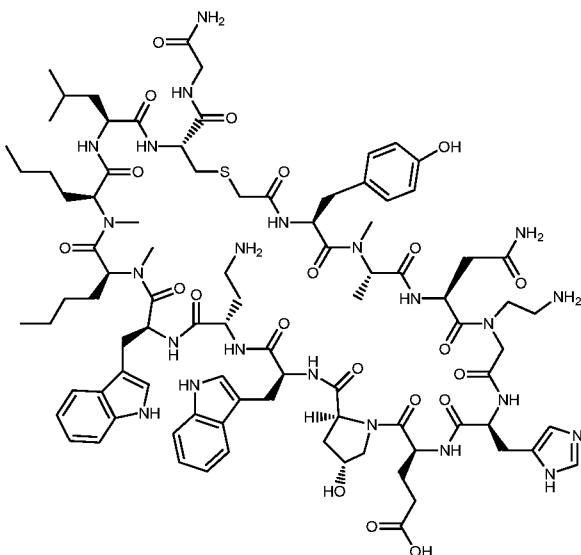
Analysis LCMS condition D: Retention time = 1.56 min; ESI-MS(+) *m/z* 943.0 (M+2H).

ESI-HRMS(+) m/z:

10 Calculated: 942.4667 (M+2H)

Found: 942.4656 (M+2H).

Preparation of Example 3014



Example 3014

15 Example 3014 was prepared following the general synthetic sequence described for the preparation of Example 3008, starting from Intermediate Resin I, using the following general procedures: "*Bromoacetic Acid Coupling*" procedure; "*N-Gly-Alkylation On-resin Method A*" procedure; "*Symphony Method A : Secondary-amine coupling without Fmoc deprotection*" procedure; *Symphony Method A : Standard coupling*" procedure; "*Symphony Method A : Secondary-amine coupling*" procedure; "*Chloroacetic Anhydride capping*" procedure ; "*Global Deprotection Method A*"; "*Cyclization Method A*".

The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A : 5:95 methanol: water with 10-mM ammonium acetate; Mobile Phase B : 95:5 methanol: water with 10-mM ammonium acetate; Gradient: 45-85% B over 40 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of product was 10.2 mg, and its estimated purity by LCMS analysis was 90% using "Analysis LCMS condition D".

5 *Analysis LCMS condition D*: Retention time = 1.75 min; ESI-MS(+) *m/z* 950.5
10 (M+2H).

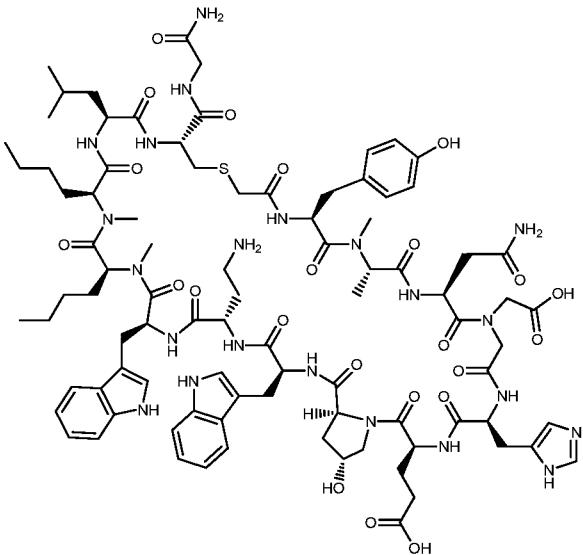
ESI-HRMS(+) m/z:

Calculated: 949.9722 (M+2H)

Found: 949.9704 (M+2H).

15

Preparation of Example 3015



Example 3015

Example 3015 was prepared following the general synthetic sequence described for the preparation of Example 3008, starting from Intermediate Resin I, using the following general procedures: "*Bromoacetic Acid Coupling*" procedure; 20 "*N-Gly-Alkylation On-resin Method B*" procedure; "*Symphony Method A : Secondary-amine coupling without Fmoc deprotection*" procedure; Symphony Method A : *Standard coupling*" procedure; "*Symphony Method A : Secondary-amine*

coupling" procedure; "*Chloroacetic Anhydride capping*" procedure ; "*Global Deprotection Method A*"; "*Cyclization Method A*".

The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge CI8, 19 x 200 mm, 5- μ m particles; Mobile Phase A : 5 5:95 methanol: water with 10-mM ammonium acetate; Mobile Phase B : 95:5 methanol: water with 10-mM ammonium acetate; Gradient: 40-80% B over 40 minutes, then a 5-minute hold at 100% B ; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of product was 3.4 mg, and its estimated purity by LCMS analysis was 90% using 10 "Analysis LCMS condition D".

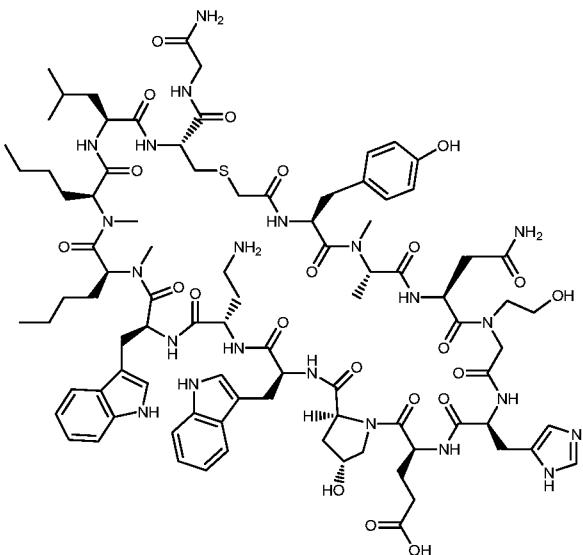
Analysis LCMS condition D : Retention time = 1.72 min; ESI-MS(+) *m/z* 958.2 (M+2H).

ESI-HRMS(+) m/z:

Calculated: 957.4538 (M+2H)

15 Found: 957.4521 (M+2H).

Preparation of Example 3016



Example 3016

Example 3016 was prepared following the general synthetic sequence 20 described for the preparation of Example 3008, starting from Intermediate Resin I, using the following general procedures: "*Bromoacetic Acid Coupling*" procedure; "*N-Gly-Alkylation On-resin Method A*" procedure; "Symphony Method A :

Secondary-amine coupling without Fmoc deprotection" procedure; Symphony Method A : *Standard coupling*" procedure; "Symphony Method A : *Secondary-amine coupling*" procedure; "*Chloroacetic Anhydride capping*" procedure ; "*Global Deprotection Method A*"; "*Cyclization Method A*".

5 The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A : 5:95 methanol: water with 10-mM ammonium acetate; Mobile Phase B : 95:5 methanol: water with 10-mM ammonium acetate; Gradient: 45-85% B over 35 minutes, then a 5-minute hold at 100% B ; Flow: 20 mL/min. Fractions containing 10 the desired product were combined and dried via centrifugal evaporation. The yield of product was 4.0 mg, and its estimated purity by LCMS analysis was 98% using "Analysis LCMS conditions D and E".

15 *Analysis LCMS condition D* : Retention time = 1.85 min; ESI-MS(+) m/z , (M+2H) not detected;

Analysis LCMS condition E : Retention time = 1.56 min; ESI-MS(+) m/z , (M+2H) not detected.

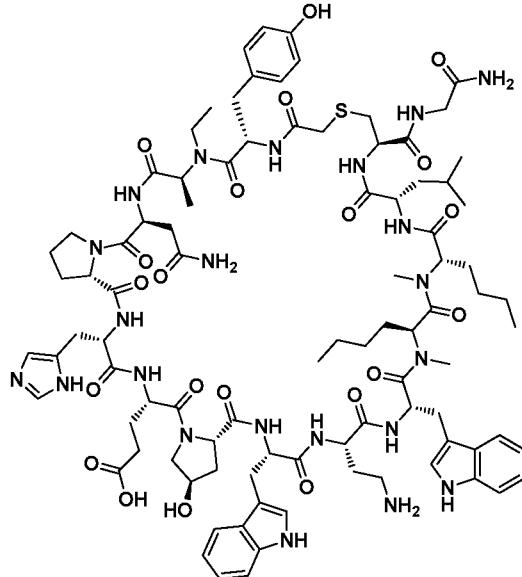
ESI-HRMS(+) m/z:

Calculated: 950.4642 (M+2H)

Found: 950.4629 (M+2H).

20

Preparation of Example 3017



Example 3017

Example 3017 was prepared following the general synthetic sequence described below, starting from Intermediate Resin E. The following procedures were performed sequentially:

"Nosylate formation", "Alkylation method B", "Nosylate removal", "Symphony Method B : *Custom amino acids-couplingprocedure* ", "Symphony Method B : *Final cappingprocedure*", "Global Deprotection Method A", and "Cyclization Method A".

The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A : 5:95 methanol: water with 10-mM ammonium acetate; Mobile Phase B : 95:5 methanol: water with 10-mM ammonium acetate; Gradient: 45-85% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The material was further purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A : 5:95 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B : 95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 10-50% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of the product was 5.1 mg, and its estimated purity by LCMS analysis was 100%.

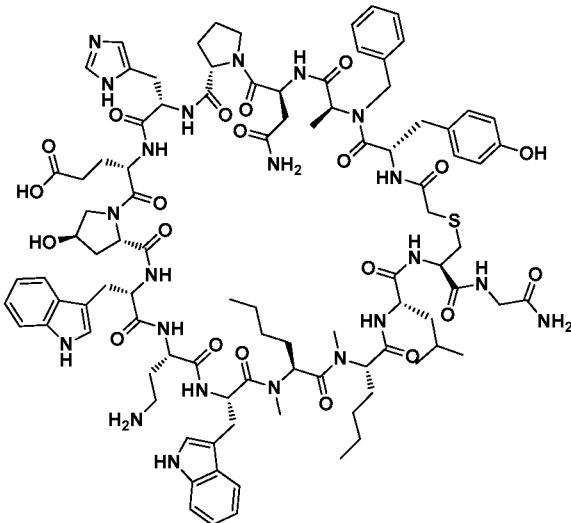
Analysis LCMS Condition H: retention time = 1.71 min.; ESI-MS(+) *m/z* 956.2 (M+2H);

Analysis LCMS Condition I: retention time = 2.73 min.; ESI-MS(+) *m/z* 956.3 (M+2H).

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Preparation of Example 3019



Example 3019 was prepared following the general synthetic sequence described for the preparation of Example 3017, starting from Intermediate Resin E.

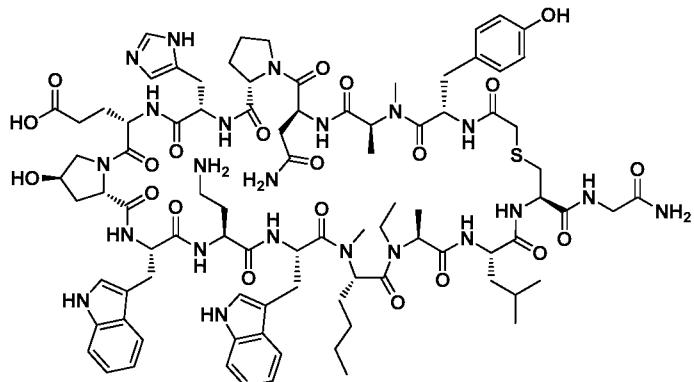
5 The following procedures were performed sequentially: "Nosylate formation", "Alkylation method A", "Nosylate removal", "Symphony Method B : *Custom amino acids-coupling procedure*", "Symphony Method B : *Final capping procedure*", "Global Deprotection Method A", and "Cyclization Method A".

The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A : 5:95 methanol: water with 10-mM ammonium acetate; Mobile Phase B : 95:5 methanol: water with 10-mM ammonium acetate; Gradient: 45-85% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield 15 of the product was 10.7 mg, and its estimated purity by LCMS analysis was 97%.

Analysis LCMS Condition H: retention time = 1.73 min.; ESI-MS(+) *m/z* 987.2 (M+2H).

Analysis LCMS Condition I: retention time = 2.81 min.; ESI-MS(+) *m/z* 987.2 (M+2H).

Preparation of Example 3020



Example 3020 was prepared following the general synthetic sequence described for the preparation of Example 3017, starting from intermediate resin A.

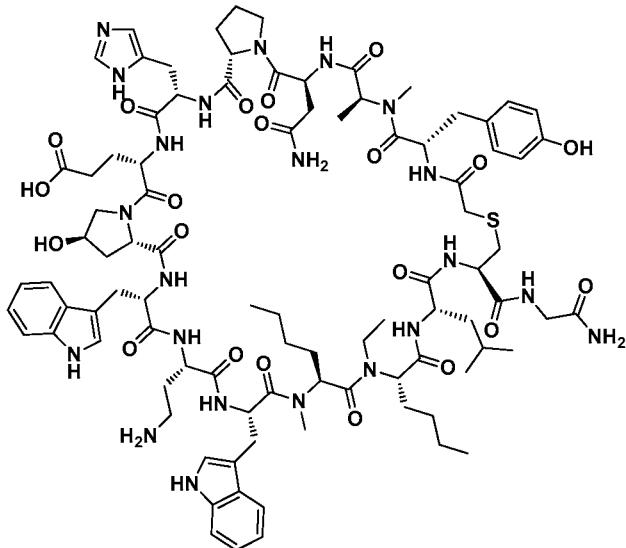
5 The following procedures were performed sequentially: "Nosylate formation", "Alkylation method B", "Nosylate removal", "Symphony Method B : *Resin-swelling procedure*", "Symphony Method B : *Standard-coupling procedure*", "Symphony Method B : *Secondary amine-coupling procedure*", "Symphony Method B : *Custom amino acids-coupling procedure*", "Symphony Method B : *Final capping procedure*", "Global Deprotection Method A", and "Cyclization Method A".

10 The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge CI8, 19 x 200 mm, 5- μ m particles; Mobile Phase A : 5:95 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B : 95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 10-50% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of the product was 14.1 mg, and its estimated purity by LCMS analysis was 97%.

15 *Analysis LCMS Condition H:* retention time = 1.5 min.; ESI-MS(+) *m/z* 935.9 (M+2H).

20 *Analysis LCMS Condition J:* retention time = 1.26 min.; ESI-MS(+) *m/z* 935.0 (M+2H).

Preparation of Example 3021



Example 3021 was prepared following the general synthetic sequence described for the preparation of Example 3017, starting from intermediate resin B.

5 The following procedures were performed sequentially: "Nosylate formation", "Alkylation method B", "Nosylate removal", "Symphony Method B : *Resin-swelling procedure*", "Symphony Method B : *Standard-coupling procedure*", "Symphony Method B : *Secondary amine-coupling procedure*", "Symphony Method B : *Custom amino acids-coupling procedure*", "Symphony Method B : *Final capping procedure*", "Global Deprotection Method A", and "Cyclization Method A".

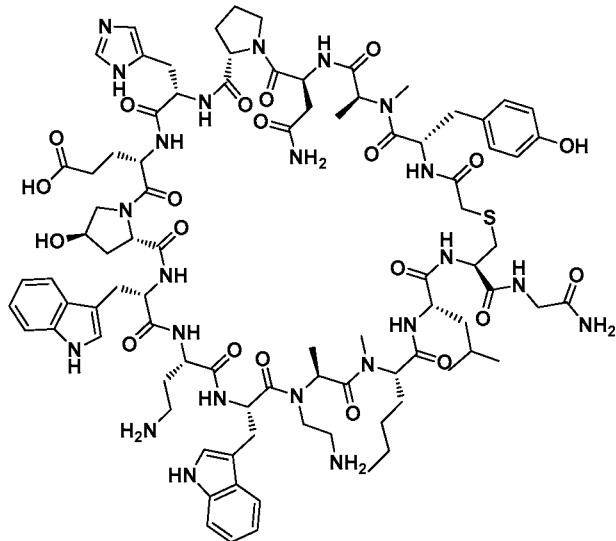
10

The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A: 5:95 acetonitrile: water with 0.1% trifluoroacetic acid; Mobile Phase B: 95:5 acetonitrile: water with 0.1% trifluoroacetic acid; Gradient: 10-50% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of the product was 6.0 mg, and its estimated purity by LCMS analysis was 100%.

15 *Analysis LCMS Condition H:* retention time = 1.77 min.; ESI-MS(+) *m/z* 956.4 (M+2H).

20 *Analysis LCMS Condition J:* retention time = 1.47 min.; ESI-MS(+) *m/z* 956.3 (M+2H).

Preparation of Example 3022



Example 3022 was prepared following the general synthetic sequence described for the preparation of Example 3017, starting from intermediate resin D.

5 The following procedures were performed sequentially: "Nosylate formation", "Alkylation method A", "Nosylate removal", "Symphony Method B :Resin-swelling procedure", "Symphony Method B :Standard-couplingprocedure ", "Symphony Method B :Secondary amine-couplingprocedure", "Symphony Method B :Custom amino acids-couplingprocedure ", "Symphony Method B :Final capping procedure", "Global Deprotection Method A ", and "Cyclization Method A".

10

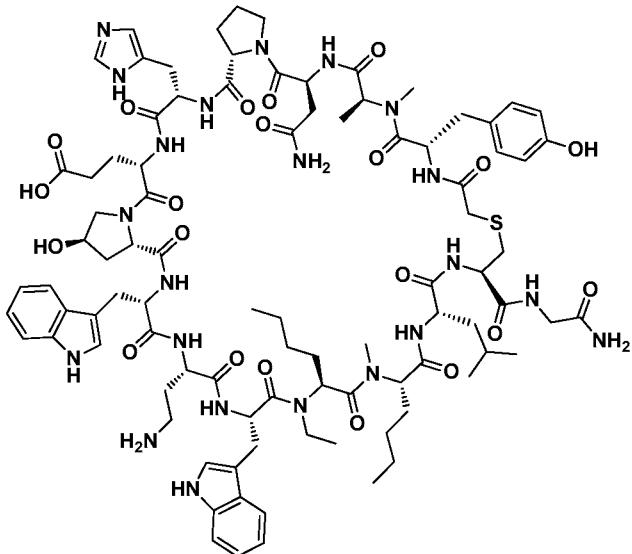
The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A : 5:95 methanol: water with 10-mM ammonium acetate; Mobile Phase B : 95:5 methanol: water with 10-mM ammonium acetate; Gradient: 35-75% B over 30 minutes, then a 5-minute hold at 100% B ; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of the product was 10.7 mg, and its estimated purity by LCMS analysis was 100%.

15

Analysis LCMS Condition H: retention time = 1.26 min.; ESI-MS(+) *m/z* 942.8 (M+2H).

20 *Analysis LCMS Condition J:* retention time = 1.0 min.; ESI-MS(+) *m/z* 942.5 (M+2H).

Preparation of Example 3023



Example 3023 was prepared following the general synthetic sequence described for the preparation of Example 3017, starting from intermediate resin C.

5 The following procedures were performed sequentially: "Nosylate formation", "Alkylation method B", "Nosylate removal", "Symphony Method B:Resin-swelling procedure", "Symphony Method B:Standard-couplingprocedure", "Symphony Method B:Secondary amine-couplingprocedure", "Symphony Method B:Custom amino acids-couplingprocedure", "Symphony Method B:Final capping procedure", "Global Deprotection Method A", and "Cyclization Method A".

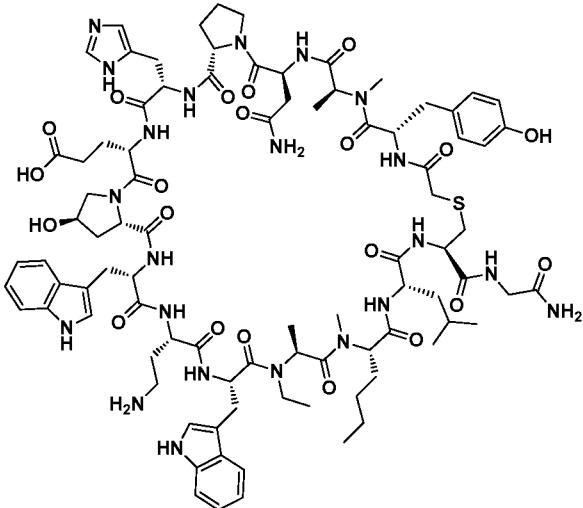
10

The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A: 5:95 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B: 95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 10-50% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of the product was 6.9 mg, and its estimated purity by LCMS analysis was 98%.

15 *Analysis LCMS Condition H:* retention time = 1.67 min.; ESI-MS(+) *m/z* 955.9 (M+2H).

20 *Analysis LCMS Condition I:* retention time = 2.73 min.; ESI-MS(+) *m/z* 956.2 (M+2H).

Preparation of Example 3024



Example 3024 was prepared following the general synthetic sequence described for the preparation of Example 3017, starting from intermediate resin D.

5 The following procedures were performed sequentially: "Nosylate formation", "Alkylation method B", "Nosylate removal", "Symphony Method B: *Resin-swelling procedure*", "Symphony Method B: *Standard-couplingprocedure*", "Symphony Method B: *Secondary amine-couplingprocedure*", "Symphony Method B: *Custom amino acids-couplingprocedure*", "Symphony Method B: *Final capping procedure*", "Global Deprotection Method A", and "Cyclization Method A".

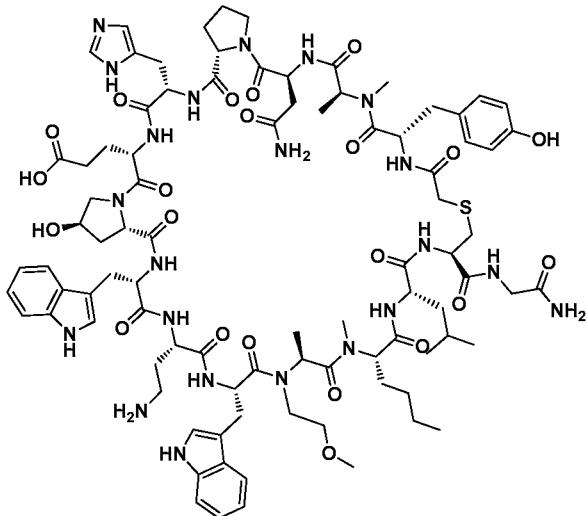
10 The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A: 5:95 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B: 95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 10-50% B over 30

15 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of the product was 9.2 mg, and its estimated purity by LCMS analysis was 100%.

Analysis LCMS Condition H: retention time = 1.59 min.; ESI-MS(+) *m/z* 935.0 (M+2H).

20 *Analysis LCMS Condition E:* retention time = 1.38 min.; ESI-MS(+) *m/z* 935.3 (M+2H).

Preparation of Example 3025



Example 3025 was prepared following the general synthetic sequence described for the preparation of Example 3017, starting from intermediate resin D.

5 The following procedures were performed sequentially: "Nosylate formation", "Alkylation method A", "Nosylate removal", "Symphony Method B : *Resin-swelling procedure*", "Symphony Method B : *Standard-couplingprocedure*", "Symphony Method B : *Secondary amine-couplingprocedure*", "Symphony Method B : *Custom amino acids-couplingprocedure*", "Symphony Method B : *Final capping procedure*", "Global Deprotection Method A", and "Cyclization Method A".

10

The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A : 5:95 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B : 95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 10-50% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of the product was 8.0 mg, and its estimated purity by LCMS analysis was 95%.

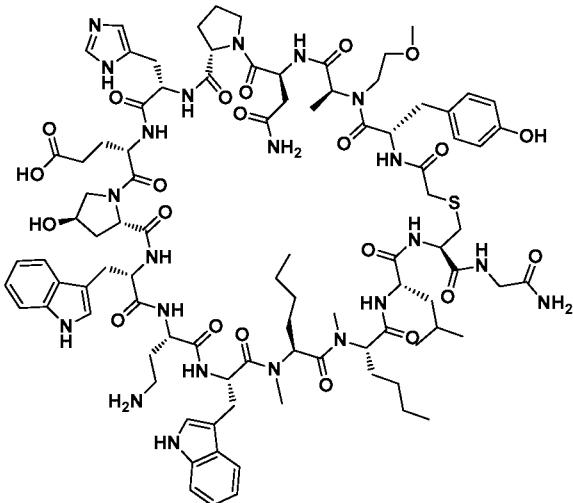
15

Analysis LCMS Condition H: retention time = 1.5 min.; ESI-MS(+) *m/z* 950.0 (M+2H).

20

Analysis LCMS Condition J: retention time = 1.37 min.; ESI-MS(+) *m/z* 949.6 (M+2H).

Preparation of Example 3026



Example 3026 was prepared following the general synthetic sequence described for the preparation of Example 3017, starting from intermediate resin E.

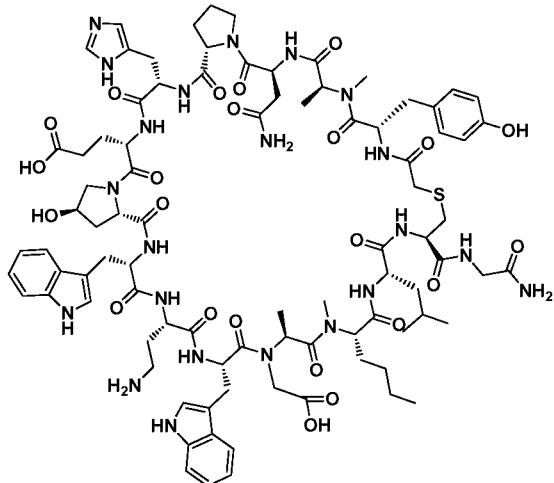
5 The following procedures were performed sequentially: "Nosylate formation", "Alkylation method A", "Nosylate removal", "Symphony Method B : *Custom amino acids-coupling procedure* ", "Symphony Method B : *Final capping procedure* ", "Global Deprotection Method A", and "Cyclization Method A".

The crude material was purified via preparative LC/MS with the following 10 conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A : 5:95 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B : 95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 10-50% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The 15 material was further purified via preparative LC/MS with the following conditions: Column: Waters CSH C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A : 5:95 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B : 95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 5-45% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the 20 desired product were combined and dried via centrifugal evaporation. The yield of the product was 10.8 mg, and its estimated purity by LCMS analysis was 78%.

Analysis LCMS Condition H: retention time = 1.74 min.; ESI-MS(+) m/z 911.1 (M+2H).

Analysis LCMS Condition J: retention time = 1.37 min.; ESI-MS(+) *m/z* 971.1 (M+2H).

Preparation of Example 3028



5

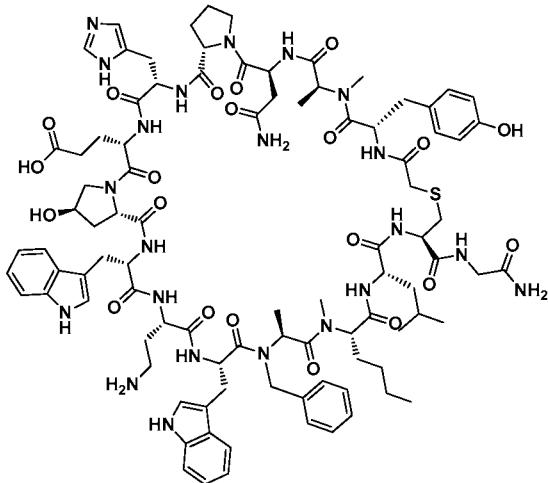
Example 3028 was prepared following the general synthetic sequence described for the preparation of Example 3017, starting from intermediate resin D. The following procedures were performed sequentially: "Nosylate formation", "Alkylation method B", "Nosylate removal", "Symphony Method B: Resin-swelling procedure", "Symphony Method B: Standard-coupling procedure", "Symphony Method B: Secondary amine-coupling procedure", "Symphony Method B: Custom amino acids-coupling procedure", "Symphony Method B: Final capping procedure", "Global Deprotection Method A", and "Cyclization Method A".

The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge CI8, 19 x 200 mm, 5- μ m particles; Mobile Phase A: 5:95 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B: 95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 5-45% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of the product was 4.1 mg, and its estimated purity by LCMS analysis was 82%.

Analysis LCMS Condition H: retention time = 1.31 min.; ESI-MS(+) *m/z* 950.0 (M+2H).

Analysis LCMS Condition J: retention time = 1.16 min.; ESI-MS(+) *m/z* 950.0 (M+2H).

Preparation of Example 3029



Example 3029 was prepared following the general synthetic sequence described for the preparation of Example 3017, starting from intermediate resin D.

5 The following procedures were performed sequentially: "Nosylate formation", "Alkylation method A", "Nosylate removal", "Symphony Method B : *Resin-swelling procedure*", "Symphony Method B : *Standard-couplingprocedure*", "Symphony Method B : *Secondary amine-couplingprocedure*", "Symphony Method B : *Custom amino acids-couplingprocedure*", "Symphony Method B : *Final capping procedure*", "Global Deprotection Method A", and "Cyclization Method A".

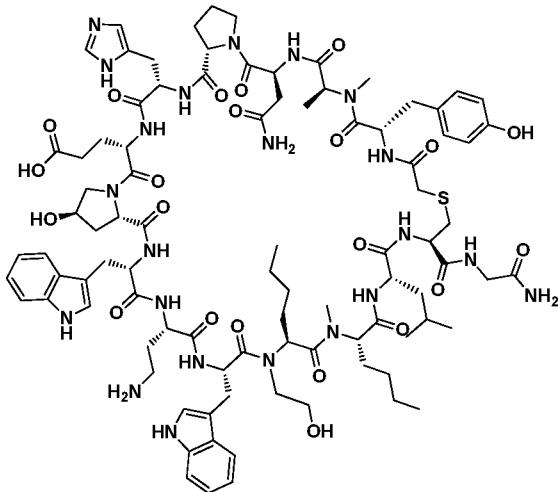
10

The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge CI8, 19 x 200 mm, 5- μ m particles; Mobile Phase A : 5:95 methanol: water with 10-mM ammonium acetate; Mobile Phase B : 95:5 methanol: water with 10-mM ammonium acetate; Gradient: 50-90% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of the product was 2.7 mg, and its estimated purity by LCMS analysis was 78%.

15 *Analysis LCMS Condition H:* retention time = 1.97 min.; ESI-MS(+) *m/z* 967.7 (M+2H).

20 *Analysis LCMS Condition J:* retention time = 1.53 min.; ESI-MS(-) *m/z* 965.5 (M+2H).

Preparation of Example 3030



Example 3030 was prepared following the general synthetic sequence described for the preparation of Example 3017, starting from intermediate resin C.

5 The following procedures were performed sequentially: "Nosylate formation", "Alkylation method A", "Nosylate removal", "Symphony Method B : *Resin-swelling procedure*", "Symphony Method B : *Standard-coupling procedure*", "Symphony Method B : *Secondary amine-coupling procedure*", "Symphony Method B : *Custom amino acids-coupling procedure*", "Symphony Method B : *Final capping procedure*", "Global Deprotection Method A", and "Cyclization Method A".

10

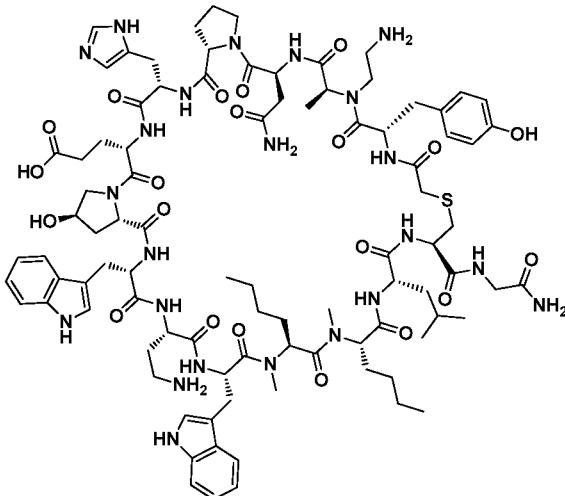
The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A: 5:95 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B : 95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 10-50% B over 40 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of the product was 3.0 mg, and its estimated purity by LCMS analysis was 86%.

Analysis LCMS Condition H: retention time = 1.59 min.; ESI-MS(+) *m/z* 964.3 (M+2H).

15

20 *Analysis LCMS Condition J:* retention time = 1.27 min.; ESI-MS(+) *m/z* 964.3 (M+2H).

Preparation of Example 3032



Example 3032 was prepared following the general synthetic sequence described for the preparation of Example 3017, starting from intermediate resin E.

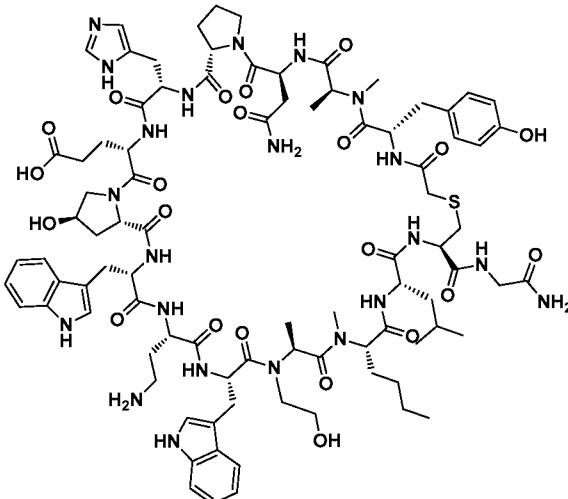
5 The following procedures were performed sequentially: "Nosylate formation", "Alkylation method A", "Nosylate removal", "Symphony Method B : *Custom amino acids-coupling procedure*", "Symphony Method B : *Final capping procedure*", "Global Deprotection Method A", and "Cyclization Method A".

The crude material was purified via preparative LC/MS with the following 10 conditions: Column: XBridge CI8, 19 x 200 mm, 5- μ m particles; Mobile Phase A : 5:95 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B : 95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 10-50% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The 15 material was further purified via preparative LC/MS with the following conditions: Column: Waters CSH C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A : 5:95 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B : 95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 0-40% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the 20 desired product were combined and dried via centrifugal evaporation. The yield of the product was 4.6 mg, and its estimated purity by LCMS analysis was 98%.

Analysis LCMS Condition H: retention time = 1.69 min.; ESI-MS(+) *m/z* 963.4 (M+2H).

Analysis LCMS Condition J: retention time = 1.14 min.; ESI-MS(+) *m/z* 964.2 (M+2H).

Preparation of Example 3033



5

Example 3033 was prepared following the general synthetic sequence described for the preparation of Example 3017, starting from Intermediate resin D. The following procedures were performed sequentially: "Nosylate formation", "Alkylation method A", "Nosylate removal", "Symphony Method B: Resin-swelling procedure", "Symphony Method B: Standard-coupling procedure", "Symphony Method B: Secondary amine-coupling procedure", "Symphony Method B: Custom amino acids-coupling procedure", "Symphony Method B: Final capping procedure", "Global Deprotection Method A", and "Cyclization Method A".

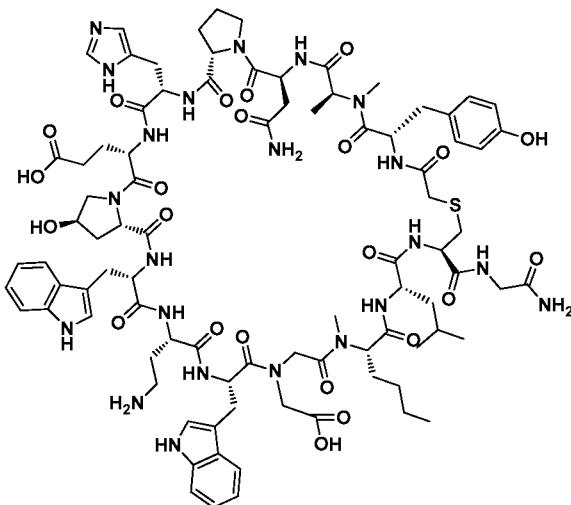
The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A: 5:95 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B: 95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 5-45% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The material was further purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A: 5:95 methanol: water with 10-mM ammonium acetate; Mobile Phase B: 95:5 methanol: water with 10-mM ammonium acetate; Gradient: 25-70% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product

were combined and dried via centrifugal evaporation. The yield of the product was 0.6 mg, and its estimated purity by LCMS analysis was 85%.

Analysis LCMS Condition H: retention time = 1.42 min.; ESI-MS(+) *m/z* 943.0 (M+2H).

5 *Analysis LCMS Condition J:* retention time = 1.13 min.; ESI-MS(+) *m/z* 1883.9 (M+H).

Preparation of Example 3037



Example 3037 was prepared following the general synthetic sequence

10 described for the preparation of Example 3017, starting with Intermediate resin F. The following procedures were performed sequentially: "*N*-Gly-Alkylation On-resin Method D", "Symphony Method B: Resin-swelling procedure", "Symphony Method B: Standard-coupling procedure", "Symphony Method B: Secondary amine-coupling procedure", "Symphony Method B: Custom amino acids-coupling procedure", "Symphony Method B: Final capping procedure", "Global Deprotection Method A", and "Cyclization Method A".

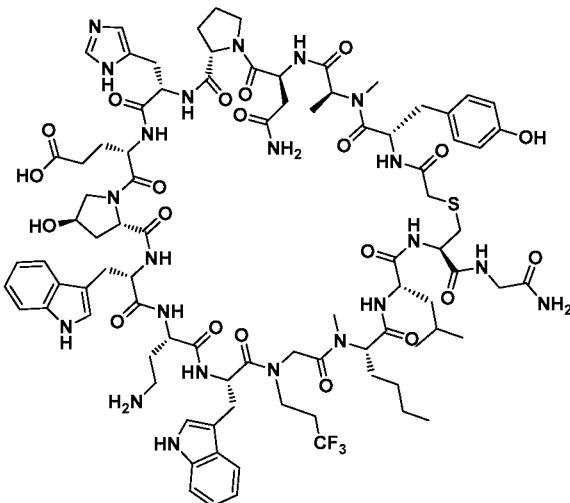
15 The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A: 5:95 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B: 95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 0-40% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of the product was 32.0 mg, and its estimated purity by LCMS analysis was 94%.

Analysis LCMS Condition H: retention time = 1.25 min.; ESI-MS(+) *m/z* 943.2 (M+2H);

Analysis LCMS Condition J: retention time = 1.14 min.; ESI-MS(+) *m/z* 943.2 (M+2H).

5

Preparation of Example 3038



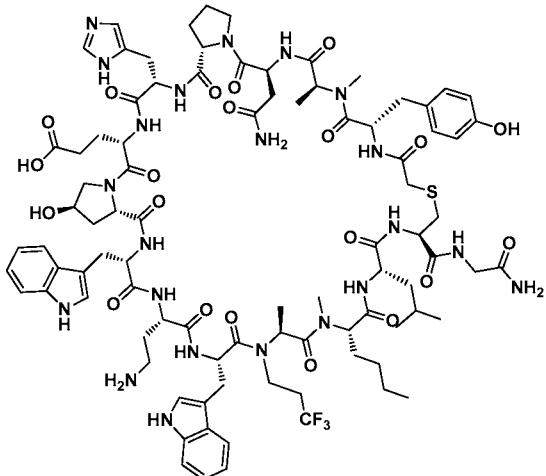
Example 3038 was prepared following the general synthetic sequence described for the preparation of Example 3017, composed of the following general procedures, starting with intermediate resin F: "N-Gly-Alkylation On-resin Method D", "Symphony Method B: Resin-swelling procedure", "Symphony Method B: Standard-coupling procedure", "Symphony Method B: Secondary amine-coupling procedure", "Symphony Method B: Custom amino acids-coupling procedure", "Symphony Method B: Final capping procedure", "Global Deprotection Method A", and "Cyclization Method A".

The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A: 5:95 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B: 95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 10-50% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of the product was 11.9 mg, and its estimated purity by LCMS analysis was 95%.

Analysis LCMS Condition H: retention time = 1.64 min.; ESI-MS(+) *m/z* 962.1 (M+2H);

Analysis LCMS Condition J: retention time = 1.33 min.; ESI-MS(+) *m/z* 962.0 (M+2H).

Preparation of Example 3041



5

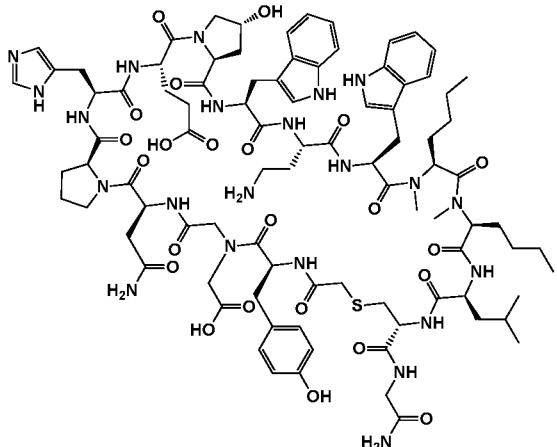
Example 3041 was prepared following the general synthetic sequence described for the preparation of Example 3017, composed of the following general procedures, starting from intermediate resin D: "Nosylate formation", "Alkylation method A", "Nosylate removal", "Symphony Method B: *Resin-swelling procedure*", "Symphony Method B: *Standard-coupling procedure*", "Symphony Method B: *Secondary amine-coupling procedure*", "Symphony Method B: *Custom amino acids-coupling procedure*", "Symphony Method B: *Final capping procedure*", "Global Deprotection Method A", and "Cyclization Method A".

The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A: 5:95 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B: 95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 10-50% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of the product was 3.8 mg, and its estimated purity by LCMS analysis was 81%.

Analysis LCMS Condition H: retention time = 1.68 min.; ESI-MS(+) *m/z* 970.0 (M+2H);

Analysis LCMS Condition J: retention time = 1.51 min.; ESI-MS(+) *m/z* 969.3 (M+2H).

Preparation of Example 3044

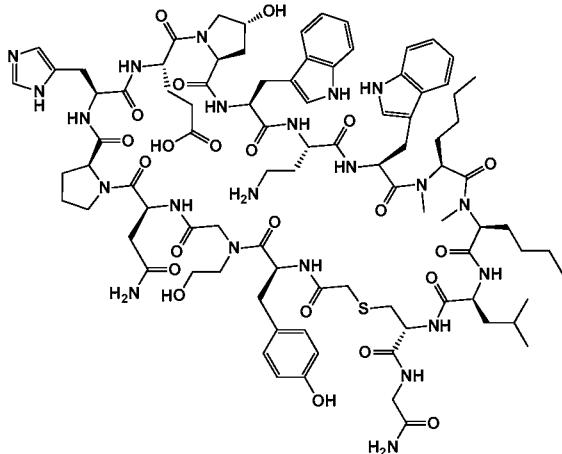


Example 3044 was prepared following the general synthetic sequence described for the preparation of Example 0001, composed of the following general 5 procedures, starting with intermediate resin G: "*N-Gly-Alkylation On-resin Method D*", "*Symphony Method B : Custom amino acids-coupling procedure*", "*Symphony Method B : Final capping procedure*", "*Global Deprotection Method A*", and "*Cyclization Method A*".

The crude material was purified via preparative LC/MS with the following 10 conditions: Column: waters CSH c-18, 19 x 200 mm, 5- μ m particles; Mobile Phase A : 5:95 acetonitrile: water with 0.1% trifluoroacetic acid; Mobile Phase B : 95:5 acetonitrile: water with 0.1% trifluoroacetic acid; Gradient: 10-50% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The 15 material was further purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A : 5:95 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B : 95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 10-50% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the 20 desired product were combined and dried via centrifugal evaporation. The yield of the product was 19.4 mg, and its estimated purity by LCMS analysis was 96%. *Analysis LCMS Condition H*: retention time = 1.55 min.; ESI-MS(+) *m/z* 964.1 (M+2H);

Analysis LCMS Condition J: retention time = 1.32 min.; ESI-MS(+) *m/z* 963.8 (M+2H).

Preparation of Example 3045



5

Example 3045 was prepared following the general synthetic sequence described for the preparation of Example 3017, composed of the following general procedures, starting with intermediate resin G: "*N-Gly-Alkylation On-resin Method D*", "*Symphony Method B : Custom amino acids-coupling procedure*", "*Symphony Method B : Final capping procedure*", "*Global Deprotection Method A*", and "*Cyclization Method A*".

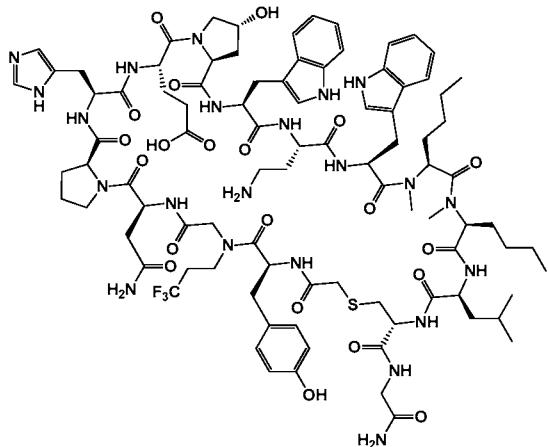
The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A: 5:95 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B: 95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 15-55% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The material was further purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A: 5:95 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B: 95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 5-45% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of the product was 1.5 mg, and its estimated purity by LCMS analysis was 94%.

Analysis LCMS Condition H: retention time = 1.60 min.; ESI-MS(+) *m/z* 957.1 (M+2H);

Analysis LCMS Condition J: retention time = 1.30 min.; ESI-MS(+) *m/z* 957.5 (M+2H).

5

Preparation of Example 3046



Example 3046 was prepared following the general synthetic sequence described for the preparation of Example 3017, composed of the following general 10 procedures, starting with intermediate resin G: "*N*-Gly-Alkylation On-resin Method D", "Symphony Method B : Custom amino acids-coupling procedure", "Symphony Method B : Final capping procedure", "Global Deprotection Method A", and "Cyclization Method A".

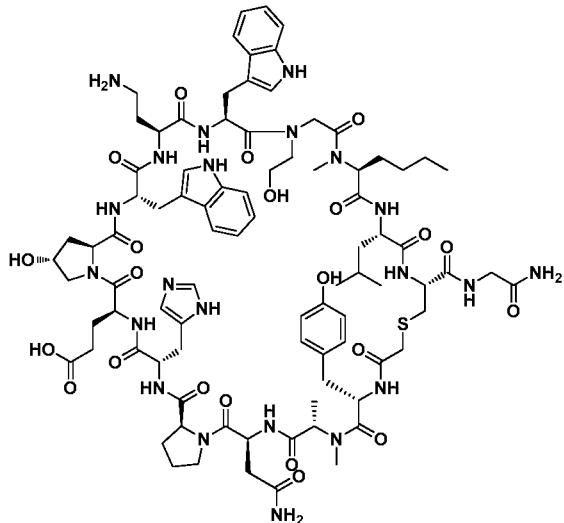
The crude material was purified via preparative LC/MS with the following 15 conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A : 5:95 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B : 95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 10-50% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The 20 material was further purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A : 5:95 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B : 95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 10-50% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the

desired product were combined and dried via centrifugal evaporation. The yield of the product was 7.2 mg, and its estimated purity by LCMS analysis was 100%.

Analysis LCMS Condition H: retention time = 1.71 min.; ESI-MS(+) *m/z* 982.9 (M+2H).

5 *Analysis LCMS Condition J:* retention time = 1.40 min.; ESI-MS(+) *m/z* 982.9 (M+2H).

Preparation of Example 3047



10 Example 3047 was prepared following the general synthetic sequence described for the preparation of Example 3017, composed of the following general procedures, starting with intermediate resin F: "*N-Gly-Alkylation On-resin Method D*", "*Symphony Method B : Resin-swelling procedure*", "*Symphony Method B : Standard-coupling procedure*", "*Symphony Method B : Secondary amine-coupling procedure*", "*Symphony Method B : Custom amino acids-coupling procedure*", "*Symphony Method B : Final capping procedure*", "*Global Deprotection Method A*", and "*Cyclization Method A*".

15

The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A: 20 5:95 methanol: water with 10-mM ammonium acetate; Mobile Phase B: 95:5 methanol: water with 10-mM ammonium acetate; Gradient: 40-80% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The

material was further purified via preparative LC/MS with the following conditions: Column: Waters CSH C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A : 5:95 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B : 95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 5-45% B over 30 5 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of the product was 1.0 mg, and its estimated purity by LCMS analysis was 97%. *Analysis LCMS Condition H:* retention time = 1.44 min.; ESI-MS(+) *m/z* 936.1 (M+2H);

10 *Analysis LCMS Condition J:* retention time = 1.21 min.; ESI-MS(+) *m/z* 936.4 (M+2H).

Experimental procedures for compounds 5001, 5002, 5003

Analytical Data:

15 Mass Spectrometry: "ESI-MS(+)" signifies electrospray ionization mass spectrometry performed in positive ion mode; "ESI-MS(-)" signifies electrospray ionization mass spectrometry performed in negative ion mode; "ESI-FIRMS(+)" signifies high-resolution electrospray ionization mass spectrometry performed in positive ion mode; "ESI-HRMS(-)" signifies high-resolution electrospray ionization mass spectrometry performed in negative ion mode. The detected masses are 20 reported following the "w/z" unit designation. Compounds with exact masses greater than 1000 were often detected as double-charged or triple-charged ions.

Analysis Condition A:

25 Column: Waters BEH C18, 2.0 x 50 mm, 1.7- μ m particles; Mobile Phase A : 5:95 acetonitrile: water with 10 mM ammonium acetate; Mobile Phase B : 95:5 acetonitrile: water with 10 mM ammonium acetate; Temperature: 50 °C; Gradient: 0% B, 0-100% B over 3 minutes, then a 0.5-minute hold at 100% B; Flow: 1 mL/min; Detection: UV at 220 nm.

Analysis Condition B:

30 Column: Waters BEH C18, 2.0 x 50 mm, 1.7- μ m particles; Mobile Phase A : 5:95 methanol: water with 10 mM ammonium acetate; Mobile Phase B : 95:5 methanol: water with 10 mM ammonium acetate; Temperature: 50 °C; Gradient:

0% B, 0-100% B over 3 minutes, then a 0.5-minute hold at 100% B; Flow: 0.5 mL/min; Detection: UV at 220 nm.

Analysis Condition C:

Column: Waters Aquity BEH CI8 2.1 X 50 mm 1.7 μ m particles; Mobile Phase A : 5 water with 0.05% TFA; Mobile Phase B : acetonitrile with 0.05% TFA; Temperature: 40 °C; Gradient: 0% B, 0-100% B over 1.5 minutes, then a 0.5-minute hold at 100% B; Flow: 0.8 mL/min; Detection: UV at 220 nm.

General Procedures:

10 *Prelude Method A:*

All manipulations were performed under automation on a Prelude peptide synthesizer (Protein Technologies). All procedures unless noted were performed in a 10 mL polypropylene tube fitted with a bottom frit; where the scale of the reaction exceeded 0.100 mmol, a 40 mL polypropylene tube fitted with a bottom frit was 15 used. The tube connects to a the Prelude peptide synthesizer through both the bottom and the top of the tube. DMF and DCM can be added through the top of the tube, which washes down the sides of the tube equally. The remaining reagents are added through the bottom of the tube and pass up through the frit to contact the resin. All 20 solutions are removed through the bottom of the tube. "Periodic agitation" describes a brief pulse of N2 gas through the bottom frit; the pulse lasts approximately 5 seconds and occurs every 30 seconds. Chloroacetyl chloride solutions in DMF were used within 24h of preparation. Amino acid solutions were generally not used beyond three weeks from preparation. HATU solutions were used within 5 days of preparation. DMF = dimethylformamide; HATU = 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid 25 hexafluorophosphate; DIPEA = diisopropylethylamine; Rink = (2,4-dimethoxyphenyl)(4-alkoxyphenyl)methanamine, where "4-alkoxy" describes the position and type of connectivity to the polystyrene resin. The resin used is Merrifield polymer (polystyrene) with a Rink linker (Fmoc-protected at nitrogen); 30 100-200 mesh, 1% DVB, 0.56 mmol/g loading. Common amino acids used are listed below with side-chain protecting groups indicated inside parenthesis.

Fmoc-Ala-OH; Fmoc-Arg(Pbf)-OH; Fmoc-Asn(Trt)-OH; Fmoc-Asp(OtBu)-OH; Fmoc-Bzt-OH; Fmoc-Cys(Trt)-OH; Fmoc-Dab(Boc)-OH; Fmoc-Dap(Boc)-OH; Fmoc-Gln(Trt)-OH; Fmoc-Gly-OH; Fmoc-His(Trt)-OH; Fmoc-Hyp(tBu)-OH; Fmoc-Ile-OH; Fmoc-Leu-OH; Fmoc-Lys(Boc)-OH; Fmoc-Nle-OH; Fmoc-Met-OH; Fmoc-[N-Me]Ala-OH; Fmoc-[N-Me]Nle-OH; Fmoc-Phe-OH; Fmoc-Pro-OH; Fmoc-Sar-OH; Fmoc-Ser(tBu)-OH; Fmoc-Thr(tBu)-OH; Fmoc-Trp(Boc)-OH; Fmoc-Tyr(tBu)-OH; Fmoc-Val-OH.

The procedures of "Prelude Method A" describe an experiment performed on a 0.100 mmol scale, where the scale is determined by the amount of Rink linker bound to the resin. This scale corresponds to approximately 178 mg of the Rink-Merrifield resin described above. All procedures can be scaled beyond 0.100 mmol scale by adjusting the described volumes by the multiple of the scale. Prior to amino acid coupling, all peptide synthesis sequences began with a resin-swelling procedure, described below as "Resin-swelling procedure". Coupling of amino acids to a primary amine N-terminus used the "Single-coupling procedure" described below. Coupling of amino acids to a secondary amine N-terminus used the "Double-coupling procedure" described below. Coupling of chloroacetylchloride to the N-terminus of the peptide is described by the "Chloroacetyl chloride coupling procedure" detailed below.

20

Resin-swelling procedure:

To a 10 mL polypropylene solid-phase reaction vessel was added Merrifield:Rink resin (178 mg, 0.100 mmol). The resin was washed (swelled) three times as follows: to the reaction vessel was added DMF (2.0 mL), upon which the mixture was periodically agitated for 10 minutes before the solvent was drained through the frit.

Single-coupling procedure:

To the reaction vessel containing resin from the previous step was added 30 piperidine:DMF (20:80 v/v, 2.0 mL). The mixture was periodically agitated for 3 minutes and then the solution was drained through the frit. To the reaction vessel was added piperidine:DMF (20:80 v/v, 2.0 mL). The mixture was periodically

agitated for 3 minutes and then the solution was drained through the frit. The resin was washed successively six times as follows: for each wash, DMF (2.0 mL) was added through the top of the vessel and the resulting mixture was periodically agitated for 30 seconds before the solution was drained through the frit. To the 5 reaction vessel was added the amino acid (0.2M in DMF, 1.0 mL, 2 eq), then HATU (0.2M in DMF, 1.0 mL, 2 eq), and finally DIPEA (0.8M in DMF, 0.5 mL, 4 eq). The mixture was periodically agitated for 15 minutes, then the reaction solution was drained through the frit. The resin was washed successively four times as follows: for each wash, DMF (2.0 mL) was added through the top of the vessel and the 10 resulting mixture was periodically agitated for 30 seconds before the solution was drained through the frit. To the reaction vessel was added acetic anhydride (2.0 mL). The mixture was periodically agitated for 10 minutes, then the solution was drained through the frit. The resin was washed successively four times as follows: for each wash, DMF (2.0 mL) was added through the top of the vessel and the resulting 15 mixture was periodically agitated for 90 seconds before the solution was drained through the frit. The resulting resin was used directly in the next step.

Double-coupling procedure:

To the reaction vessel containing resin from the previous step was added 20 piperidine:DMF (20:80 v/v, 2.0 mL). The mixture was periodically agitated for 3 minutes and then the solution was drained through the frit. To the reaction vessel was added piperidine:DMF (20:80 v/v, 2.0 mL). The mixture was periodically agitated for 3 minutes and then the solution was drained through the frit. The resin was washed successively six times as follows: for each wash, DMF (2.0 mL) was 25 added through the top of the vessel and the resulting mixture was periodically agitated for 30 seconds before the solution was drained through the frit. To the reaction vessel was added the amino acid (0.2M in DMF, 1.0 mL, 2 eq), then HATU (0.2M in DMF, 1.0 mL, 2 eq), and finally DIPEA (0.8M in DMF, 0.5 mL, 4 eq). The mixture was periodically agitated for 15 minutes, then the reaction solution was 30 drained through the frit. The resin was twice washed as follows: for each wash, DMF (2.0 mL) was added through the top of the vessel and the resulting mixture was periodically agitated for 30 seconds before the solution was drained through the frit.

To the reaction vessel was added the amino acid (0.2M in DMF, 1.0 mL, 2 eq), then HATU (0.2M in DMF, 1.0 mL, 2 eq), and finally DIPEA (0.8M in DMF, 0.5 mL, 4 eq). The mixture was periodically agitated for 15 minutes, then the reaction solution was drained through the frit. The resin was twice washed as follows: for each wash, 5 DMF (2.0 mL) was added through the top of the vessel and the resulting mixture was periodically agitated for 30 seconds before the solution was drained through the frit. To the reaction vessel was added acetic anhydride (2.0 mL). The mixture was periodically agitated for 10 minutes, then the solution was drained through the frit. The resin was washed successively four times as follows: for each wash, DMF (2.0 10 mL) was added through the top of the vessel and the resulting mixture was periodically agitated for 90 seconds before the solution was drained through the frit. The resulting resin was used directly in the next step.

Chloroacetyl chloride coupling procedure:

15 To the reaction vessel containing the resin from the previous step was added piperidine:DMF (20:80 v/v, 2.0 mL). The mixture was periodically agitated for 3 minutes and then the solution was drained through the frit. To the reaction vessel was added piperidine:DMF (20:80 v/v, 2.0 mL). The mixture was periodically agitated for 3 minutes and then the solution was drained through the frit. The resin 20 was washed successively six times as follows: for each wash, DMF (2.0 mL) was added through the top of the vessel and the resulting mixture was periodically agitated for 30 seconds before the solution was drained through the frit. To the reaction vessel was added DIPEA (0.8M in DMF, 3.0 mL, 24 eq), then chloroacetyl chloride (0.8M in DMF, 1.65 mL, 13.2 eq). The mixture was periodically agitated 25 for 30 minutes, then the solution was drained through the frit. The resin was washed successively three times as follows: for each wash, DMF (2.0 mL) was added to top of the vessel and the resulting mixture was periodically agitated for 90 seconds before the solution was drained through the frit. The resin was washed successively four times as follows: for each wash, CH₂Cl₂ (2.0 mL) was added to top of the vessel 30 and the resulting mixture was periodically agitated for 90 seconds before the solution was drained through the frit. The resulting resin was placed under a N₂ stream for 15 minutes.

Symphony Method A:

This collection of procedures is identical that of "Prelude Method A" except as noted. For all procedures a Symphony X peptide synthesizer (Protein Technologies) was used instead of a Prelude peptide synthesizer and all reagents 5 were added through the top of the reaction vessel.

Resin-swelling procedure:

This procedure is identical to "Prelude Method A : Resin-swelling procedure".

Single-coupling procedure:

This procedure is identical to "Prelude Method A : Single-coupling procedure" 10 except that the concentration of DIPEA solution was 0.4M and 1.0 mL of this solution was delivered to the reaction.

Double-coupling procedure:

This procedure is identical to "Prelude Method A : Double-coupling procedure" except that the concentration of DIPEA solution was 0.4M and 1.0 mL of 15 this solution was delivered to the reaction.

Chloroacetyl chloride coupling procedure:

This procedure is identical to "Prelude Method A : Chloroacetyl chloride coupling procedure".

Global Deprotection Method A:

20 All manipulations were performed manually unless noted. The procedure of "Global Deprotection Method A" describes an experiment performed on a 0.100 mmol scale, where the scale is determined by the amount of Rink linker bound to the resin. The procedure can be scaled beyond 0.100 mmol scale by adjusting the described volumes by the multiple of the scale. A "deprotection solution" was 25 prepared by combining in a 40 mL glass vial trifluoroacetic acid (22 mL), phenol (1.325 g), water (1.25 mL) and triisopropylsilane (0.5 mL). The resin was removed from the reaction vessel and transferred to a 4 mL glass vial. To the vial was added the "deprotection solution" (2.0 mL). The mixture was vigorously mixed in a shaker (1000 RPM for 1 minute, then 500 RPM for 1-2h). The mixture was filtered through 30 a 0.2 micron syringe filter and the solids were extracted with the "deprotection solution" (1.0 mL) or TFA (1.0 mL). To a 24 mL test tube charged with the combined filtrates was added Et₂O (15 mL). The mixture was vigorously mixed

upon which a significant amount of a white solid precipitated. The mixture was centrifuged for 5 minutes, then the solution was decanted away from the solids and discarded. The solids were suspended in Et₂O (20 mL); then the mixture was centrifuged for 5 minutes; and the solution was decanted away from the solids and discarded. For a final time, the solids were suspended in Et₂O (20 mL); the mixture was centrifuged for 5 minutes; and the solution was decanted away from the solids and discarded to afford the crude peptide as a white to off-white solid.

Cyclization Method A :

All manipulations were performed manually unless noted. The procedure of "Cyclization Method B" describes an experiment performed on a 0.100 mmol scale, where the scale is determined by the amount of Rink linker bound to the resin that was used to generate the peptide. This scale is not based on a direct determination of the quantity of peptide used in the procedure. The procedure can be scaled beyond 0.100 mmol scale by adjusting the described volumes by the multiple of the scale.

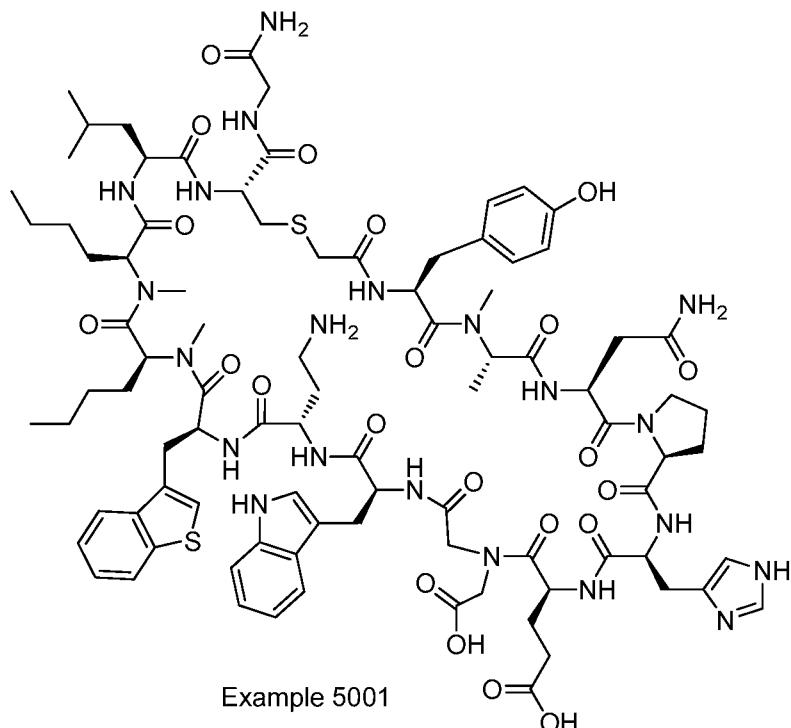
The crude peptide solids were dissolved in MeCN:aq. 0.1M NFl₄OAc (1:1) to a total volume of 18-22 mL, and the solution was carefully then adjusted to pH = 8.5-9.0 using aq NaOH (1.0M). The solution was then allowed to stand without stirring for 6 days. The reaction solution was concentrated and the residue was then dissolved in DMSO:MeOH. This solution was subjected to reverse-phase FIPLC purification to afford the desired cyclic peptide.

General Synthetic Sequence A :

"General Synthetic Sequence A" describes a general sequence of procedures that were used to afford the cyclic peptides described herein. For the purposes of this general procedure, the procedures of "Symphony Method A" are interchangeable with those of "Prelude Method A". To a 10 mL polypropylene solid-phase reaction vessel was added Rink-Merrifield resin (178 mg, 0.100 mmol), and the reaction vessel was placed on the Prelude peptide synthesizer. "Prelude Method A : Resin-swelling procedure" was followed. Then a series of amino acids couplings was sequentially performed on the Prelude following "Prelude Method A : Single-coupling procedure" if the N-terminus of the resin-bound peptide was a primary amine or "Prelude Method A : Double-coupling procedure" if the N-terminus of the resin-bound peptide was a secondary amine. "Prelude Method A : Chloroacetyl

chloride coupling procedure" was followed; then "Global Deprotection Method A" was followed; then "Cyclization Method A" was followed.

Preparation of Example 5001



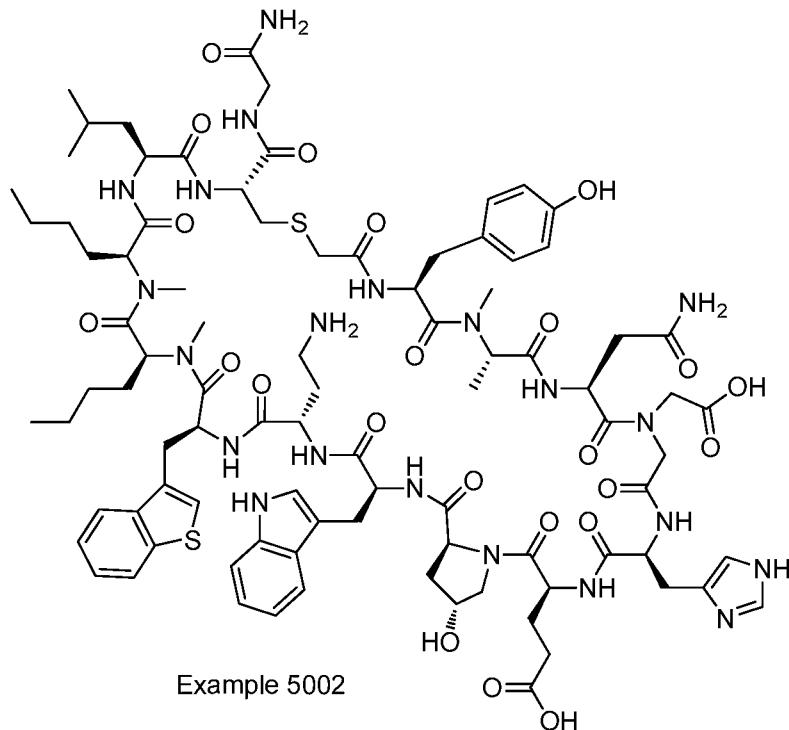
5

Example 5001 was prepared following "General Synthetic Sequence A. In the synthesis 2-(((9H-fluoren-9-yl)methoxy)carbonyl)(2-(tert-butoxy)-2-oxoethyl)amino acid was used as indicated by the sequence. The crude material was purified via preparative LC/MS with the following conditions: Column: 10 XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A: 5:95 methanol: water with 10-mM ammonium acetate; Mobile Phase B: 95:5 methanol: water with 10-mM ammonium acetate; Gradient: 50-90% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of the product was 7.8 mg, and its estimated purity by LCMS analysis was 100%.

15 *Analysis condition A*: Retention time = 1.48 min; ESI-MS(+) *m/z* 956.8.1 (M-2H)

Analysis condition B: Retention time = 2.82 min; ESI-MS(+) *m/z* 958.2 (M+2H).

Preparation of Example 5002

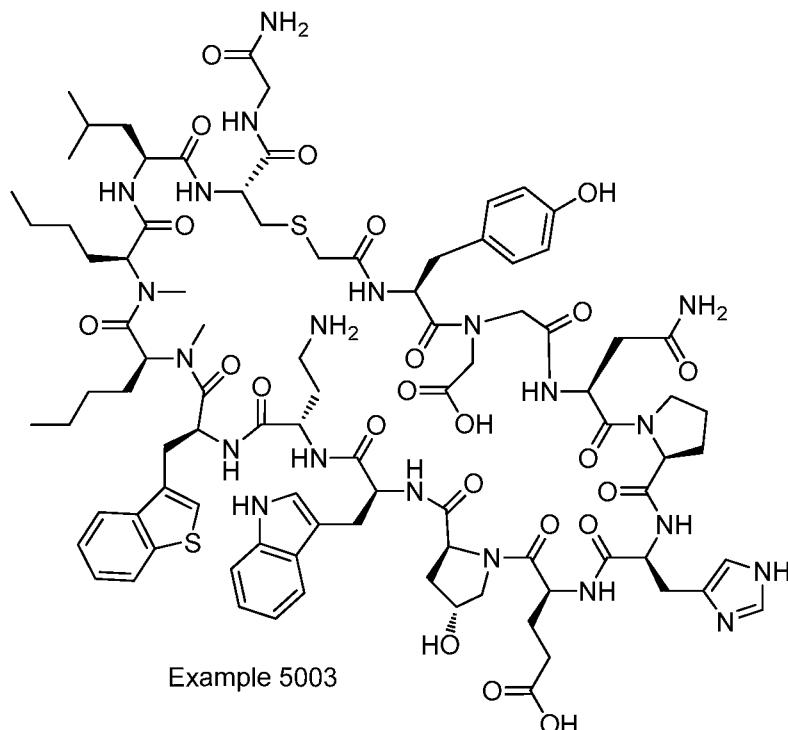


Example 5002 was prepared following "General Synthetic Sequence A". In the synthesis 2-(((9H-fluoren-9-yl)methoxy)carbonyl)(2-(tert-butoxy)-2-5 oxoethyl)amino)acetic acid was used as indicated by the sequence. The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A: 5:95 methanol: water with 10-mM ammonium acetate; Mobile Phase B: 95:5 methanol: water with 10-mM ammonium acetate; Gradient: 55-95% B over 30 minutes, then a 5-minute hold at 10 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of the product was 2.2 mg, and its estimated purity by LCMS analysis was 81%>.

Analysis condition A: Retention time = 1.51 min; ESI-MS(+) *m/z* 964.1 (M-2H)

Analysis condition B: Retention time = 2.88 min; ESI-MS(+) *m/z* 966.5 (M+2H).

Preparation of Example 5003



Example 5003 was prepared following "General Synthetic Sequence A". In the synthesis 2-(((9H-fluoren-9-yl)methoxy)carbonyl)(2-(tert-butoxy)-2-5 oxoethyl)amino acetic acid was used as indicated by the sequence. The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A : 5:95 methanol: water with 10-mM ammonium acetate; Mobile Phase B : 95:5 methanol: water with 10-mM ammonium acetate; Gradient: 60-100% B over 30 minutes, then a 5-minute hold at 10 100% B ; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The material was further purified via preparative LC/MS with the following conditions: Column: XBridge Phenyl, 19 x 200 mm, 5- μ m particles; Mobile Phase A : 5:95 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B : 95:5 acetonitrile: water with 10-mM 15 ammonium acetate; Gradient: 5-40% B over 30 minutes, then a 5-minute hold at 100% B ; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of the product was 4.0 mg, and its estimated purity by LCMS analysis was 100%.

Analysis condition A : Retention time = 1.777 min; ESI-MS(+) *m/z* 972.40 (M+2H)

Analysis condition B: Retention time = 2.393 min; ESI-MS(+) *m/z* 968.25 (M-2H)
ESI-HRMS(+) *m/z*: Calculated: 971.9344 (M+2H) Found: 971.9311
(M+2H).

5 METHODS FOR TESTING THE ABILITY OF MACROCYCLIC PEPTIDES TO
COMPETE FOR THE BINDING OF PD-1 TO PD-L1 USING HOMOGENOUS
TIME-RESOLVED FLUORESCENCE (HTRF) BINDING ASSAYS

10 The ability of the macrocyclic peptides of the present disclosure to bind to
PD-L1 was investigated using a PD-1/PD-L1 Homogenous Time-Resolved
Fluorescence (HTRF) binding assay.

Methods

Homogenous Time-Resolved Fluorescence (HTRF) Assays of Binding of Soluble PD-1 to Soluble PD-L1. Soluble PD-1 and soluble PD-L1 refers to proteins with carboxyl-end truncations that remove the transmembrane-spanning regions and are fused to heterologous sequences, specifically the Fc portion of the human immunoglobulin G sequence (Ig) or the hexahistidine epitope tag (His). All binding studies were performed in an HTRF assay buffer consisting of dPBS supplemented with 0.1% (w/v) bovine serum albumin and 0.05% (v/v) Tween-20. For the PD-1-Ig/PD-L1-His binding assay, inhibitors were pre-incubated with PD-L1-His (10 nM final) for 15m in 4 μ l of assay buffer, followed by addition of PD-1-Ig (20 nM final) in 1 μ l of assay buffer and further incubation for 15m. PD-L1 fusion proteins from either human, cynomolgous macaques, mouse, or other species were used. HTRF detection was achieved using europium cryptate-labeled anti-Ig monoclonal antibody (1 nM final) and allophycocyanin (APC) labeled anti-His monoclonal antibody (20 nM final). Antibodies were diluted in HTRF detection buffer and 5 μ l was dispensed on top of binding reaction. The reaction was allowed to equilibrate for 30 minutes and signal (665nm/620nm ratio) was obtained using an EnVision fluorometer. Additional binding assays were established between PD-1-Ig/PD-L2-His (20 and 5 nM, respectively), CD80-His/PD-L1-Ig (100 and 10 nM, respectively) and CD80-His/CTLA4-Ig (10 and 5 nM, respectively). Binding/competition studies between biotinylated Compound No. 71 and human PD-L1-His were performed as follows.

Macrocyclic peptide inhibitors were pre-incubated with PD-L1-His (10 nM final) for 60 minutes in 4 μ l of assay buffer followed by addition of biotinylated Compound No. 71 (0.5 nM final) in 1 μ l of assay buffer. Binding was allowed to equilibrate for 30 minutes followed by addition of europium crypted labeled Streptavidin (2.5 pM final) and APC-labeled anti-His (20 nM final) in 5 μ l of HTRF buffer. The reaction was allowed to equilibrate for 30m and signal (665nm/620nm ratio) was obtained using an EnVision fluorometer.

Recombinant Proteins. Carboxyl -truncated human PD-1 (amino acids 25-167) with a C-terminal human Ig epitope tag [hPD-1 (25-167)-3S-IG] and human PD-L1 (amino acids 18-239) with a C-terminal His epitope tag [hPD-L1(19-239)-tobacco vein mottling virus protease cleavage site (TVMV)-His] were expressed in HEK293T cells and purified sequentially by recombinant Protein A affinity chromatography and size exclusion chromatography. Human PD-L2-His (Sino Biologicals), CD80-His (Sino Biologicals), CTLA4-Ig (RnD Systems) were all obtained through commercial sources.

Sequence of Recombinant Human PD-1-Ig

hPD1(25-167)-3S-IG

X	LDSFDRPWNP	PTFSFALLVV	TEGDNATFTC	SFSNTSESFV	LNWYRMSPSN
S1	QTDKLAAPFE	DRSQPGQDCR	F&VTQLPNGR	DFHMSVVEAR	RNDSGTYLCG
101	AISLAPKAQI	KESLRAELRV	TERRASVPTA	HPSPSPRFAG	QFQGSPGJGG
I S1	GRSPKS8 DKT	MTSPPSPAPI	LLOGSSYFLF	PPKPKDTLM	SRTPRTVCVV
201	VDVSHEDPEV	KFNWYVDGVE	VHNAAKTKPRE	EQYNSTYRVV	SVLTVLIHQDW
251	iNGKRYKCRV	SNKALPASIE	KTISKAKGQP	REPQVYTLPP	SRDELTSGNQV
301	SLTCLVEGFY	PSDXAVENSS	NGQPENNYKT	TFPVLDSDGS	FFLYSKLTVD
351	KS RWQQGNVF	SCSVNREALH	NHYTQKSLSI	SPGK	

(SEQ ID NO:1)

Sequence of Recombinant Human PD-L1-TVMV-His (PD-L1-His)

hPDL1(19-239)-TVMV-His

1	FTVTVPKDLY	VVSYG6NMTI	BCKFPVBRQL	DIAALIVYWB	MBDKNXIQFV
51	HG1EDLKVQH	SSYRQRARLL	KDQLSLGNAA	LQITDVKLQD	AGVYRCMISY
101	GGADYKRITV	KVNAPYNKIN	QRXLVVDFVT	SEHELTQAE	GYFNAEVINT
151	SSDHQVLSGK	TTTTNSKRBE	KLFNVTSTLR	INTTTNBIFY	CTFRRRLDPEB
201	NHTAELVIFS	LPLAHPPNER	TGSSSETVRFQ	GHHHHHH	

(SEQ ID NO:2)

The results are shown in Table 1. As shown, the macrocyclic peptides of the present disclosure demonstrated potent inhibition of PD-1-Ig binding activity to PD-L1-TVMV-His (PD-L1-His). Ranges are as follows: A = 0.10-10 μ M; B = 0.01-0.099 μ M; C = 0.005 - 0.0099 μ M.

5

Table 1

<i>Example Number</i>	<i>HTRF IC50 (μM)</i>
3003	B
3004	B
3005	0.03
3006	B
3007	B
3008	0.20
3009	B
3010	B
3011	6.12E-03
3012	B
3013	B
3014	B
3015	B
3016	B
3017	C
3019	B
3020	C
3021	C
3022	B
3023	B
3024	B
3025	B
3026	B
3028	A
3029	A

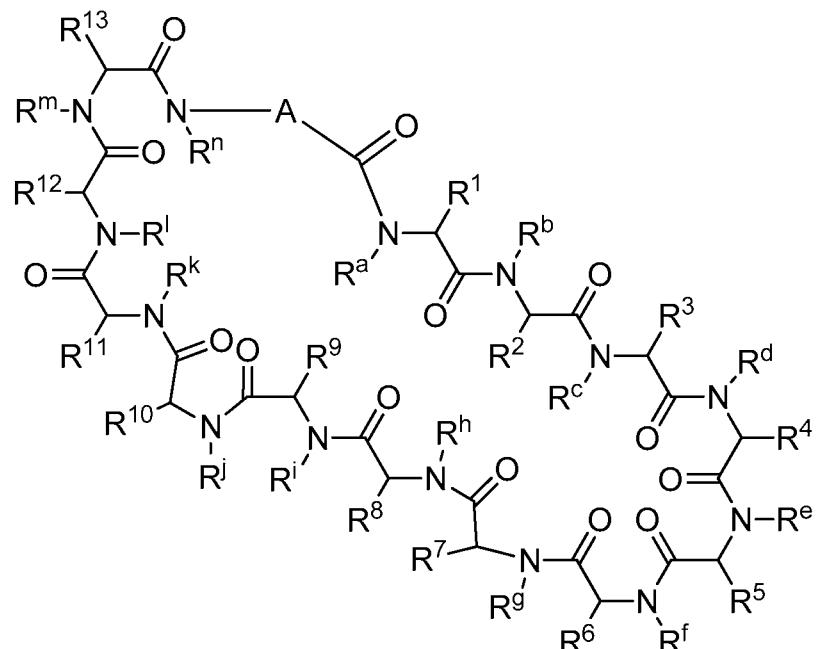
3030	B
3032	B
3033	A
3037	A
3038	2.36
3041	B
3044	B
3045	B
3046	B
3047	—
3048	A
3049	A
3050	A
5001	C
5002	C
5003	B

It will be evident to one skilled in the art that the present disclosure is not limited to the foregoing illustrative examples, and that it can be embodied in other specific forms without departing from the essential attributes thereof. It is therefore
5 desired that the examples be considered in all respects as illustrative and not restrictive, reference being made to the appended claims, rather than to the foregoing examples, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.

CLAIMS

WHAT IS CLAIMED IS:

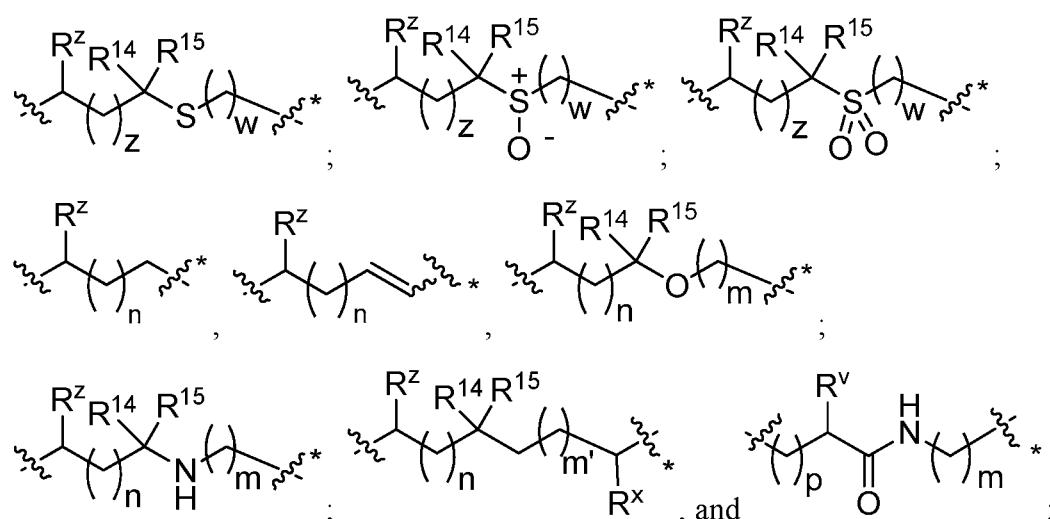
1. A compound of formula (I)



(I),

or a pharmaceutically acceptable salt thereof, wherein:

A is selected from a bond,



wherein:

~~~~\* denotes the point of attachment to the carbonyl group and ~~~ denotes the point of attachment to the nitrogen atom;

z is 0, 1, or 2;

w is 1 or 2;

n is 0 or 1;

m is 1 or 2;

m' is 0 or 1;

p is 0, 1, or 2;

R<sup>x</sup> is selected from hydrogen, amino, hydroxy, and methyl;

R<sup>14</sup> and R<sup>15</sup> are independently selected from hydrogen and methyl; and

R<sup>z</sup> is selected from hydrogen and -C(0)NHR<sup>16</sup>; wherein R<sup>16</sup> is selected from hydrogen, -CHR<sup>17</sup>C(0)NH<sub>2</sub>, -CHR<sup>17</sup>C(0)NHCHR<sup>18</sup>C(0)NH<sub>2</sub>, and -CHR<sup>17</sup>C(0)NHCHR<sup>18</sup>C(0)NHCH<sub>2</sub>C(0)NH<sub>2</sub>; wherein R<sup>17</sup> is selected from hydrogen and -CH<sub>2</sub>OH and wherein R<sup>18</sup> is selected from hydrogen and methyl;

R<sup>y</sup> is hydrogen or a natural amino acid side chain;

R<sup>c</sup>, R<sup>f</sup>, R<sup>h</sup>, R<sup>j</sup>, and R<sup>m</sup> are hydrogen;

R<sup>n</sup> is hydrogen or methyl or R<sup>y</sup> and R<sup>n</sup> form a pyrrolidine ring;

R<sup>a</sup> is hydrogen or methyl;

R<sup>j</sup> is selected from hydrogen, Ci-C<sub>6</sub>alkoxyCi-C<sub>6</sub>alkyl, Ci-C<sub>6</sub>alkyl, carboxyCi-Cealkyl, haloCi-Cealkyl, hydroxyCi-Cealkyl, (NR<sup>a'</sup>R<sup>b'</sup>)Ci-C<sub>6</sub>alkyl wherein R<sup>a'</sup> and R<sup>b'</sup> are independently selected from hydrogen and Ci-C<sub>6</sub>alkyl; and phenylCi-C<sub>6</sub>alkyl wherein the phenyl is optionally substituted with one, two, three, four or five groups independently selected from Ci-C<sub>6</sub>alkoxy, Ci-C<sub>6</sub>alkyl, cyano, halo, and nitro;

R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, R<sup>4</sup>, R<sup>5</sup>, R<sup>6</sup>, R<sup>7</sup>, R<sup>8</sup>, R<sup>9</sup>, R<sup>10</sup>, R<sup>11</sup>, R<sup>12</sup>, and R<sup>13</sup> are independently selected from a natural amino acid side chain and an unnatural amino acid side chain; or

R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, R<sup>4</sup>, R<sup>5</sup>, R<sup>6</sup>, R<sup>7</sup>, R<sup>8</sup>, R<sup>9</sup>, R<sup>10</sup>, R<sup>11</sup>, R<sup>12</sup>, and R<sup>13</sup> can each independently form a ring with the corresponding vicinal R group as described below;

R<sup>b</sup> is selected from Ci-C<sub>6</sub>alkoxyCi-C<sub>6</sub>alkyl, Ci-C<sub>6</sub>alkyl, carboxyCi-Cealkyl, haloCi-Cealkyl, hydroxyCi-Cealkyl, (NR<sup>a'</sup>R<sup>b'</sup>)Ci-C<sub>6</sub>alkyl wherein R<sup>a'</sup> and R<sup>b'</sup> are

independently selected from hydrogen and Ci-C<sub>6</sub>alkyl; and phenylCi-C<sub>6</sub>alkyl wherein the phenyl is optionally substituted with one, two, three, four or five groups independently selected from Ci-C<sub>6</sub>alkoxy, Ci-C<sub>6</sub>alkyl, cyano, halo, and nitro; or, R<sup>b</sup> and R<sup>2</sup>, together with the atoms to which they are attached, form a ring selected from azetidine, pyrrolidine, morpholine, piperidine, piperazine, and tetrahydrothiazole; wherein each ring is optionally substituted with one to four groups independently selected from amino, cyano, methyl, halo, and hydroxy;

R<sup>d</sup> is selected from hydrogen, Ci-C<sub>6</sub>alkoxyCi-C<sub>6</sub>alkyl, Ci-C<sub>6</sub>alkyl, carboxyCi-Cealkyl, haloCi-Cealkyl, hydroxyCi-Cealkyl, (NR<sup>a'</sup>R<sup>b'</sup>)Ci-C<sub>6</sub>alkyl wherein R<sup>a'</sup> and R<sup>b'</sup> are independently selected from hydrogen and Ci-C<sub>6</sub>alkyl; and phenylCi-C<sub>6</sub>alkyl wherein the phenyl is optionally substituted with one, two, three, four or five groups independently selected from Ci-C<sub>6</sub>alkoxy, Ci-C<sub>6</sub>alkyl, cyano, halo, and nitro; or, R<sup>d</sup> and R<sup>4</sup>, together with the atoms to which they are attached, can form a ring selected from azetidine, pyrrolidine, morpholine, piperidine, piperazine, and tetrahydrothiazole; wherein each ring is optionally substituted with one to four groups independently selected from amino, cyano, methyl, halo, hydroxy, and phenyl;

R<sup>e</sup> is selected from hydrogen, Ci-C<sub>6</sub>alkoxyCi-C<sub>6</sub>alkyl, Ci-C<sub>6</sub>alkyl, carboxyCi-Cealkyl, haloCi-Cealkyl, hydroxyCi-Cealkyl, (NR<sup>a'</sup>R<sup>b'</sup>)Ci-C<sub>6</sub>alkyl wherein R<sup>a'</sup> and R<sup>b'</sup> are independently selected from hydrogen and Ci-C<sub>6</sub>alkyl; and phenylCi-C<sub>6</sub>alkyl wherein the phenyl is optionally substituted with one, two, three, four or five groups independently selected from Ci-C<sub>6</sub>alkoxy, Ci-C<sub>6</sub>alkyl, cyano, halo, and nitro; or R<sup>e</sup> and R<sup>5</sup> together with the atoms to which they are attached, can form a ring selected from azetidine, pyrrolidine, morpholine, piperidine, piperazine, and tetrahydrothiazole; wherein each ring is optionally substituted with one to four groups independently selected from amino, cyano, methyl, halo, and hydroxy;

R<sup>g</sup> is selected from hydrogen, Ci-C<sub>6</sub>alkoxyCi-C<sub>6</sub>alkyl, Ci-C<sub>6</sub>alkyl, carboxyCi-Cealkyl, haloCi-Cealkyl, hydroxyCi-Cealkyl, (NR<sup>a'</sup>R<sup>b'</sup>)Ci-C<sub>6</sub>alkyl wherein R<sup>a'</sup> and R<sup>b'</sup> are independently selected from hydrogen and Ci-C<sub>6</sub>alkyl; and phenylCi-C<sub>6</sub>alkyl wherein the phenyl is optionally substituted with one, two, three, four or five groups independently selected from Ci-C<sub>6</sub>alkoxy, Ci-C<sub>6</sub>alkyl, cyano, halo, and nitro; or R<sup>g</sup> and R<sup>7</sup>, together with the atoms to which they are attached, can form a ring

selected from azetidine, pyrrolidine, morpholine, piperidine, piperazine, and tetrahydrothiazole; wherein each ring is optionally substituted with one to four groups independently selected from amino, benzyl optionally substituted with a halo group, benzyloxy, cyano, cyclohexyl, methyl, halo, hydroxy, isoquinolinylxy optionally substituted with a methoxy group, quinolinylxy optionally substituted with a halo group, and tetrazolyl; and wherein the pyrrolidine and the piperidine ring are optionally fused to a cyclohexyl, phenyl, or indole group;

provided that at least one of  $R^a$ ,  $R^b$ ,  $R^d$ ,  $R^e$ ,  $R^g$ ,  $R^j$ ,  $R^k$ , and  $R^l$  is selected from,  $Ci-C_6alkoxyCi-C_6alkyl$ ,  $C_2-C_6alkyl$ ,  $carboxyCi-Cealkyl$ ,  $haloCi-Cealkyl$ ,  $hydroxyCi-C_6alkyl$ ,  $(NR^{a'}R^{b'})Ci-C_6alkyl$  wherein  $R^{a'}$  and  $R^{b'}$  are independently selected from hydrogen and  $Ci-C_6alkyl$ ; and  $phenylCi-C_6alkyl$  wherein the phenyl is optionally substituted with one, two, three, four or five groups independently selected from  $Ci-C_6alkoxy$ ,  $Ci-C_6alkyl$ , cyano, halo, and nitro.

2. A compound of claim 1, or a pharmaceutically acceptable salt thereof, wherein

$R^e$  is selected from hydrogen and methyl, or  $R^e$  and  $R^{5'}$  together with the atoms to which they are attached, can form a ring selected from azetidine, pyrrolidine, morpholine, piperidine, piperazine, and tetrahydrothiazole; wherein each ring is optionally substituted with one to four groups independently selected from amino, cyano, methyl, halo, and hydroxy; and

$R^j$  is hydrogen or methyl.

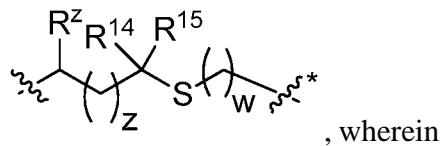
3. A compound of claim 2, or a pharmaceutically acceptable salt thereof, wherein

$R^k$  is selected from hydrogen and methyl, or  $R^k$  and  $R^{11}$ , together with the atoms to which they are attached, can form a ring selected from azetidine, pyrrolidine, morpholine, piperidine, piperazine, and tetrahydrothiazole; wherein each ring is optionally substituted with one to four groups independently selected from amino, cyano, methyl, halo, and hydroxy; and

$R^l$  is methyl, or,  $R^l$  and  $R^{12}$ , together with the atoms to which they are attached, form a ring selected from azetidine and pyrrolidine, wherein each ring is

optionally substituted with one to four groups independently selected from amino, cyano, methyl, halo, and hydroxy.

4. A compound of claim 3, or a pharmaceutically acceptable salt thereof, wherein A is



z and w are each 1;

R<sup>14</sup> and R<sup>15</sup> are hydrogen; and

R<sup>z</sup> is -C(0)NHR<sup>16</sup>; wherein R<sup>16</sup> is -CHR<sup>17</sup>C(0)NH<sub>2</sub>;

R<sup>17</sup> is hydrogen;

R<sup>1</sup> is benzyl optionally substituted with hydroxy;

R<sup>2</sup> is hydrogen or methyl;

R<sup>8</sup> is -(CH<sub>2</sub>)indolyl;

R<sup>10</sup> is selected from -(CH<sub>2</sub>)indolyl and -(CH<sub>2</sub>)benzothienyl, each optionally substituted with -CH<sub>2</sub>C(0)H;

R<sup>11</sup> is butyl; and

R<sup>12</sup> is butyl.

5. A compound selected from:

Example 3003, Example 3004, Example 3005, Example 3006, Example 3007, Example 3008, Example 3009, Example 3010, Example 3011, Example 3012, Example 3013, Example 3014, Example 3015, Example 3016, Example 3017, Example 3019, Example 3020, Example 3021, Example 3022, Example 3023, Example 3024, Example 3025, Example 3026, Example 3028, Example 3029, Example 3030, Example 3032, Example 3033, Example 3037, Example 3038, Example 3041, Example 3044, Example 3045, Example 3046, Example 3047, Example 5001, Example 5002, and Example 5003; or a pharmaceutically acceptable salt thereof.

6. A method of enhancing, stimulating, and/or increasing the immune response in a subject in need thereof, said method comprising administering to the subject a therapeutically effective amount of a compound of claim 1 or a therapeutically acceptable salt thereof.

7. The method of claim 6 further comprising administering an additional agent prior to, after, or simultaneously with the compound of claim 1 or a therapeutically acceptable salt thereof.

8. The method of claim 7 wherein the additional agent is an antimicrobial agent, an antiviral agent, a cytotoxic agent, and/or an immune response modifier.

9. The method of claim 7 wherein the additional agent is an HDAC inhibitor.

10. The method of claim 7 wherein the additional agent is a TLR7 and/or TLR8 agonist.

11. A method of inhibiting growth, proliferation, or metastasis of cancer cells in a subject in need thereof, said method comprising administering to the subject a therapeutically effective amount a compound of claim 1 or a therapeutically acceptable salt thereof.

12. The method of claim 11 wherein the cancer is selected from melanoma, renal cell carcinoma, squamous non-small cell lung cancer (NSCLC), non-squamous NSCLC, colorectal cancer, castration-resistant prostate cancer, ovarian cancer, gastric cancer, hepatocellular carcinoma, pancreatic carcinoma, squamous cell carcinoma of the head and neck, carcinomas of the esophagus, gastrointestinal tract and breast, and hematological malignancies.

13. A method of treating an infectious disease in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of a compound of claim 1 or a therapeutically acceptable salt thereof.

14. The method of claim 13 wherein the infectious disease is caused by a virus.
15. The method of claim 14 wherein the virus is selected from HIV, Hepatitis A, Hepatitis B, Hepatitis C, herpes viruses, and influenza.
16. A method of treating septic shock in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of a compound of claim 1 or a therapeutically acceptable salt thereof.
17. A method blocking the interaction of PD-L1 with PD-1 and/or CD80 in a subject, said method comprising administering to the subject a therapeutically effective amount of a compound of claim 1 or a therapeutically acceptable salt thereof.

# INTERNATIONAL SEARCH REPORT

International application No  
PCT/US2015/065723

A. CLASSIFICATION OF SUBJECT MATTER  
INV. C07K7/54 A61K38/12 A61P35/00  
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                              | Relevant to claim No. |
|-----------|-----------------------------------------------------------------------------------------------------------------|-----------------------|
| X         | WO 2014/151634 A1 (SQUIBB BRISTOL MYERS CO [US] ) 25 September 2014 (2014-09-25)<br>table 6; compounds<br>----- | 1-17                  |



Further documents are listed in the continuation of Box C.



See patent family annex.

\* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

|                                                           |                                                    |
|-----------------------------------------------------------|----------------------------------------------------|
| Date of the actual completion of the international search | Date of mailing of the international search report |
| 24 March 2016                                             | 05/04/2016                                         |

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Authorized officer

Fuhr, Christian

**INTERNATIONAL SEARCH REPORT**

## Information on patent family members

International application No  
PCT/US2015/065723

| Patent document cited in search report | Publication date | Patent family member(s) |    |              | Publication date |
|----------------------------------------|------------------|-------------------------|----|--------------|------------------|
| wo 2014151634                          | AI               | 25-09-2014              | AU | 2014233675   | AI               |
|                                        |                  |                         | CA | 2905453      | AI               |
|                                        |                  |                         | CN | 105209479    | A                |
|                                        |                  |                         | EA | 201591822    | AI               |
|                                        |                  |                         | EP | 2970390      | AI               |
|                                        |                  |                         | KR | 20150133215  | A                |
|                                        |                  |                         | PE | 16712015     | AI               |
|                                        |                  |                         | PH | 12015502027  | AI               |
|                                        |                  |                         | SG | 11201506917Y | A                |
|                                        |                  |                         | TW | 201502141    | A                |
|                                        |                  |                         | US | 2014294898   | AI               |
|                                        |                  |                         | UY | 35448        | A                |
|                                        |                  |                         | WO | 2014151634   | AI               |