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- *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*
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(54) Title: IMMUNOMODULATORS

(57) **Abstract:** The present disclosure provides novel macrocyclic compounds 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 APPLICATION

This application claims the benefit of United States serial number 62/303,673
 5 filed March 4, 2016 the entire contents of which is incorporated in its entirety.

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

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.*
 15 *Immunol.*, 170:711-718 (2003)).

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.*,
 20 181:1953-1956 (1995); Vivier, E. et al., *Immunol. Today*, 18:286-291 (1997)).
 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
 25 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)).
 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
 30 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.*

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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);

5 Brown et al., *J. Immunol.*, 170:1257-1266 (2003)).

PD-L1 has also been shown to interact with CD80 (Butte MJ et al, *Immunity*;27:111-122 (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.*,

10 187:1097-1105 (2011); Yang J, et al. *J Immunol.* Aug 1;187(3):1113-9 (2011)).

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

15 development of self tolerance (Keir, M.E. et al., *Annu. Rev. Immunol.*, 26:Epub (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

20 display PD-1/PD-ligand suppression and "exhaustion".

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

25 infections have shown that blockade of the PD-1/PD-L1 pathway by monoclonal 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.*,

30 81(5):281-287 (2003); Dong, H. et al., "Tumor-associated B7-H1 promotes T-cell 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.

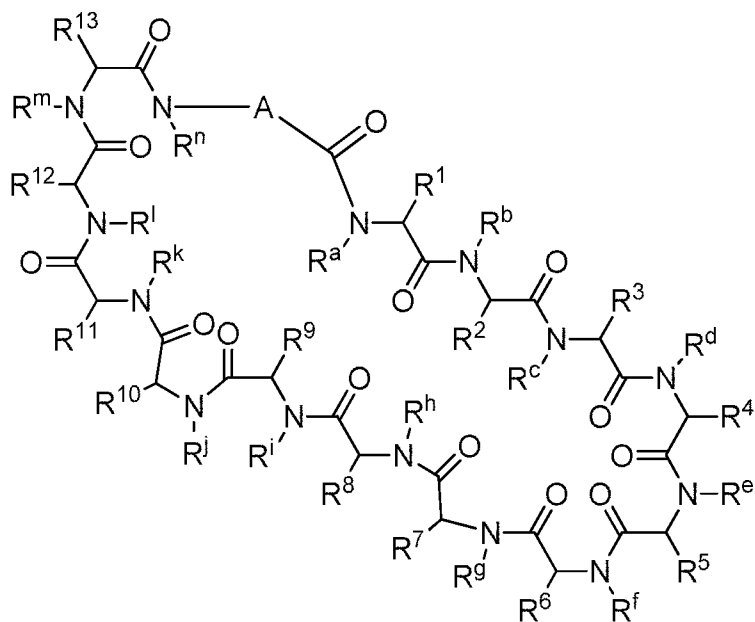
Yang J., et al.. *J Immunol.* Aug 1;187(3):1113-9 (2011)). Studies have revealed that blockade of the interactions of PD-L1 using antibodies can suppress inappropriate immune responses and ameliorate disease signs.

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)).

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.

The macrocyclic compounds 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.

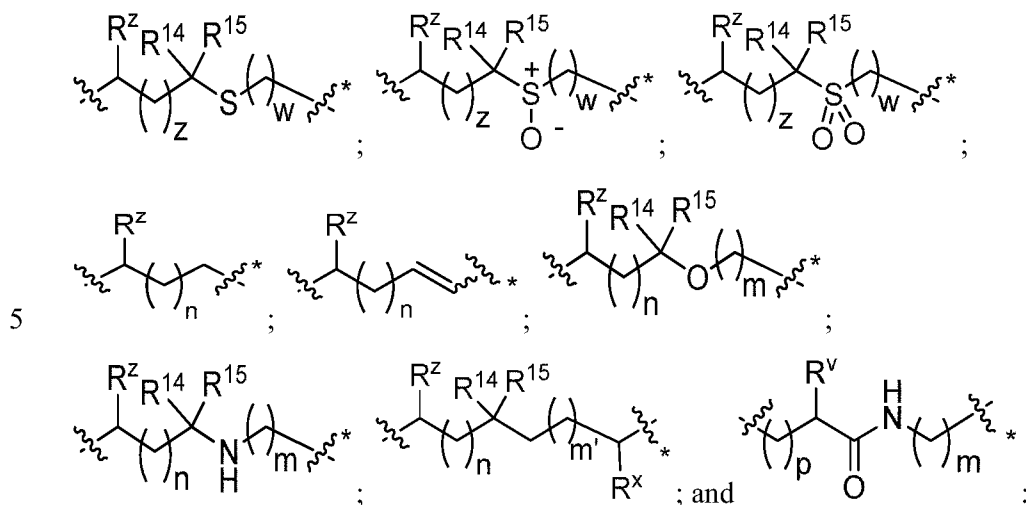
In one aspect the present disclosure provides a compound of formula (I)



(I),

or a pharmaceutically acceptable salt thereof, wherein:

A is selected from a bond,



wherein:

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

10 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;

15 p is 0, 1, or 2;

R^x is selected from hydrogen, amino, hydroxy, and methyl;R¹⁴ and R¹⁵ are independently selected from hydrogen and methyl; and

R^z is selected from hydrogen and -C(O)NHR¹⁶; wherein R¹⁶ is selected from hydrogen, -CHR¹⁷C(O)NH₂, -CHR¹⁷C(O)NHCHR¹⁸C(O)NH₂, and

20 -CHR¹⁷C(O)NHCHR¹⁸C(O)NHCH₂C(O)NH₂; wherein R¹⁷ is selected from hydrogen and -CH₂OH and wherein R¹⁸ is selected from hydrogen and methyl;

R^v is hydrogen or a natural amino acid side chain;R^a and R^j are each independently selected from hydrogen and methyl;R^c, R^f, R^h, Rⁱ, R^m, and Rⁿ are hydrogen;

R¹, R⁸, and R¹⁰ are independently selected from a natural amino acid side chain and an unnatural amino acid side chain;

R³ and R⁶ are independently selected from a natural amino acid side chain and an unnatural amino acid side chain, or, R³ and R⁶ together form a bridge containing
5 between 2 and 7 carbon atoms, optionally one double bond, and optionally one -C(O)NH- or -NHC(O)- group;

R⁹ and R¹³ are independently selected from a natural amino acid side chain and an unnatural amino acid side chain, or, R⁹ and R¹³ together form a bridge containing between 2 and 7 carbon atoms, optionally one double bond, and optionally
10 one -C(O)NH- or -NHC(O)- group;

R⁷ is selected from a natural amino acid side chain and an unnatural amino acid side chain; or R⁷ and R¹⁶ together form a bridge containing between 2 and 7 carbon atoms, optionally one double bond, and optionally one -C(O)NH- or -NHC(O)- group; or R⁷ and R^g can form a ring as described below;
15 provided that at least one of R³ and R⁶; R⁹ and R¹³, and R⁷ and R¹⁶ form a bridge;

R⁵ is a natural amino acid side chain or an unnatural amino acid side chain, or, R³ and R⁵ together form a bridge containing between 2 and 7 carbon atoms, optionally one double bond, and optionally one -C(O)NH- or -NHC(O)- group; or R⁵ and R^e can form a ring as described below;

20 R², R⁴, R¹¹, and R¹² are independently selected from a natural amino acid side chain and an unnatural amino acid side chain or form a ring with the corresponding vicinal R group as described below;

R^b is methyl or, R^b and R², together with the atoms to which they are attached, form a ring selected from azetidine, pyrrolidine, morpholine, piperidine,
25 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 hydrogen or methyl, or, R^d and R⁴, together with the atoms to which they are attached, can form a ring selected from azetidine, pyrrolidine, morpholine,
30 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;

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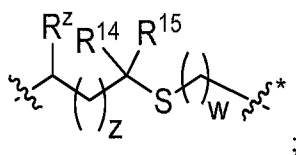
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compound of formula (I), or a pharmaceutically acceptable salt thereof, wherein A is



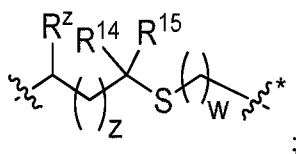
z is 0;

w is 1;

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

5 R^Z is ---C(O)NHR^{16} .

In a third embodiment of the first aspect the present disclosure provides a compound of formula (I), or a pharmaceutically acceptable salt thereof, wherein A is



z is 0;

10 w is 1;

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

R^Z is ---C(O)NHR^{16} ;

R^1 is phenyl $\text{C}_1\text{--C}_3$ alkyl wherein the phenyl is optionally substituted with hydroxy;

15 R^2 is $\text{C}_1\text{--C}_7$ alkyl;

R^3 is $\text{---CH}_2\text{C(O)NH}_2$ or R^3 and R^6 together form a bridge containing between 2 and 7 carbon atoms, optionally one double bond, and optionally one ---C(O)NH--- or ---NHC(O)--- group, or R^3 and R^5 together form a bridge containing between 2 and 7 carbon atoms, optionally one double bond, and optionally one ---C(O)NH--- or ---NHC(O)--- group,

R^4 and R^d , together with the atoms to which they are attached, form a pyrrolidine ring;

R^5 is selected from $\text{C}_1\text{--C}_7$ alkyl and $\text{---CH}_2(\text{imidazolyl})$ optionally substituted with $\text{CF}_3\text{C(O)---}$, or R^3 and R^5 together form a bridge containing between 2 and 7 carbon atoms, optionally one double bond, and optionally one ---C(O)NH--- or ---NHC(O)--- group;

R^6 is selected from C_1 - C_7 alkyl and $-(CH_2)_2C(O)NH_2$, or R^3 and R^6 together form a bridge containing between 2 and 7 carbon atoms, optionally one double bond, and optionally one $-C(O)NH-$ or $-NHC(O)-$ group;

R^7 is hydrogen or R^7 and R^8 , together with the atoms to which they are attached, form a pyrrolidine ring optionally substituted with a hydroxy group or R^7 and R^{16} together form a bridge containing between 2 and 7 carbon atoms, optionally one double bond, and optionally one $-C(O)NH-$ or $-NHC(O)-$ group;

R^8 is $-(CH_2)$ indolyl;

R^9 is selected from hydroxymethyl and $-CH_2CH_2CO_2H$ or R^9 and R^{13} together form a bridge containing between 2 and 7 carbon atoms, optionally one double bond, and optionally one $-C(O)NH-$ or $-NHC(O)-$ group;

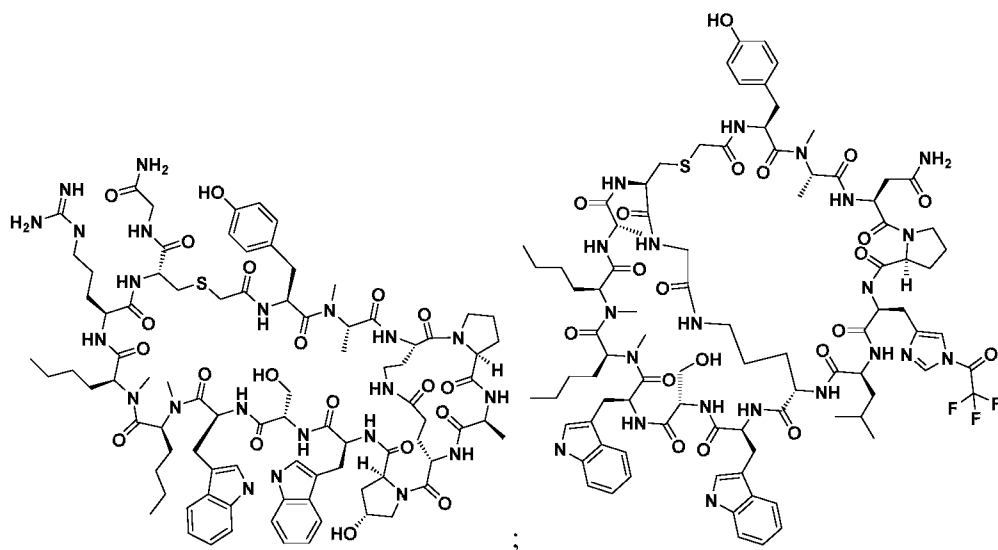
R^{10} is selected from $-(CH_2)$ indolyl and $-(CH_2)$ benzothieryl, each optionally substituted with $-CH_2CO_2H$;

R^{11} is C_1 - C_7 alkyl;

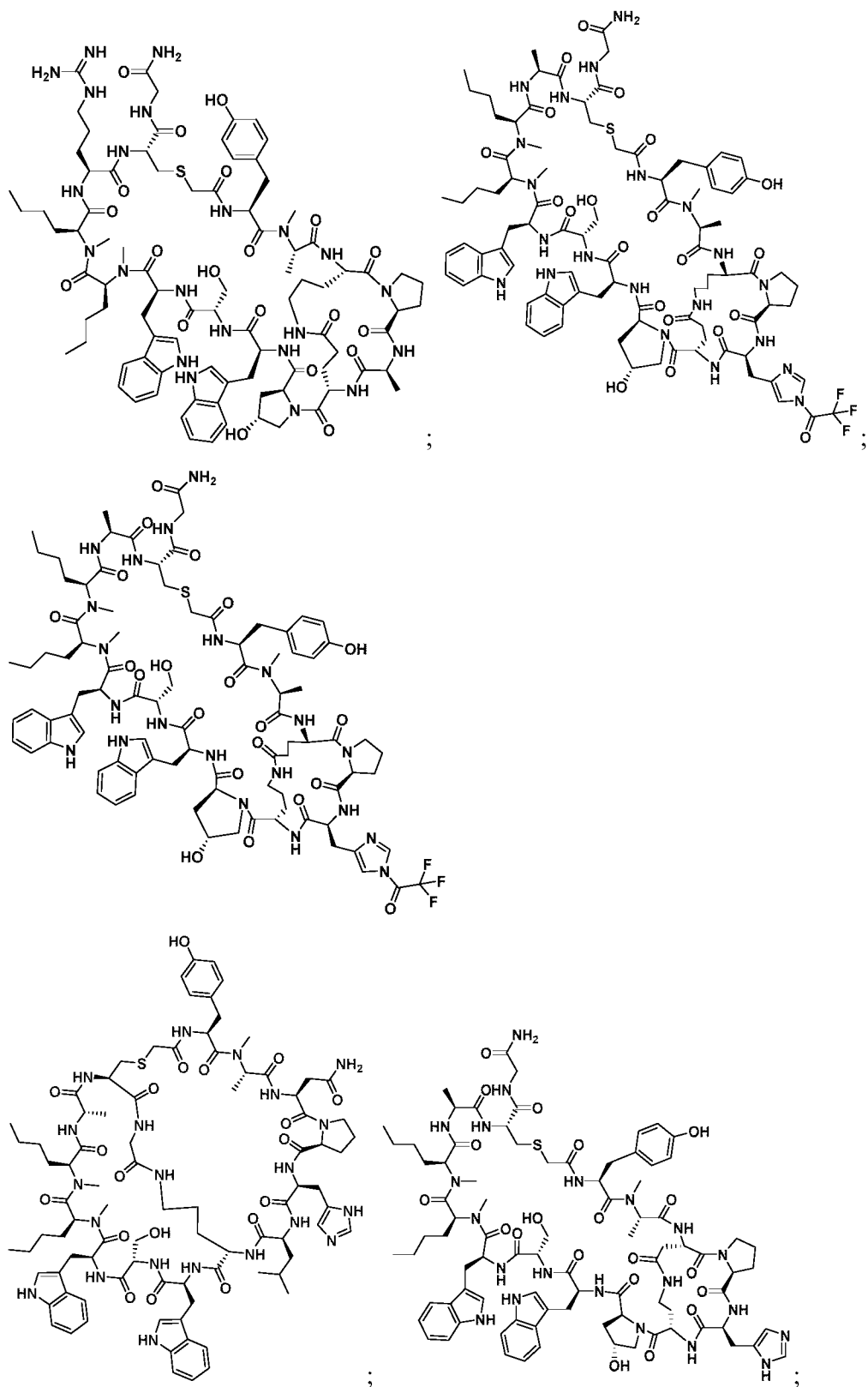
R^{12} is C_1 - C_7 alkyl; and

R^{13} is selected from C_1 - C_7 alkyl and $-(CH_2)_3NHC(NH)NH_2$ or R^9 and R^{13} together form a bridge containing between 2 and 7 carbon atoms, optionally one double bond, and optionally one $-C(O)NH-$ or $-NHC(O)-$ group.

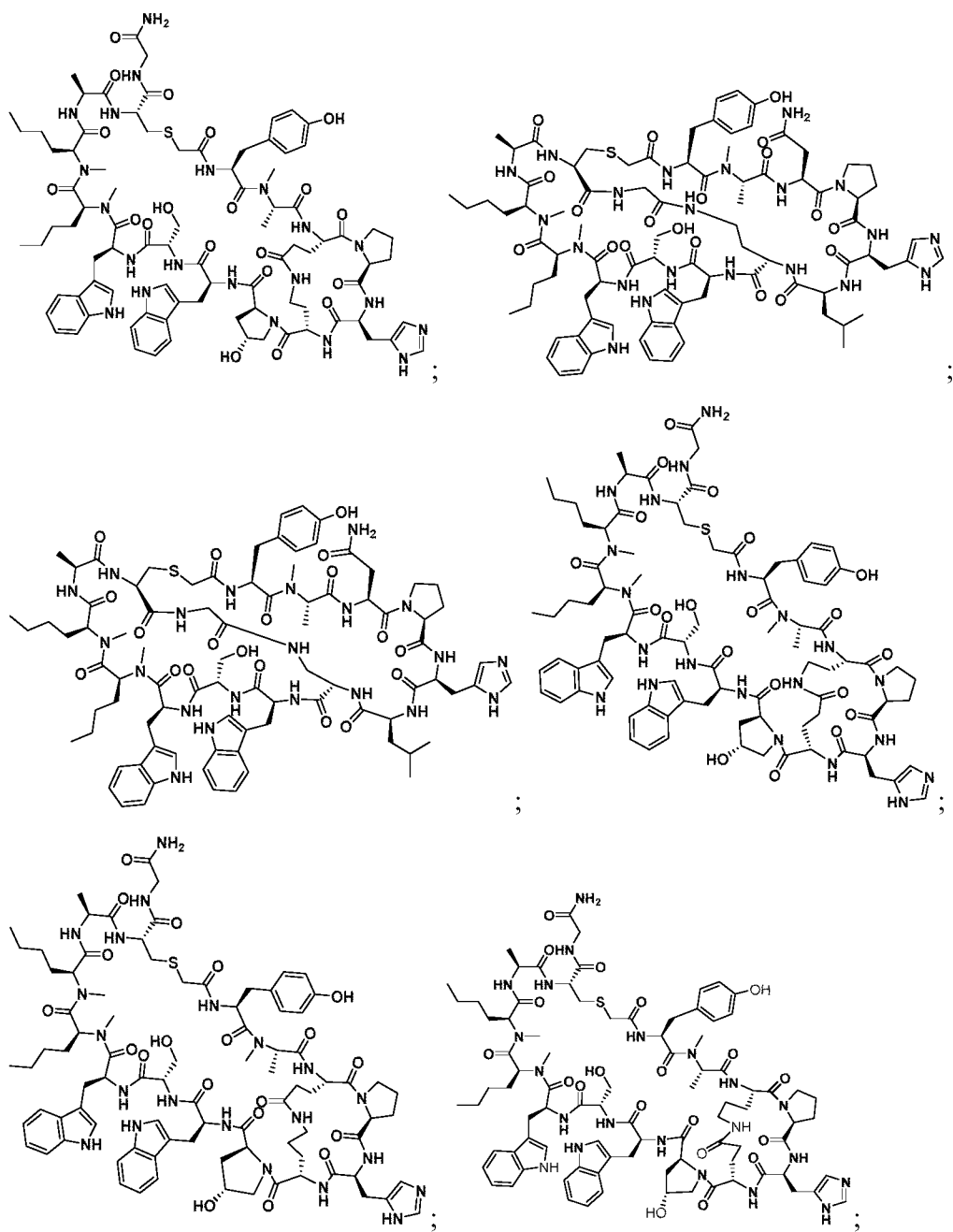
In a second aspect the present disclosure provides a compound selected from:



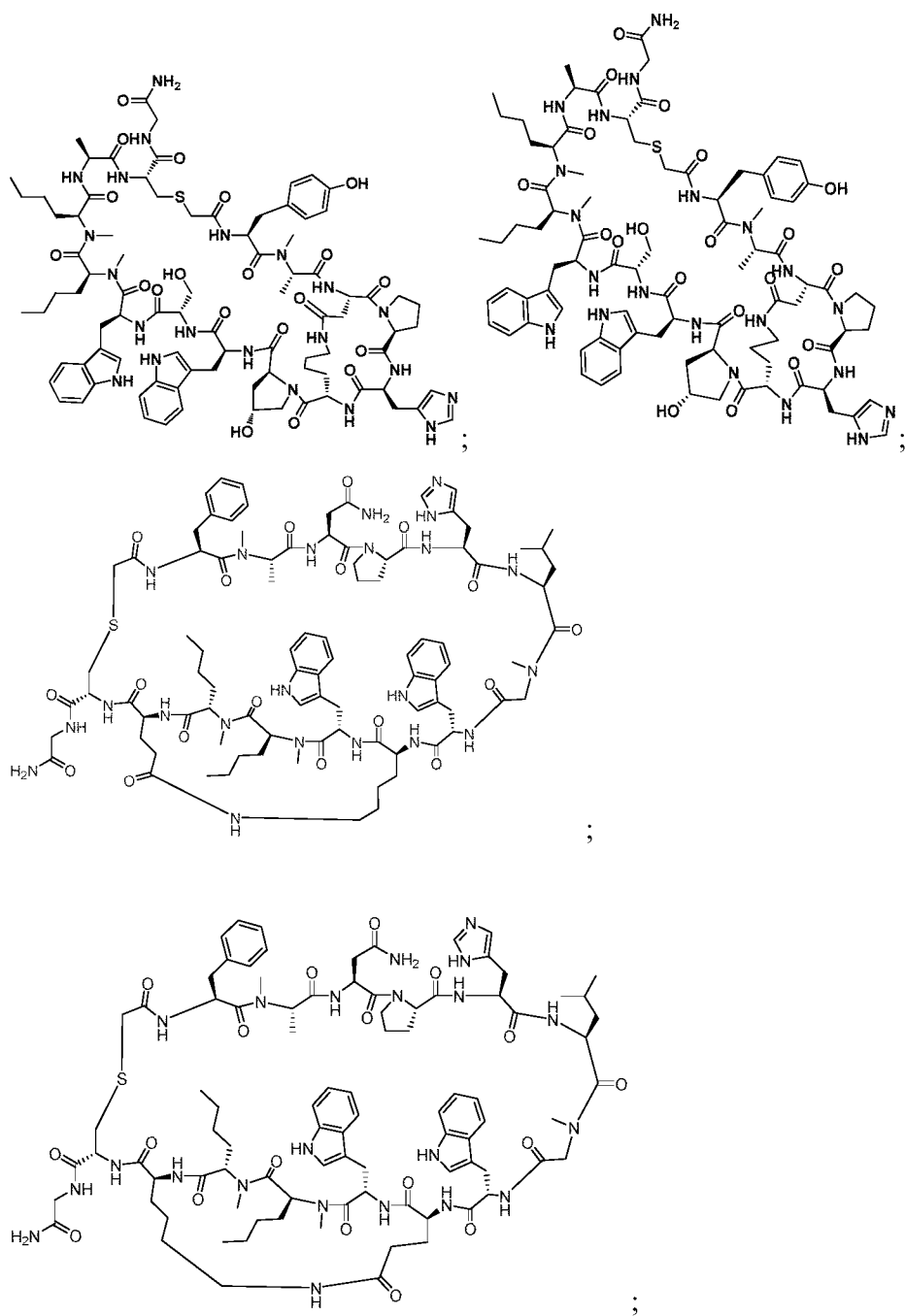
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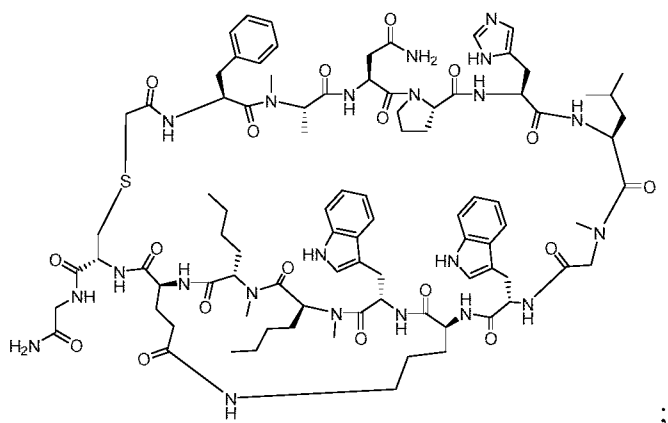
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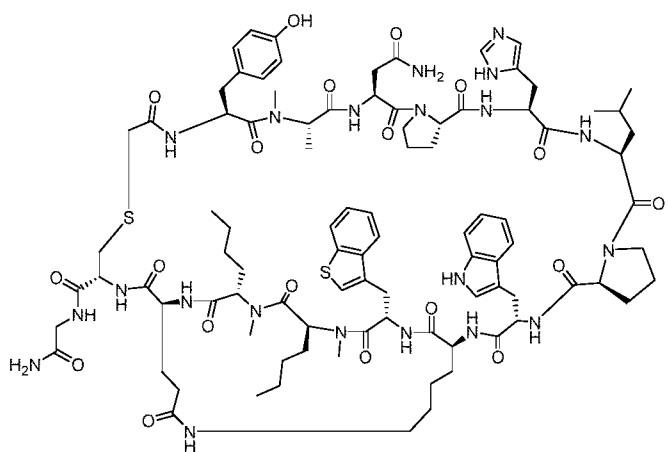
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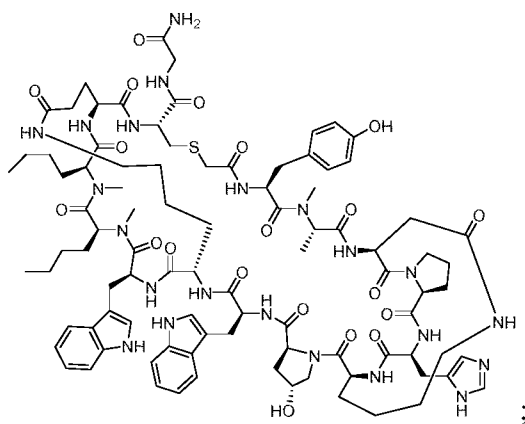
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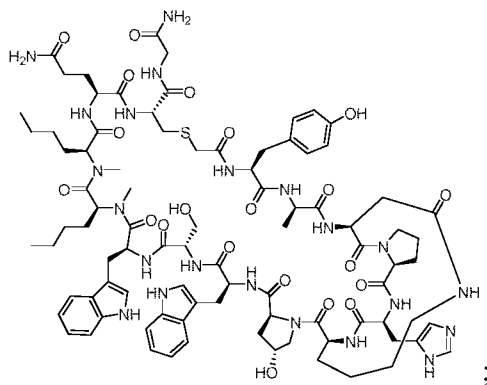
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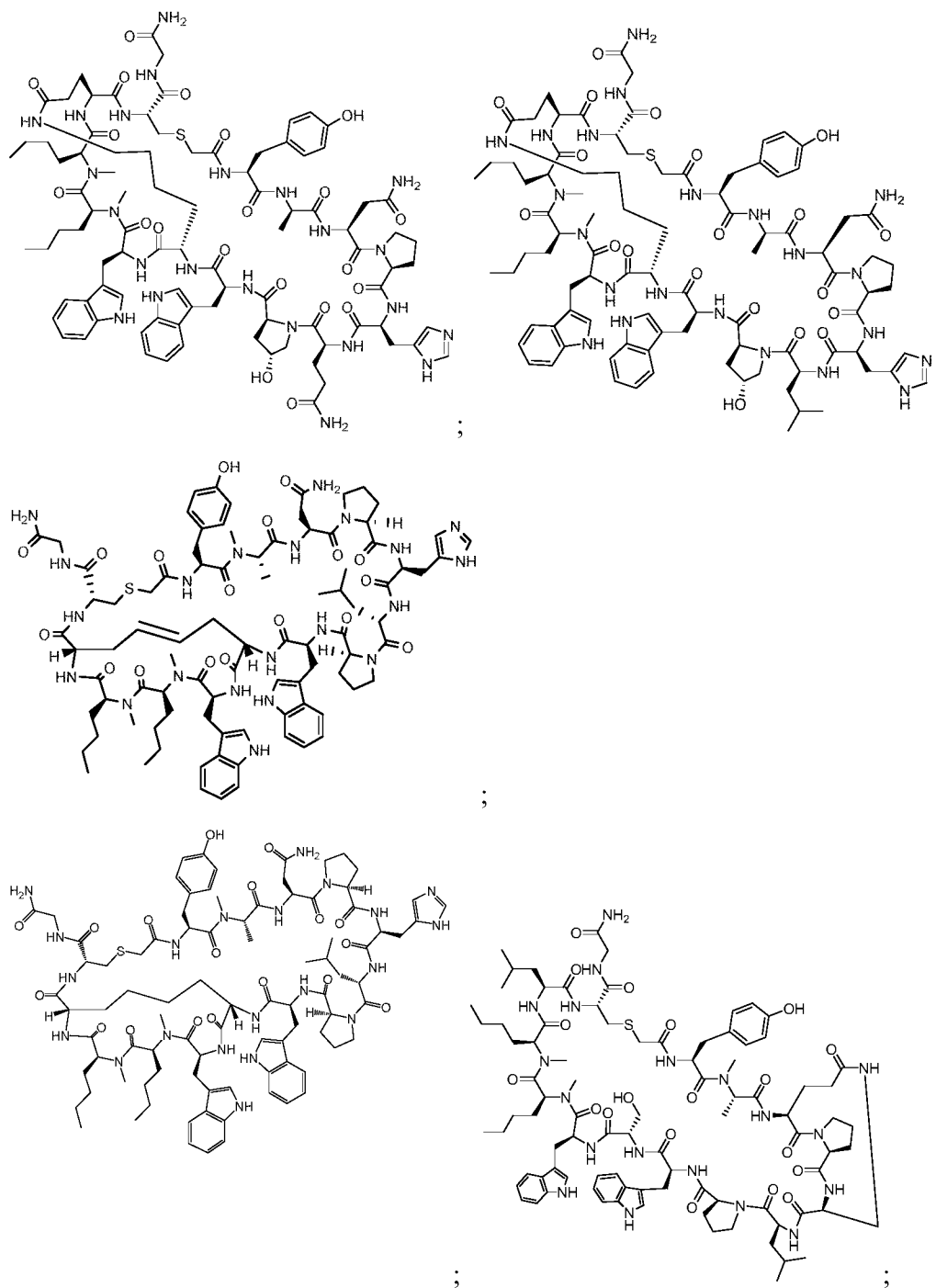


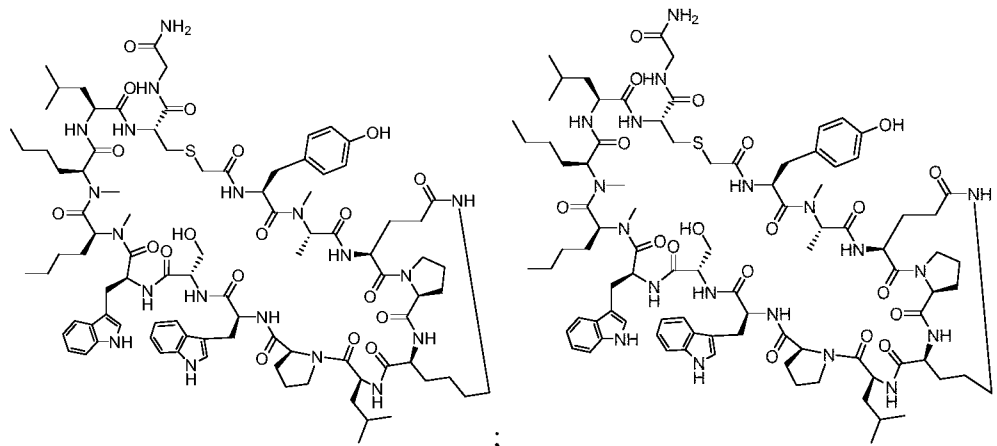
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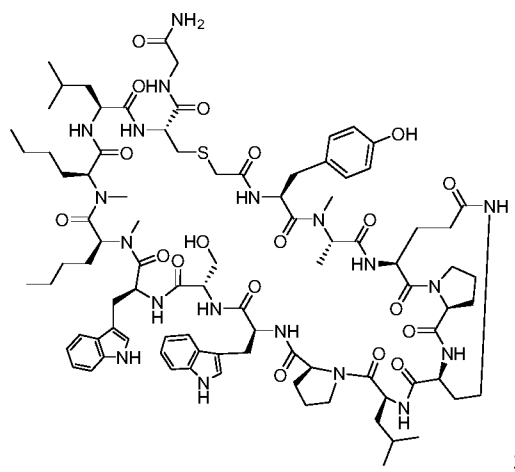
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and



or a pharmaceutically acceptable salt thereof.

- 5 In a third aspect the present disclosure provides a method of enhancing, stimulating, and/or increasing an immune response mediated by the interaction of PD-L1 with PD-1 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. In a first embodiment of the third aspect the
- 10 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 a second embodiment of the third aspect the additional agent is an antimicrobial agent, an antiviral agent, a cytotoxic agent, and/or an immune response modifier. In a third embodiment of the third aspect embodiment the additional agent
- 15 is an HDAC inhibitor. In a fourth embodiment of the third aspect the additional agent is a TLR7 and/or TLR8 agonist.

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In a fourth aspect the present disclosure provides a method of inhibiting growth, proliferation, or metastasis of cancer cells mediated by the interaction of PD-L1 with PD-1 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 a first embodiment of the fourth aspect 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 a hematological malignancy.

In a fifth aspect the present disclosure provides a method of treating an infectious disease mediated by the interaction of PD-L1 with PD-1 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 a first embodiment of the fifth aspect the infectious disease is caused by a virus. In a second embodiment of the fifth aspect the virus is selected from HIV, Hepatitis A, Hepatitis B, Hepatitis C, herpes virus, and influenza.

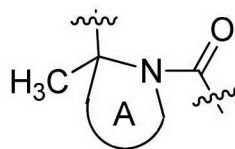
In a sixth aspect the present disclosure provides a method of treating septic shock mediated by the interaction of PD-L1 with PD-1 in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of one or more macrocyclic compounds described herein.

In a seventh aspect 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 macrocyclic compound 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. In the compounds of the present disclosure, there is at least one one carbon on the backbone of the ring that has four substituents other than hydrogen and is not an alpha-methyl-substituted ring of formula (II)

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(II)

wherein A is a ring as described in the claims and wherein \sim indicates the points of attachment to the rest of the macrocyclic structure.

- 5 In compounds of formula (I), preferred R^1 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.

In compounds of formula (I) where R^2 is not part of a ring, preferred R^2 side chains are: alanine, serine, and glycine.

- 15 In compounds of formula (I), preferred R^3 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 R^4 is not part of a ring, preferred R^4 side chains are: valine, alanine, isoleucine, and glycine.

- 20 In compounds of formula (I), preferred R^5 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 R^6 side chains are: leucine, aspartic acid, asparagine, glutamic acid, glutamine, serine, lysine, 3-cyclohexane, threonine, ornithine, 2,4-diaminobutane, alanine, arginine, and ornithine (COCH_3).

- 25 In compounds of formula (I) where R^7 is not part of a ring, preferred R^7 side chains are: glycine, 2,4-diaminobutane, serine, lysine, arginine, ornithine, histidine, asparagine, glutamine, alanine, and 2,4-diaminobutane (C(O)cyclobutane).

- 30 In compounds of formula (I) preferred R^8 side chains are tryptophan and 1,2-benzisothiazolylalanine.

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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.

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

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.

10 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.

15 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.

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

25 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 compound that specifically binds to protein binding site A will reduce the amount of labeled compound bound to the protein. In
30 contrast, nonspecific binding of a compound to the protein does not result in a concentration-dependent displacement of the labeled compound from the protein.

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The present disclosure is intended to include all isotopes of atoms occurring in the present compounds. Isotopes include those atoms having the same atomic 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-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.

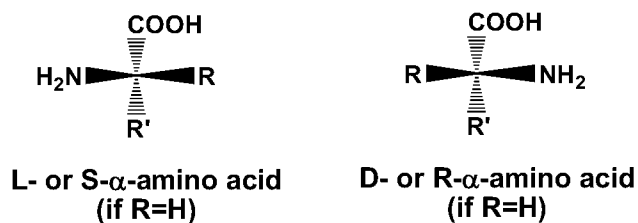
An additional aspect of the subject matter described herein is the use of the disclosed compounds as radiolabeled ligands for development of ligand binding assays or for monitoring of *in vivo* adsorption, metabolism, distribution, receptor binding or occupancy, or compound disposition. For example,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

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

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

The definitions provided herein apply, without limitation, to the terms as used 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 " α " carbon, where R and/or R' can be a natural or an un-natural side chain, including hydrogen. The absolute "S" configuration at the " α " carbon is commonly referred to as the "L" or "natural" 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, 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 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, C₁-C₃alkoxyC₁-C₃alkyl, C₁-C₆alkoxycarbonylC₁-C₃alkyl, C₁-C₇alkyl, C₁-C₃alkylsulfanylC₁-C₃alkyl, amidoC₁-C₃alkyl, aminoC₁-C₃alkyl, azaindolyC₁-C₃alkyl, benzothiazolyC₁-C₃alkyl, benzothienylC₁-C₃alkyl, benzyloxyC₁-C₃alkyl, carboxyC₁-C₃alkyl, C₃-C₁₄cycloalkylC₁-C₃alkyl, diphenylmethyl, furanylC₁-C₃alkyl, imidazolyC₁-C₃alkyl, naphthylC₁-C₃alkyl, pyridinylC₁-C₃alkyl, thiazolyC₁-C₃alkyl, thienylC₁-C₃alkyl;

biphenylC₁-C₃alkyl wherein the biphenyl is optionally substituted with a methyl group;

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heterorocyclyl optionally substituted with one, two, three, four, or five groups independently selected from C₁-C₄alkoxy, C₁-C₄alkyl, C₁-C₃alkylsulfonylamino, amido, amino, aminoC₁-C₃alkyl, aminosulfonyl, carboxy, cyano, halo, haloC₁-C₃alkyl, hydroxy, -NC(NH₂)₂, nitro, and -OP(O)(OH)₂;

5 indolylC₁-C₃alkyl, wherein the indolyl part is optionally substituted with one group selected from C₁-C₃alkyl, carboxyC₁-C₃alkyl, halo, hydroxy, and phenyl, wherein the phenyl is further optionally substituted by one, two, or three groups independently selected from C₁-C₃alkoxy, C₁-C₃alkyl, and halo;

phenyl optionally substituted with one, two, three, four, or five groups
10 independently selected from C₁-C₄alkoxy, C₁-C₄alkyl, C₁-C₃alkylsulfonylamino, amido, amino, aminoC₁-C₃alkyl, aminosulfonyl, carboxy, cyano, halo, haloC₁-C₃alkyl, hydroxy, -NC(NH₂)₂, nitro, and -OP(O)(OH)₂;

NR^{a'}R^{b'}(C₁-C₇alkyl), wherein R^{a'} and R^{b'} are independently selected from hydrogen, C₂-C₄alkenyloxycarbonyl, C₁-C₃alkyl, C₁-C₃alkylcarbonyl, C₃-
15 C₆cycloalkylcarbonyl, furanylcarbonyl, and phenylcarbonyl. When the alkyl linker contains more than one carbon an additional NR^{a'}R^{b'} group can be on the chain.

NR^{c'}R^{d'}carbonylC₁-C₃alkyl, wherein R^{c'} and R^{d'} are independently selected from hydrogen, C₁-C₃alkyl, and triphenylmethyl;

phenylC₁-C₃alkyl wherein the phenyl part is optionally substituted with one,
20 two, three, four, or five groups independently selected from C₁-C₄alkoxy, C₁-C₄alkyl, C₁-C₃alkylsulfonylamino, amido, amino, aminoC₁-C₃alkyl, aminosulfonyl, carboxy, cyano, halo, haloC₁-C₃alkyl, hydroxy, -NC(NH₂)₂, nitro, and -OP(O)(OH)₂; and

phenoxyC₁-C₃alkyl wherein the phenyl is optionally substituted with a C₁-C₃alkyl group.

25 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.

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
30 bond.

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

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The term "C₁-C₃alkoxy," as used herein, refers to a C₁-C₃alkyl group attached to the parent molecular moiety through an oxygen atom.

The term "C₁-C₄alkoxy," as used herein, refers to a C₁-C₄alkyl group attached to the parent molecular moiety through an oxygen atom.

5 The term "C₁-C₆alkoxy," as used herein, refers to a C₁-C₆alkyl group attached to the parent molecular moiety through an oxygen atom.

The term "C₁-C₃alkoxyC₁-C₃alkyl," as used herein, refers to a C₁-C₃alkoxy group attached to the parent molecular moiety through a C₁-C₃alkyl group.

10 The term "C₁-C₆alkoxycarbonyl," as used herein, refers to a C₁-C₆alkoxy group attached to the parent molecular moiety through a carbonyl group.

The term "C₁-C₆alkoxycarbonylC₁-C₃alkyl," as used herein, refers to a C₁-C₆alkoxycarbonyl group attached to the parent molecular moiety through a C₁-C₃alkyl group.

15 The term "C₁-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 "C₁-C₄alkyl," as used herein, refers to a group derived from a straight or branched chain saturated hydrocarbon containing from one to four carbon atoms.

20 The term "C₁-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 "C₁-C₃alkylamino," as used herein, refers to -NHR¹ wherein R¹ is a C₁-C₃alkyl group.

25 The term "C₁-C₃alkylcarbonyl," as used herein, refers to a C₁-C₃alkyl group attached to the parent molecular moiety through a carbonyl group.

The term "C₁-C₃alkylsulfanyl," as used herein, refers to a C₁-C₃alkyl group attached to the parent molecular moiety through a sulfur atom.

30 The term "C₁-C₃alkylsulfanylC₁-C₃alkyl," as used herein, refers to a C₁-C₃alkylsulfanyl group attached to the parent molecular moiety through a C₁-C₃alkyl group.

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The term “C₁-C₃alkylsulfonyl,” as used herein, refers to a C₁-C₃alkyl group attached to the parent molecular moiety through a sulfonyl group.

The term “C₁-C₃alkylsulfonylamino,” as used herein, refers to a C₁-C₃alkylsulfonyl group attached to the parent molecular moiety through an amino group.

The term “amido,” as used herein, refers to -C(O)NH₂.

The term “amidoC₁-C₃alkyl,” as used herein, refers to an amido group attached to the parent molecular moiety through a C₁-C₃alkyl group.

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

The term “aminoC₁-C₃alkyl,” as used herein, refers to an amino group attached to the parent molecular moiety through a C₁-C₃alkyl group.

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

The term “azaindolylC₁-C₃alkyl,” as used herein, refers to an azaindolyl group attached to the parent molecular through a C₁-C₃alkyl group. The azaindolyl group can be attached to the alkyl moiety through any substitutable atom in the group.

The term “benzothiazolylC₁-C₃alkyl,” as used herein, refers to an benzothiazolyl group attached to the parent molecular through a C₁-C₃alkyl group.

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

The term “benzothienylC₁-C₃alkyl,” as used herein, refers to a benzothienyl group attached to the parent molecular through a C₁-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 “benzyloxyC₁-C₃alkyl,” as used herein, refers to a benzyloxy group attached to the parent molecular moiety through a C₁-C₃alkyl group.

The term “biphenylC₁-C₃alkyl,” as used herein, refers to a biphenyl group attached to the parent molecular moiety through a C₁-C₃alkyl group. The biphenyl

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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)- .

The term “carboxy,” as used herein, refers to $\text{-CO}_2\text{H}$.

5 The term “carboxy $\text{C}_1\text{-C}_3$ alkyl,” as used herein, refers to a carboxy group attached to the parent molecular moiety through a $\text{C}_1\text{-C}_3$ alkyl group.

The term “cyano,” as used herein, refers to -CN .

The term “ $\text{C}_3\text{-C}_{14}$ cycloalkyl,” as used herein, refers to a saturated monocyclic, bicyclic, or tricyclic hydrocarbon ring system having three to fourteen carbon atoms
10 and zero heteroatoms. The bicyclic and tricyclic rings may be fused, spirocyclic, or bridged. Representative examples of cycloalkyl groups include, but are not limited to, cyclopropyl, cyclopentyl, bicyclo[3.1.1]heptyl, and adamantyl.

The term “ $\text{C}_3\text{-C}_{14}$ cycloalkyl $\text{C}_1\text{-C}_3$ alkyl,” as used herein, refers to a $\text{C}_3\text{-C}_{14}$ cycloalkyl group attached to the parent molecular moiety through a $\text{C}_1\text{-C}_3$ alkyl
15 group.

The term “ $\text{C}_3\text{-C}_{14}$ cycloalkylcarbonyl,” as used herein, refers to a $\text{C}_3\text{-C}_{14}$ cycloalkyl group attached to the parent molecular moiety through a carbonyl group.

The term “ $\text{C}_1\text{-C}_3$ dialkylamino,” as used herein, refers to $\text{-NR}^1\text{R}^2$, wherein R^1 and R^2 are each alkyl groups containing one to three carbon atoms. The groups may
20 be the same or different.

The term “furanyl $\text{C}_1\text{-C}_3$ alkyl,” as used herein, refers to a furanyl group attached to the parent molecular moiety through a $\text{C}_1\text{-C}_3$ alkyl group. The furanyl group can be attached to the alkyl moiety through any substitutable atom in the group.

25 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 “halo $\text{C}_1\text{-C}_3$ alkyl,” as used herein, refers to a $\text{C}_1\text{-C}_3$ alkyl group substituted with one, two, or three halogen atoms.

30 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, indolyl, isothiazolyl, isoxazolyl, morpholinyl, oxazolyl, piperazinyl, piperidinyl, pyrazolyl, pyridinyl, pyrrolidinyl, pyrrolopyridinyl, pyrrolyl, thiazolyl, thienyl, and thiomorpholinyl.

The term “hydroxy,” as used herein, refers to -OH .

The term “imidazolyl $\text{C}_1\text{-C}_3$ alkyl,” as used herein, refers to an imidazolyl group attached to the parent molecular moiety through a $\text{C}_1\text{-C}_3$ alkyl group. The imidazolyl group can be attached to the alkyl moiety through any substitutable atom in the group.

The term “indolyl $\text{C}_1\text{-C}_3$ alkyl,” as used herein, refers to an indolyl group attached to the parent molecular moiety through a $\text{C}_1\text{-C}_3$ alkyl group. The indolyl group can be attached to the alkyl moiety through any substitutable atom in the group.

The term “naphthyl $\text{C}_1\text{-C}_3$ alkyl,” as used herein, refers to a naphthyl group attached to the parent molecular moiety through a $\text{C}_1\text{-C}_3$ alkyl 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 -NO_2 .

The term “ $\text{NR}^{\text{a}}\text{R}^{\text{b}}$,” as used herein, refers to two groups, R^{a} and R^{b} , which are attached to the parent molecular moiety through a nitrogen atom. R^{a} and R^{b} are independently selected from hydrogen, $\text{C}_2\text{-C}_4$ alkenyloxycarbonyl, $\text{C}_1\text{-C}_3$ alkylcarbonyl, $\text{C}_3\text{-C}_6$ cycloalkylcarbonyl, furanylcabonyl, and phenylcarbonyl.

The term “ $\text{NR}^{\text{a}}\text{R}^{\text{b}}(\text{C}_1\text{-C}_3)\text{alkyl}$,” as used herein, refers to an $\text{NR}^{\text{a}}\text{R}^{\text{b}}$ group attached to the parent molecular moiety through a $\text{C}_1\text{-C}_3$ alkyl group.

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The term “NR^cR^d,” as used herein, refers to two groups, R^c and R^d, which are attached to the parent molecular moiety through a nitrogen atom. R^c and R^d are independently selected from hydrogen, C₁-C₃alkyl, and triphenylmethyl.

5 The term “NR^cR^dcarbonyl,” as used herein, refers to an NR^cR^d group attached to the parent molecular moiety through a carbonyl group.

The term “NR^cR^dcarbonylC₁-C₃alkyl,” as used herein, refers to an NR^cR^dcarbonyl group attached to the parent molecular moiety through a C₁-C₃alkyl group.

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

The term “phenoxyC₁-C₃alkyl,” as used herein, refers to a phenoxy group attached to the parent molecular moiety through a C₁-C₃alkyl group.

The term “phenylC₁-C₃alkyl,” as used herein, refers to a phenyl group attached to the parent molecular moiety through a C₁-C₃alkyl group.

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

The term “pyridinylC₁-C₃alkyl,” as used herein, refers to a pyridinyl group attached to the parent molecular moiety through a C₁-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-.

20 The term “thiazolylC₁-C₃alkyl,” as used herein, refers to a thiazolyl group attached to the parent molecular moiety through a C₁-C₃alkyl group. The thiazolyl group can be attached to the alkyl moiety through any substitutable atom in the group.

25 The term “thienylC₁-C₃alkyl,” as used herein, refers to a thienyl group attached to the parent molecular moiety through a C₁-C₃alkyl group. The thienyl group can be attached to the alkyl moiety through any substitutable atom in the group.

30 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

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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.

5 Binding of the macrocyclic compounds 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 compounds to PD-L1 expressed on the surface of cells can be measured
10 as described herein in cellular binding assays.

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
15 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 effective amount for a subject will depend upon the subject's size and health, the
20 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 compounds of the present disclosure.
25 As demonstrated herein, the macrocyclic compounds 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 IFN γ secretion, and enhancement of HIV-specific T cell IFN γ secretion. As a result, the
30 macrocyclic compounds 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 compounds with an antigen of interest).

In order that the present disclosure may be more readily understood, certain 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-LI", "CD274" and "B7-H1" are used interchangeably, and include variants, isoforms, species 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 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, 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 macromolecules produced by the above cells or the liver (including macrocycles, 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.

An "adverse event" (AE) as used herein is any unfavorable and generally unintended, even undesirable, sign (including an abnormal laboratory finding),

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5 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 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.

10 As used herein, "hyperproliferative disease" refers to conditions wherein cell 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).

15 As used herein, "about" or "comprising essentially of" mean within an 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 range of up to 20%. Furthermore, particularly with respect to biological systems or
20 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.

25 The term "comprising" as used in this specification and claims means "consisting at least in part of". When interpreting statements in this specification, and claims which include the term "comprising", it is to be understood that other features that are additional to the features prefaced by this term in each statement or claim may also be present. Related terms such as "comprise" and "comprised" are to be interpreted in similar manner.

30 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

5 The present disclosure is also directed to macrocyclic compounds 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 compounds may share structural
10 homology with one or more macrocyclic compounds disclosed herein, including mutant, conservative substitution, functional substitution, and deletion forms, provided they specific bind to PD-L1. For example, if a macrocyclic compound binds substantially to the same region of PD-L1 as a reference anti-PD-L1 antibody, the macrocyclic compound should bind to an epitope of PD-L1 that at least overlaps
15 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 compound 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
20 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-1105 (BMS); L01X-C (Serono), L1X3 (Serono), MSB-0010718C (Serono), and PD-L1 Probody (CytomX),
25 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, 4A11, 7D3 and 5F4 each disclosed in co-owned U.S. Patent No. 8,008,449
30 (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.

Pharmaceutical Compositions

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

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 compound 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 uses of the macrocyclic compounds 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, parenteral, spinal or epidermal administration (*e.g.*, by injection or infusion). Depending on the route of administration, the active compound, *i.e.*, a macrocyclic compound, 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.

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,

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phosphoric, sulfuric, hydrobromic, hydroiodic, phosphorous and the like, as well as from nontoxic organic acids such as aliphatic mono- and dicarboxylic acids, phenyl-substituted alkanolic acids, hydroxy alkanolic acids, aromatic acids, aliphatic and aromatic sulfonic acids and the like. Base addition salts include those derived from
5 alkaline earth metals, such as sodium, potassium, magnesium, calcium and the like, 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
10 pharmaceutically acceptable anti-oxidant. Examples of pharmaceutically acceptable antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol,
15 and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine 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
20 mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such 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
25 agents, emulsifying agents and dispersing agents. Prevention of presence of 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.
30 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.

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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 the pharmaceutical compositions of the disclosure is contemplated. Supplementary active compounds can also be incorporated into the compositions.

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 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.

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 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

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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 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 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 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 individuals.

For administration of the macrocyclic compound, 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 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 compound 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

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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 compounds with different binding specificities are administered simultaneously, in which case the dosage of each compound administered falls within the ranges indicated. The compounds are 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 compound 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.

Alternatively, the macrocyclic compound 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 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.

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 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,

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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 compound 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 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 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, the severity of the subject's symptoms, and the particular composition or route of administration selected.

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

25 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 will vary depending upon the desired results. Preferred routes of administration for macrocyclic compounds 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

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topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal
5 injection and infusion.

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

10 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.

Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid.

15 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).

Therapeutic compositions can be administered with medical devices known in the art. For example, in a preferred embodiment, a therapeutic composition of the
20 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 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
25 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 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
30 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

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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 compounds of the disclosure can be 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 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)); macrocycles (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)); p120 (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).

Uses and Methods of the Disclosure

The macrocyclic compounds, 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 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 describes a method of modifying an immune response in a subject comprising administering to the subject the macrocyclic compound 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 compound 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,

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chickens, woodchuck, amphibians, and reptiles, although mammals are preferred, 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 vivo*. To achieve antigen-specific enhancement of immunity, the macrocyclic compounds can be administered together with an antigen of interest. When macrocyclic compounds to PD-L1 are administered together with another agent, the two can be administered in either order or simultaneously.

The disclosure further describes methods for detecting the presence of human, 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 compound 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, 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 compounds of the disclosure for PD-L1, compared to CD28, ICOS and CTLA-4, the macrocyclic compounds 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.

25

Cancer

Blockade of PD-1 by macrocyclic compounds 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

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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 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 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 compound such that growth of cancerous tumors is inhibited. A macrocyclic compound may be used alone to inhibit the growth of cancerous tumors. Alternatively, a macrocyclic compound may be used in conjunction with other immunogenic agents, standard cancer treatments, or other macrocyclic compounds, as described below.

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

Preferred cancers whose growth may be inhibited using the macrocyclic compounds 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), 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 compounds of the disclosure.

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

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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 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 cancers including those induced by asbestos, and combinations of said cancers. The 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 compounds to PD-L1 can be combined with an immunogenic agent, such as cancerous cells, purified tumor antigens (including recombinant proteins, compounds, 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, 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 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.,

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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 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 (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 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 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*, 266:2011-2013 (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.

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 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*, 278:117-120 (1997)).

Dendritic cells (DC) are potent antigen presenting cells that can be used to prime antigen-specific responses. DC's 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 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 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 compound in combination with decarbazine for the treatment of melanoma. Another example of such a combination is a macrocyclic compound in combination with interleukin-2 (IL-2) for the treatment of melanoma. The 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 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 compounds can also be used in combination with bispecific macrocycles 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 macrocycles can be used to target two separate antigens. For example anti-Fc receptor/anti tumor antigen (*e.g.*, Her-2/neu) bispecific macrocycles have been used to target macrophages to sites of tumor. This targeting may more

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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 delivered directly to DCs by the use of bispecific macrocycles which bind to tumor antigen and a dendritic cell specific cell surface marker.

5 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 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
10 (Hahne, M. et al., *Science*, 274:1363-1365 (1996)). Macrocyclic compounds 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.

Other macrocyclic compounds which may be used to activate host immune
15 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 compounds are able to substitute effectively for T cell helper activity (Ridge, J. et al., *Nature*, 393:474-478 (1998)) and can be used in conjunction with PD-1 antibodies (Ito, N. et al., *Immunobiology*, 201(5):527-
20 540 (2000)). Activating macrocyclic compounds 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 increased levels of T cell activation.

25 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.

30 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.

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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 compounds may be expected to increase the frequency and activity of the adoptively transferred T cells.

5

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 disclosure describes a method of treating an infectious disease in a subject comprising administering to the subject a macrocyclic compound 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 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., *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, thus provoking a strong T cell response that is not dampened 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, flaviviruses, echovirus, rhinovirus, coxsackie virus, cornovirus, respiratory syncytial virus, mumps virus, rotavirus, measles virus, rubella virus, parvovirus, vaccinia virus, HTLV virus, dengue virus, papillomavirus, molluscum virus, poliovirus, rabies virus, JC virus and arboviral encephalitis virus.

Some examples of pathogenic bacteria causing infections treatable by methods of the disclosure include chlamydia, rickettsial bacteria, mycobacteria, staphylococci, streptococci, pneumonococci, meningococci and conococci, klebsiella, proteus, serratia, pseudomonas, legionella, diphtheria, salmonella, bacilli, cholera, 5 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*, *krusei*, *glabrata*, *tropicalis*, etc.), *Cryptococcus neoformans*, *Aspergillus* (*fumigatus*, *niger*, etc.), Genus *Mucorales* (*mucor*, *absidia*, *rhizopus*), *Sporothrix schenckii*, *Blastomyces dermatitidis*, 10 *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*, *Naegleria fowleri*, *Acanthamoeba* sp., *Giardia lamblia*, *Cryptosporidium* sp., *Pneumocystis carinii*, *Plasmodium vivax*, *Babesia microti*, *Trypanosoma brucei*, 15 *Trypanosoma cruzi*, *Leishmania donovani*, *Toxoplasma gondi*, and *Nippostrongylus brasiliensis*.

In all of the above methods, PD-L1 blockade can be combined with other 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 20 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*, 2:1121-1123 (1994)).

Autoimmune Reactions

25 The macrocyclic compounds may provoke and amplify autoimmune responses. Indeed, induction of anti-tumor responses using tumor cell and peptide vaccines 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 30 evoked by TRAMP tumor cell vaccines (Hurwitz, A., *supra* (2000)), melanoma

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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 with various self proteins in order to devise vaccination protocols to efficiently
 5 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)).

Other self proteins may also be used as targets such as IgE for the treatment
 10 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 soluble factors that are required for the growth of particular tumors may also be
 15 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 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
 20 IgE.

Vaccines

The macrocyclic compounds may be used to stimulate antigen-specific
 immune responses by coadministration of an anti-PD-1 macrocycle with an antigen of interest (*e.g.*, a vaccine). Accordingly, in another aspect the disclosure describes a
 25 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 tumor antigen, a viral antigen, a bacterial antigen or an antigen from a pathogen. Non-limiting examples of such antigens include those discussed in
 30 the sections above, such as the tumor antigens (or tumor vaccines) discussed above, or antigens from the viruses, bacteria or other pathogens described above.

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Suitable routes of administering the compositions (*e.g.*, macrocyclic compounds, 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
5 (*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 compounds of the disclosure can be co-administered with one or other more therapeutic agents, *e.g.*, a cytotoxic agent, a
10 radiotoxic agent or an immunosuppressive agent. The compound can be linked to the agent (as an immunocomplex) or can be administered separate from the agent. In the latter case (separate administration), the compound 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
15 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
20 every 21 days. Co-administration of the macrocyclic compounds 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
25 unreactive with the compounds.

Also within the scope of the present disclosure are kits comprising the compositions of the disclosure (*e.g.*, macrocyclic compounds, 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
30 compounds 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.

Combination Therapy

5 The combination of the macrocyclic compounds 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 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.

10 Accordingly, in one aspect, the disclosure describes a method of modifying an immune response in a subject comprising administering to the subject a macrocyclic compound of the disclosure such that the immune response in the subject is modified. Preferably, the response is enhanced, stimulated or up-regulated. In another

15 embodiment, the instant disclosure describes a method of altering adverse events associated with treatment of a hyperproliferative disease with an immunostimulatory therapeutic agent, comprising administering a macrocyclic compound of the present disclosure and a subtherapeutic dose of another immunomodulator to a subject.

 Blockade of PD-L1 by macrocyclic compounds can enhance the immune response to cancerous cells in the patient. Cancers whose growth may be inhibited

20 using the macrocyclic compounds 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 endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the

30 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,

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5 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 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 present disclosure is also useful for treatment of metastatic cancers.

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

30 Optionally, the combination of the macrocyclic compound and a second immunomodulator can be further combined with an immunogenic agent, such as cancerous cells, purified tumor antigens (including recombinant proteins, peptides,

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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, MAGE antigens, Trp-2, MART1 and/or

5 tyrosinase, or tumor cells transfected to express the cytokine GM-CSF (discussed further below).

A combined PD-L1 macrocyclic compound 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

10 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 DeVita et al., eds., *Cancer: Principles and Practice of Oncology*, Fifth Edition

15 (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 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)).

20 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, *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,

25 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. In certain embodiments, a combined PD-L1 macrocyclic compound 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

30 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

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85% of human cancers and in only a limited number of somatic tissues (Kim et al., *Science*, 266:2011-2013 (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.

Other tumor vaccines may include the proteins from viruses implicated in human cancers such as 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 compound 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 et al., *Science*, 269:1585-1588 (1995); Tamura et al., *Science*, 278:117-120 (1997)).

Dendritic cells (DC) are potent antigen presenting cells that can be used to prime antigen-specific responses. DC's 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 method of vaccination, DC immunization may be effectively further combined with a combined anti-PD-L1 macrocyclic compound and a second immunomodulator to activate more potent anti-tumor responses.

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

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combination is a combination of a macrocyclic compound and a second immunomodulator further in combination with decarbazine for the treatment of melanoma. Another example is a combination of a macrocyclic compound 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 compound 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 compound 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 macrocycles 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 macrocycles can be used to target two separate antigens. For example anti-Fc receptor/anti tumor antigen (*e.g.*, Her-2/neu) bispecific macrocycles 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 macrocycles which bind to tumor antigen and a dendritic cell specific cell surface marker.

In another example, a combination of a macrocyclic compound 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 (eptuzumab), AVASTIN® (bevacizumab), and TARCEVA® (erlotinib), and the like. By way of example and not wishing to be bound by theory,

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5 treatment with an anti-cancer antibody or an anti-cancer antibody conjugated to a 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 compound and a second immunomodulator concurrently or sequentially or any combination thereof, which may potentiate an anti-tumor immune responses by the host.

10 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, among others, TGF- β . (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 compound and another immunomodulator to counteract the effects of immunosuppressive agents and 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 compound of the present disclosure. 20 These include molecules on the surface of dendritic cells that 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 conjunction with the macrocyclic compounds 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 compounds to T cell costimulatory molecules, such as 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 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.

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A macrocyclic compound of the present disclosure, either alone or in combination with 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 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 compound 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.

In certain embodiments, the present disclosure describes a method for altering an adverse event associated with treatment of a hyperproliferative disease with an immunostimulatory agent, comprising administering a macrocyclic compound of the present disclosure in combination with a subtherapeutic dose of another immunomodulator to a subject. For example, the methods of the present disclosure describes 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 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.

In further embodiments, a macrocyclic compound 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

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steroid is budesonide. Budesonide is a locally-acting glucocorticosteroid, which is 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: sulfasalazine (AZULFIDINE®, Pharmacia & Upjohn); olsalazine (DIPENTUM®, Pharmacia & Upjohn); balsalazide (COLAZAL®, Salix Pharmaceuticals, Inc.); and mesalamine (ASACOL®, Procter & Gamble Pharmaceuticals; PENTASA®, Shire US; CANASA®, Axcan Scandipharm, Inc.; ROWASA®, Solvay).

Dosage and Formulation

A suitable compound of Formula I, or more specifically a macrocyclic compound 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

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to mammals, including humans, in need of such treatment. The route of administration 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); *Remington's Pharmaceutical Sciences*, 18th Edition, Mack Publishing Co., Easton, PA (1990)).

The pharmaceutically acceptable compound 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, 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. 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 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 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

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0.001ng to 100.0 ng per min/per Kg of body weight during a constant rate infusion. 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 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 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 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 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 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,

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magnesium stearate, sodium benzoate, sodium acetate, and sodium chloride. Disintegrants include, but are not limited to, starch, methyl cellulose, agar, bentonite, and xanthan gum.

5 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 from a variety of phospholipids, such as cholesterol, stearylamine, or phosphatidylcholines. Permeation enhancers may be added to enhance drug absorption.

10 Since prodrugs are known to enhance numerous desirable qualities of pharmaceuticals (*i.e.*, solubility, bioavailability, manufacturing, etc.) the compounds 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.

15 The compositions described herein may also be coupled with soluble polymers as targetable drug carriers. Such polymers can include polyvinylpyrrolidone, 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 polylactic and polyglycolic acid, polyepsilon caprolactone, polyhydroxy butyric acid, polyorthoesters, polyacetals, polydihydropyrans, polycyanoacylates, and crosslinked or amphipathic block copolymers of hydrogels.

20 Dosage forms (pharmaceutical compositions) suitable for administration may contain from about 0.01 milligram to about 500 milligrams of active ingredient per 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.

25 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

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can be manufactured as sustained release products to provide for continuous release 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.

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

 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
10 preferably contains a water-soluble salt of the active ingredient, suitable stabilizing agents, and if necessary, buffer substances. Antioxidizing agents such as sodium 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
15 chloride, methyl- or propyl-paraben, and chlorobutanol.

 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
20 compounds described herein can be illustrated as follows:

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
25 milligrams of lactose, 50 milligrams of cellulose, and 6 milligrams magnesium stearate.

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
30 displacement pump into gelatin to form soft gelatin capsules containing 100 milligrams of the active ingredient. The capsules should be washed and dried.

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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 compound 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

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

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incorporated, and removing the solvent from the matrix thereby forming the matrix of the polymer with the active agent distributed throughout the matrix.

Compound Synthesis

5 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 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
10 chemical bonding and stability.

Chemical synthesis of a macrocyclic compound of the present disclosure can be carried out using a variety of art recognized methods, including stepwise solid phase synthesis, semi-synthesis through the conformationally-assisted re-ligation of peptide fragments, enzymatic ligation of cloned or synthetic peptide segments, and
15 chemical ligation. A preferred method to synthesize the macrocyclic compounds 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*, Oxford University Press, Oxford (2000); Barany, G. et al., *The Peptides: Analysis, Synthesis, Biology*, Vol. 2: "Special Methods in Peptide Synthesis, Part
20 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-oxycarbonyl) group for temporary protection of the α -amino group, in combination with the *tert*-butyl group for temporary protection of the
25 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).

30 The compounds can be synthesized in a stepwise manner on an insoluble polymer support (also referred to as "resin") starting from the C-terminus of the compound. A synthesis is begun by appending the C-terminal amino acid of the compound to the resin through formation of an amide or ester linkage. This allows

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the eventual release of the resulting compound as a C-terminal amide or carboxylic acid, respectively.

The C-terminal amino acid and all other amino acids used in the synthesis are required to have their α -amino groups and side chain functionalities (if present) differentially protected such that the α -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 α -amino group of the N-terminal amino acid appended to the resin. The sequence of α -amino group deprotection and coupling is repeated until the entire compound sequence is assembled. The compound 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 compound is finally purified by reverse phase HPLC.

The synthesis of the peptidyl-resins required as precursors to the final compounds 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 HOBt, 6-Cl-HOBt or HOAt active esters produced from DIC/HOBt, HBTU/HOBt, BOP, PyBOP, or from DIC/6-Cl-HOBt, HCTU, DIC/HOAt or HATU, respectively. Preferred solid supports are: 2-Chlorotrityl chloride resin and 9-Fmoc-amino-xanthen-3-yloxy-Merrifield resin (Sieber amide resin) for protected compound fragments. Loading of the first amino acid onto the 2-chlorotrityl 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.

The syntheses of the compound analogs described herein can be carried out by using a single or multi-channel peptide synthesizer, such as an CEM Liberty

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Microwave synthesizer, or a Protein Technologies, Inc. Prelude (6 channels), Symphony (12 channels) or Symphony-X (12 or 24 channels) synthesizer.

The peptidyl-resin precursors may be cleaved and deprotected using any standard procedure (see, for example, King, D.S. et al., *Int. J. Peptide Protein Res.*, 5 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 compound is either precipitated and washed 10 with Et₂O or is redissolved directly into DMSO or 50% aqueous acetic acid for purification by preparative HPLC.

Compounds with the desired purity can be obtained by purification using preparative HPLC, for example, on a Waters Model 4000 or a Shimadzu Model LC-8A liquid chromatograph. The solution of crude compound is injected into a YMC 15 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 compounds can be confirmed by electro-spray MS analysis.

In this specification where reference has been made to patent specifications, 20 other external documents, or other sources of information, this is generally for the purpose of providing a context for discussing the features of the invention. Unless specifically stated otherwise, reference to such external documents is not to be construed as an admission that such documents, or such sources of information, in any jurisdiction, are prior art, or form part of the common general knowledge in the 25 art.

In the description in this specification reference may be made to subject matter that is not within the scope of the claims of the current application. That subject matter should be readily identifiable by a person skilled in the art and may assist in putting into practice the invention as defined in the claims of this 30 application.

Examples

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: min or mins for minutes; h or hr or hrs for hours; RT or rt for room temperature or retention time (context will dictate); sat. for saturated; TFA for trifluoroacetic acid; DMF for N,N-dimethylformamide; DCM for dichloromethane; Fmoc for 9-fluorenylmethyloxycarbonyl; HATU for (1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate); DIEA or DIPEA for diisopropylethylamine; NMP for N-methylpyrrolidone; EDC for 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; DMSO for dimethylsulfoxide; MeOH for methanol; EtOAc for ethyl acetate; Et₃N for triethylamine; MeCN or ACN for acetonitrile; DIAD for diethyl azodicarboxylate; and TMSI for trimethylsilyl iodide.

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 “*m/z*” unit designation. Compounds with exact masses greater than 1000 were often detected as double-charged or triple-charged ions. The following analytical protocols and synthetic methods apply to Examples 1400-1508.

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 “*m/z*” unit designation. Compounds with exact masses greater than 1000 were often detected as double-charged or triple-charged ions.

Analysis LCMS Condition A:

- 5 Column: 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 minutes, then a 0.5 minutes hold at 98% B; Flow: 0.8 mL/min; Detection: UV at 220 nm.

10 *Analysis LCMS Condition B:*

 Column: BEH C18, 2.1 x 50 mm, 1.7- μ m particles; Mobile Phase A: 5:95 acetonitrile:water with 0.05% TFA; Mobile Phase B: 95:5 acetonitrile:water with 0.05% TFA; Temperature: 50 °C; Gradient: 0-100% B over 3 minutes, then a 0.75-minute hold at 100% B; Flow: 1.11 mL/min.

15

Analysis LCMS Condition C:

- Column: 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 minutes, 80% B to 98% B over 0.1 minute then a 0.5 minutes hold at 98% B; Flow: 0.8 mL/min; Detection: UV at 220 nm.
- 20

Analysis LCMS Condition D:

- Column: Waters Acquity UPLC 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: 50 °C; Gradient: 0-100% B over 3 minutes, then a 0.75-minute hold at 100% B; Flow: 1.11 mL/min; Detection: UV at 220 nm.
- 25

30 *Analysis LCMS Condition E:*

 Column: Waters Acquity UPLC 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;
Gradient: 0-100% B over 3 minutes, then a 0.75-minute hold at 100% B; Flow: 1.11 mL/min; Detection: UV at 220 nm.

Analysis LCMS Condition F:

- 5 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 minutes, then a 1-minute hold at 100% B; Flow: 4 mL/min;
Detection: UV at 220 nm.

10

Analysis LCMS Condition G:

- Finnigan LTQ Mass Spectrometer; column: Phenomenex Jupiter C4, 1 x 50
mm; Mobile Phase A: 1% formic acid in water; Mobile Phase B: 0.1% formic acid in
acetonitrile; Temperature: 30 °C; Gradient: 1% B, 1 min. hold; 1-95% B over 3 min.,
15 then a 3-min. hold at 95% B; Flow: 0.15 mL/min.

Analysis LCMS Condition H:

- Column: Waters BEH C18, 2.0 x 50 mm, 1.7-µm particles; Mobile Phase A:
5:95
20 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: 1.0 mL/min;
Detection: UV at 220 nm.

25 *Analysis LCMS Condition I:*

- Column: Waters BEH C18, 2.0 x 50 mm, 1.7-µm 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-
100% B over 3 minutes,
30 then a 0.5-minute hold at 100% B; Flow: 0.5 mL/min; Detection: UV at 220 nm.

Analysis HPLC Condition A:

Column: YMC Pack ODS-AQ 3um 150x4.6mm Mobile Phase A: water with 0.1% TFA; Mobile Phase B: Acetonitrile with 0.1% TFA; Temperature: 60 °C; Gradient: from 35% B to 80% B over 25 min.; Flow: 1 mL/min; Detection: UV at 217 nm.

5

Analysis HPLC Condition B:

Column: YMC Pack ODS-AQ 3um 150x4.6mm Mobile Phase A: water with 0.1% TFA; Mobile Phase B: Acetonitrile with 0.1% TFA; Temperature: 60 °C; Gradient: from 25% B to 75% B over 25 min.; Flow: 1 mL/min; Detection: UV at 217 nm.

10

Analysis HPLC Condition C:

Column: YMC Pack ODS-AQ 3um 150x4.6mm Mobile Phase A: water with 0.1% TFA; Mobile Phase B: Acetonitrile with 0.1% TFA; Temperature: 60 °C; Gradient: from 20% B to 70% B over 25 min.; Flow: 1 mL/min; Detection: UV at 217 nm.

15

Analysis HPLC Condition D:

Column: YMC Pack ODS-AQ 3um 150x4.6mm Mobile Phase A: water with 0.1% TFA; Mobile Phase B: Acetonitrile with 0.1% TFA; Temperature: 60 °C; Gradient: from 15% B to 65% B over 25 min.; Flow: 1 mL/min; Detection: UV at 217 nm.

20

Analysis HPLC Condition E:

Column: YMC Pack ODS-AQ 3um 150x4.6mm Mobile Phase A: water with 0.1% TFA; Mobile Phase B: Acetonitrile with 0.1% TFA; Temperature: 60 °C; Gradient: from 25% B to 60% B over 20 min.; Flow: 1.25 mL/min; Detection: UV at 217 nm.

25

Analysis HPLC Condition F:

Column: YMC Pack ODS-AQ 3um 150x4.6mm Mobile Phase A: water with 0.1% TFA; Mobile Phase B: Acetonitrile with 0.1% TFA; Temperature: 60 °C;

30

Gradient: from 25% B to 65% B over 20 min.; Flow: 1.25 mL/min; Detection: UV at 217 nm.

Analysis HPLC Condition G:

- 5 Column: Sunfire C18 3.5um, 3.0x150mm; Mobile Phase A: 5:95
 acetonitrile:water with 0.05% trifluoroacetic acid; Mobile Phase B: 95:5
 acetonitrile:water with 0.05% trifluoroacetic acid; temperature: 50 °C; Gradient: 10-
 100% B over 12 minutes, then a 3-minute hold at 100% B; Flow: 1 mL/min;
 Detection: UV at 220 nm.

10 *Analysis HPLC Condition H:*

Column: Xbridge Phenyl 3.5x150um, Mobile Phase A: 5:95 acetonitrile:water
 with 0.05% trifluoroacetic acid; Mobile Phase B: 95:5 acetonitrile:water with 0.05%
 trifluoroacetic acid; temperature: 50 °C; Gradient: 10-100% B over 12 minutes, then
 a 3-minute hold at 100% B; Flow: 1 mL/min; Detection: UV at 220 nm.

15

Analysis HPLC Condition I:

- Column: Phenomenex Luna 5u C18(2) 150 x 4.6 mm; mobile Phase A: water
 with 0.1% trifluoroacetic acid, mobile Phase B: acetonitrile with 0.1% trifluoroacetic
 acid, Gradient 5-100% B over 20min, then a 5 minute hold at 100% B; Flow
 20 1mL/min, Detection: UV at 220

Analysis HPLC Condition J:

- Column: Phenomenex Luna 5u C18(2) 150 x 4.6 mm; mobile Phase A: water
 with 0.1% trifluoroacetic acid, mobile Phase B: acetonitrile with 0.1% trifluoroacetic
 25 acid, Gradient 10-100% B over 20min, then a 5 minute hold at 100% B; Flow
 1mL/min, Detection: UV at 220

General Procedures:

30 *Prelude Method A:*

All manipulations were performed under automation on a Prelude peptide
 synthesizer (Protein Technologies). All procedures unless noted were performed in a

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10 or 45 mL polypropylene tube fitted with a bottom frit. The tube connects to 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 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. Amino acid solutions were generally not used beyond three weeks from preparation 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; 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 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 compound 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 Fmoc-N-methyl amino acids and coupling to a secondary amine N-terminus used the "Secondary amine-coupling procedure" described below. Coupling of chloroacetyl group to the N-terminus of the compound is described by

the “Chloroacetyl chloride coupling procedure” or “Chloroacetic acid coupling procedure” detailed below.

Resin-swelling procedure:

5 To a 40 mL polypropylene solid-phase reaction vessel was added Merrifield:Sieber resin (140 mg, 0.100 mmol). The resin was washed three times as follows: to the reaction vessel was added DMF (5.0 mL) and DCM (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.

10

Single-coupling procedure:

 To the reaction vessel containing resin from the previous step 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. To the reaction vessel
15 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 added through the top of the vessel and the resulting mixture was periodically agitated for 60 seconds before the solution was drained through the frit. To the
20 reaction vessel was added a solution of the amino acid and HOAt (0.2M in DMF, 5.0 mL, 10 eq), then DIC (0.2M in DMF, 5.0 mL, 10 eq). The mixture was periodically agitated for 60 min, then the reaction solution was drained through the frit. The resin was washed successively four times as follows: for each wash, DMF (4.0 mL) was added through the top of the vessel and the resulting mixture was
25 periodically agitated for 30 seconds before the solution was drained through the frit. 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 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 (4.0 mL) was added through the 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 used directly in the next step.

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Secondary amine-coupling procedure:

To the reaction vessel containing resin from the previous step 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. To the reaction vessel
5 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 added through the top of the vessel and the resulting mixture was periodically agitated for 60 seconds before the solution was drained through the frit. To the
10 reaction vessel was added a solution of the amino acid and HOAt (0.2M in DMF, 5.0 mL, 5 eq), then DIC (0.2M in DMF, 5.0 mL, 5 eq). The mixture was periodically agitated for 300 min, then the reaction solution was drained through the frit. The resin was washed successively four times as follows: for each wash, DMF (4.0 mL) was added through the top of the vessel and the resulting mixture was periodically
15 agitated for 30 seconds before the solution was drained through the frit. 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 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 (4.0 mL) was added through the top of the vessel and the
20 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:

To the reaction vessel containing the resin from the previous step was added
25 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
30 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

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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. 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. The resin was washed successively four times as follows: for each wash, DCM (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.

10 *Prelude Method B:*

All manipulations were performed under automation on a Prelude peptide synthesizer (Protein Technologies). All procedures were performed in a 10 or 45 mL polypropylene tube fitted with a bottom frit. 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 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. Amino acid solutions were generally not used beyond three weeks from preparation. 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; 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.

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The procedures of "Prelude Method B" 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 compound 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 amine-coupling procedure" described below. Coupling of chloroacetyl group to the N-terminus of the compound is described by the "Chloroacetyl chloride coupling procedure" or "Chloroacetic acid coupling procedure" detailed below.

Resin-swelling procedure:

To a 40 mL polypropylene solid-phase 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 (5.0 mL) and DCM (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.

Single-coupling procedure:

To the reaction vessel containing resin from the previous step 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. 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 added through the top of the vessel and the resulting mixture was periodically agitated for 60 seconds before the solution was drained through the frit. To the reaction vessel was added the amino acid (0.2M in DMF, 5.0 mL, 10 eq), then HCTU (0.2M in DMF, 5.0 mL, 10 eq), and finally DIPEA (0.8M in DMF, 2.5 mL, 20 eq).

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The mixture was periodically agitated for 30 minutes, then the reaction solution was drained through the frit. The resin was washed successively four times as follows: for each wash, DMF (4.0 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 a solution of acetic anhydride:DIEA:DMF (10:1:89 v/v/v, 5.0 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 (4.0 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.

Double-coupling procedure:

To the reaction vessel containing resin from the previous step 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. 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 added through the top of the vessel and the resulting mixture was periodically agitated for 60 seconds before the solution was drained through the frit. To the reaction vessel was added the amino acid (0.2M in DMF, 5.0 mL, 10 eq), then HCTU (0.2M in DMF, 5.0 mL, 10 eq), and finally DIPEA (0.8M in DMF, 2.5 mL, 20 eq). The mixture was periodically agitated for 15 minutes, then the reaction solution was drained through the frit. The resin was washed successively 3 times with DMF (4.0 mL) through the top of the vessel and the resulting mixture was periodically agitated for 60 seconds before the solution was drained through the frit. To the reaction vessel was added the amino acid (0.2M in DMF, 5.0 mL, 10 eq), then HCTU (0.2M in DMF, 5.0 mL, 10 eq), and finally DIPEA (0.8M in DMF, 2.5 mL, 20 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 (4.0 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. The resulting resin was used directly in the next step.

5 *Secondary amine-coupling procedure:*

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To the reaction vessel containing resin from the previous step 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. To the reaction vessel was added piperidine:DMF (20:80 v/v, 5.0 mL). The mixture was periodically
10 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 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, 2.5 mL, 10 eq), then HCTU
15 (0.2M in DMF, 2.5 mL, 10 eq), and finally NMM (0.8M in DMF, 1.5 mL, 12 eq). The mixture was periodically agitated for 12 hrs, then the reaction solution was drained through the frit. The resin was washed successively four times as follows: for each wash, DMF (4.0 mL) was added through the top of the vessel and the resulting mixture was periodically agitated for 90 seconds before the solution was
20 drained through the frit. The resulting resin was used directly in the next step.

Chloroacetyl chloride coupling procedure A:

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 3
25 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 added through the top of the vessel and the resulting mixture was periodically
30 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

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was periodically agitated for 12 to 18 hours, then the solution was drained through 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.

- 5 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 agitated for 90 seconds before the solution was drained through the frit.

Chloroacetic acid coupling procedure B:

- 10 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 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
- 15 was washed successively five times as follows: for each wash, DMF (4.0 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 DMF (2.0 mL), chloroacetic acid (1.2 mmol, 113 mg, 12 eq), and N,N'-diisopropylcarbodiimide (1.2 mmol, 0.187 mL, 12 eq). The mixture
- 20 was periodically agitated for 12 to 18 hours, then the solution was drained through 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
- 25 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.

Prelude Method C:

- 30 All manipulations were performed under automation on a Prelude peptide synthesizer (Protein Technologies). All procedures unless noted were performed in a 10 or 45 mL polypropylene tube fitted with a bottom frit. The tube connects to a the Prelude peptide synthesizer through both the bottom and the top of the tube. DMF

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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 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. Amino acid solutions were generally not used beyond three weeks from preparation. HATU solution were used within 5 days of preparation. 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; 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 C" 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 compound 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 amine-coupling procedure" described below. The Final wash of the Resin used the "*Final wash procedure*" described below

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Resin-swelling procedure:

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To a 40 mL polypropylene solid-phase 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 (5.0 mL) and DCM (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.

Single-coupling procedure:

To the reaction vessel containing resin from the previous step 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. 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 added through the top of the vessel and the resulting mixture was periodically agitated for 60 seconds before the solution was drained through the frit. To the reaction vessel was added the amino acid (0.2M in DMF, 5.0 mL, 10 eq), then HATU (0.2M in DMF, 5.0 mL, 10 eq), and finally DIPEA (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 four times as follows: for each wash, DMF (4.0 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 a solution of acetic anhydride:DIEA:DMF (10:1:89 v/v/v, 5.0 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 (4.0 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.

Secondary amine-coupling procedure:

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To the reaction vessel containing resin from the previous step 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. 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 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, 2.5 mL, 5 eq), then HATU (0.2M in DMF, 2.5 mL, 5 eq), and finally DIPEA (0.8M in DMF, 1.5 mL, 12 eq). The mixture was periodically agitated for 300 minutes, then the reaction solution was drained through the frit. The resin was twice washed as follows: for each wash, DMF (4.0 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, 2.5 mL, 5 eq), then HATU (0.2M in DMF, 2.5 mL, 5 eq), and finally DIPEA (0.8M in DMF, 1.5 mL, 12 eq). The mixture was periodically agitated for 300 minutes, then the reaction solution was drained through the frit. The resin was twice washed as follows: for each wash, DMF (4.0 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 a solution of acetic anhydride:DIEA:DMF (10:1:89 v/v/v, 5.0 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 (4.0 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.

Custom amino acids-coupling procedure:

To the reaction vessel containing resin from the previous step 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. To the reaction vessel

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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 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, 0.5 to 2.5 mL, 1 to 5 eq), then HATU (0.2M in DMF, 0.5 to 2.5 mL, 1 to 5 eq), and finally DIPEA (0.8M in DMF, 0.5 to 1.5 mL, 4 to 12 eq). The mixture was periodically agitated for 60 minutes to 600 minutes, then the reaction solution was 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 a solution of acetic anhydride:DIEA:DMF (10:1:89 v/v/v, 5.0 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 (4.0 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.

20 *Final wash procedure:*

The resin was washed successively two times as follows: for each wash, DMF (4.0 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 resin was washed successively four times as follows: for each wash, DCM (4.0 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.

Chloroacetic acid coupling procedure:

30 Note manual step. 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 shaken at room temperature for 5 minutes and then the solution was drained through

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the frit. The resin was washed successively five times as follows: for each wash, DMF (4.0 mL) was added through the top of the vessel and the resulting mixture was agitated before the solution was drained through the frit. To the reaction vessel was added DMF (2.0 mL), chloroacetic acid (1.2 mmol, 113 mg, 12 eq), and N,N'-diisopropylcarbodiimide (1.2 mmol, 0.187 mL, 12 eq). The mixture was shaken at room temperature for 12 to 18 hours, then the solution was drained through 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 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 (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.

Prelude Method D:

All manipulations were performed under automation on a Prelude peptide synthesizer (Protein Technologies). All procedures unless noted were performed in a 10 or 45 mL polypropylene tube fitted with a bottom frit. The tube connects to 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 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. Amino acid solutions were generally not used beyond three weeks from preparation. HATU solution were used within 5 days of preparation. 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; Fmoc-Bzt-OH; Fmoc-Cys(Trt)-OH; Fmoc-Dab(Boc)-OH; Fmoc-Dap(Boc)-OH; Fmoc-Gln(Trt)-OH; Fmoc-Gly-OH; Fmoc-

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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.

- 5 The procedures of “Prelude Method D” 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 compound 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 amine-coupling procedure” described below. The Final wash of the Resin used the “*Final wash procedure*” described below
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- 15

Resin-swelling procedure:

- To a 40 mL polypropylene solid-phase 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 (5.0 mL) and DCM (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.
- 20

25 *Single-coupling procedure:*

- To the reaction vessel containing resin from the previous step 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. 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 added through the top of the vessel and the resulting mixture was periodically
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agitated for 60 seconds before the solution was drained through the frit. To the reaction vessel was added the amino acid (0.2M in DMF, 1.25 mL, 2.5 eq), then HATU (0.2M in DMF, 1.25 mL, 2.5 eq), and finally DIPEA (0.8M in DMF, 0.75 mL, 5 eq). The mixture was periodically agitated for 30 minutes, then the reaction solution was drained through the frit. The resin was washed successively four times as follows: for each wash, DMF (4.0 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 a solution of acetic anhydride:DIEA:DMF (10:1:89 v/v/v, 5.0 mL). The mixture was periodically agitated for 15 minutes, then the solution was drained through the frit. The resin was washed successively four times as follows: for each wash, DMF (4.0 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.

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Secondary amine-coupling procedure:

To the reaction vessel containing resin from the previous step 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. 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 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, 1.25 mL, 2.5 eq), then HATU (0.2M in DMF, 1.25 mL, 2.5 eq), and finally DIPEA (0.8M in DMF, 0.75 mL, 5 eq). The mixture was periodically agitated for 30 minutes, then the reaction solution was drained through the frit. The resin was twice washed as follows: for each wash, DMF (4.0 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 (0.2M in DMF, 1.25 mL, 2.5 eq), then HATU (0.2M in DMF, 1.25 mL, 2.5 eq), and finally DIPEA (0.8M

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in DMF, 0.75 mL, 5 eq). The mixture was periodically agitated for 30 minutes, then the reaction solution was drained through the frit. The resin was twice washed as follows: for each wash, DMF (4.0 mL) was added through the top of the vessel and the resulting mixture was periodically agitated for 30 seconds before the solution was
5 drained through the frit. 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 agitated for 15 minutes, then the solution was drained through the frit. The resin was twice washed as follows: for each wash, DMF (4.0 mL) was added through the top of the vessel and the resulting mixture was periodically agitated for 30 seconds before
10 the solution was drained through the frit. 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 agitated for 15 minutes, then the solution was drained through the frit. The resin was washed successively four times as follows: for each wash, DMF (4.0 mL) was added through the top of the vessel and the resulting mixture was
15 periodically agitated for 90 seconds before the solution was drained through the frit. The resulting resin was used directly in the next step.

Final wash procedure:

The resin was washed successively two times as follows: for each wash, DMF
20 (4.0 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 resin was washed successively four times as follows: for each wash, DCM (4.0 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.
25 The resulting resin was used directly in the next step.

Chloroacetic acid coupling procedure:

Note manual step. 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
30 shaken at room temperature for 5 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 added through the top of the vessel and the resulting mixture was

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agitated before the solution was drained through the frit. To the reaction vessel was added DMF (2.0 mL), chloroacetic acid (1.2 mmol, 113 mg, 12 eq), and N,N'-diisopropylcarbodiimide (1.2 mmol, 0.187 mL, 12 eq). The mixture was shaken at room temperature for 12 to 18 hours, then the solution was drained through the frit.

5 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 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 (4.0 mL) was added to top of the vessel and the resulting mixture was periodically agitated for 90 seconds

10 before the solution was drained through the frit.

CEM Method A:

All manipulations were performed under automation on a CEM Liberty microwave peptide synthesizer (CEM Corporation). All procedures unless noted

15 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

20 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 were 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-

25 1,2,3-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. Other Common resins

30 Such as Rink, ChloroTrityl, or other acid sensitive linkers can be employed in the synthesis, Seiber amide resin is used unless otherwise noted in specific examples. Common amino acids used are listed below with side-chain protecting groups

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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-Orn(Boc)-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 “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 compound 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 amine-coupling procedure” described below. Coupling of chloroacetyl group to the N-terminus of the compound is described by the “Chloroacetyl chloride coupling procedure” or “Chloroacetic acid coupling procedure” detailed above.

Resin-swelling procedure:

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 of the reaction vessel for 15 minutes before the solvent was drained through the frit.

Standard Coupling procedure:

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

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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 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 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 and finally with DMF (7 mL) wash from top. The resulting resin was used directly in the next step.

Double-couple coupling procedure:

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 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 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

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- 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-
- 5 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 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
- 10 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 and finally with DMF (7 mL) wash from top. The resulting resin was used directly in the next step.
- 15 *Secondary amine coupling procedure:*
- To the reaction vessel containing resin from the previous step was added a solution of 5% piperazine and 0.1 M HOBt in DMF (7 mL). The mixture was periodically agitated for 3 minutes at 75 °C and then the solution was drained. This procedure was repeated one more time. The resin was washed successively three
- 20 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 in DMF, 2.5 mL, 5 eq), HCTU (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 (50 °C for Fmoc-Cys(Trt)-OH
- 25 and Fmoc-His(Trt)-OH), followed by 6 hrs with no heating. After draining, 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
- 30 bubbled for 2 minutes at 65 °C, then the solution was drained. 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:

5 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
10 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
15 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
20 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 and finally with DMF (7 mL) wash from top. The resulting resin was used directly in the next step.

25 *Symphony Method A:*

All manipulations were performed under automation on a Symphony peptide synthesizer (Protein Technologies). All procedures unless noted were performed in a Symphony polypropylene tube fitted with a bottom frit. The tube connects to the Symphony peptide synthesizer through both the bottom and the top of the tube. All
30 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

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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 were 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-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate; NMM= n-Methylmorpholine; 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. 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. 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 “Symphony Method A” describes an experiment performed on a 0.050 mmol scale, where the scale is determined by the amount of Sieber linker bound to the resin. This scale corresponds to approximately 70 mg of the Sieber-Merrifield resin described above. All procedures can be scaled beyond 0.050 mmol scale by adjusting the described volumes by the multiple of the scale. Prior to amino acid coupling, all compound 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. Coupling of amino acids to a secondary amine N-terminus used the “Double-coupling”, custom amino acids are coupled via a manual Blank addition of the amino acid “Blank coupling” described below.

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Swelling procedure:

To a Symphony polypropylene solid-phase reaction vessel was added Merrifield:Sieber resin (70 mg, 0.050 mmol). The resin was washed (swelled) 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 10 minutes before the solvent was drained through the frit. To the reaction vessel was added piperidine:DMF (20:80 v/v, 2.5 mL). The mixture was periodically agitated for 2.5 minutes and then the 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 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 mL, 5 eq), then HATU (0.2M in DMF, 1.25 mL, 5 eq), and finally NMM (0.8M in DMF, 1.25 mL, 10 eq). The mixture was periodically agitated for 10 minutes, then the reaction solution was drained through the frit. The resin was washed with DMF (6.25 mL) 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 mL, 5 eq), then HATU (0.2M in DMF, 1.25 mL, 5 eq), and finally NMM (0.8M in DMF, 1.25 mL, 10 eq). The mixture was periodically agitated for 10 minutes, then the reaction solution was drained through the frit. 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. The resulting resin was used directly in the next step.

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, 2.5 mL). The mixture was periodically agitated for 2.5 minutes and then the solution was drained through the frit. The resin was washed 6 times as follows: for each

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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, 1.25 mL, 5 eq), then HATU (0.2M in DMF, 1.25 mL, 5 eq), and finally NMM (0.8M in DMF, 1.25 mL, 10 eq). The mixture was periodically agitated for 10 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 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 mL, 5 eq), then HATU (0.2M in DMF, 1.25 mL, 5 eq), and finally NMM (0.8M in DMF, 1.25 mL, 10 eq). The mixture was periodically agitated for 10 minutes, then the reaction solution was drained through the frit. The resin was washed successively three 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 resulting resin was used directly in the next step.

Secondary amine-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, 2.5 mL). The mixture was periodically agitated for 2.5 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, 1.25 mL, 5 eq), then HATU (0.2M in DMF, 1.25 mL, 5 eq), and finally NMM (0.8M in DMF, 1.25 mL, 10 eq). The mixture was periodically agitated for 300 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 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 mL, 5 eq),

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then HATU (0.2M in DMF, 1.25 mL, 5 eq), and finally NMM (0.8M in DMF, 1.25 mL, 10 eq). The mixture was periodically agitated for 300 minutes, then the reaction solution was drained through the frit. The resin was washed successively three 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 resulting resin was used directly in the next step.

Custom amino acids-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, 2.5 mL). The mixture was periodically agitated for 2.5 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. The synthesis was paused by the Symphony software to add to the reaction vessel manually the custom amino acid (0.2M in DMF, 1.25 mL, 5 eq), then restart automation: to add HATU (0.2M in DMF, 1.25 mL, 5 eq), and finally NMM (0.8M in DMF, 1.25 mL, 10 eq). The mixture was periodically agitated for 300 minutes, then the reaction solution was drained through the frit. The resin was washed six times as follows with 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 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 three 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.

Symphony Method B:

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All manipulations were performed under automation on a Symphony peptide synthesizer (Protein Technologies). All procedures unless noted were performed in a Symphony polypropylene tube fitted with a bottom frit. The tube connects to a the Symphony peptide synthesizer through both the bottom and the top of the tube. All

5 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

10 preparation. HATU solution were 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-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate; NMM= n-Methyl morpholine; DIPEA = diisopropylethylamine; Sieber = Fmoc-amino-xanthen-3-

15 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. 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

20 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-

25 [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 "Symphony Method B" describes an experiment performed on a 0.050 mmol scale, where the scale is determined by the amount of Sieber linker bound to the resin. This scale corresponds to approximately

30 70 mg of the Sieber-Merrifield resin described above. All procedures can be scaled beyond 0.050 mmol scale by adjusting the described volumes by the multiple of the scale. Prior to amino acid coupling, all compound synthesis sequences began with a

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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. 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:

10 To a Symphony polypropylene solid-phase reaction vessel was added Merrifield:Sieber resin (70 mg, 0.050 mmol). The resin was washed (swelled) 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 10 minutes before the solvent was drained through the frit. To the reaction
15 vessel was added piperidine:DMF (20:80 v/v, 2.5 mL). The mixture was periodically agitated for 2.5 minutes and then the 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 30 seconds before the solution was drained through the frit. To the
20 reaction vessel was added the amino acid (0.2M in DMF, 1.25 mL, 5 eq), then HATU (0.2M in DMF, 1.25 mL, 5 eq), and finally NMM (0.8M in DMF, 1.25 mL, 10 eq). The mixture was periodically agitated for 10 minutes, then the reaction solution was drained through the frit. The resin was washed with DMF (6.25 mL) added through the bottom of the vessel and the resulting mixture was periodically agitated for 30
25 seconds before the solution was drained through the frit. To the reaction vessel was added the amino acid (0.2M in DMF, 1.25 mL, 5 eq), then HATU (0.2M in DMF, 1.25 mL, 5 eq), and finally NMM (0.8M in DMF, 1.25 mL, 10 eq). The mixture was periodically agitated for 10 minutes, then the reaction solution was drained through the frit. The resin was washed three times as follows: to the reaction vessel was
30 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. The resulting resin was used directly in the next step.

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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, 2.5 mL). The mixture was periodically agitated for 2.5 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, 1.25 mL, 5 eq), then HATU (0.2M in DMF, 1.25 mL, 5 eq), and finally NMM (0.8M in DMF, 1.25 mL, 10 eq). The mixture was periodically agitated for 15 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:

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 mL). The mixture was periodically agitated for 2.5 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

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through the frit. To the reaction vessel was added the amino acid (0.2M in DMF, 1.25 mL, 5 eq), then HATU (0.2M in DMF, 1.25 mL, 5 eq), and finally NMM (0.8M in DMF, 1.25 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 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 mL, 5 eq), then HATU (0.2M in DMF, 1.25 mL, 5 eq), and finally NMM (0.8M in DMF, 1.25 mL, 10 eq). The mixture was periodically agitated for 15 minutes, then the reaction solution was drained through the frit. The resin was washed successively three 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 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.

20

Custom amino acids-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, 2.5 mL). The mixture was periodically agitated for 2.5 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. The System was paused by the system for the manual addition of the custom amino acid to the reaction vessel (0.2M in DMF, 1.25 mL, 5 eq), then the automation was restarted to add to the reaction vesicle HATU (0.2M in DMF, 1.25

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mL, 5 eq), and finally NMM (0.8M in DMF, 1.25 mL, 10 eq). The mixture was periodically agitated for 15 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
5 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
10 for 90 seconds before the solution was drained through the frit. The resulting resin was used directly in the next step.

Final capping procedure:

The resin was washed three times as follows: to the reaction vessel was added
15 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 mL). The mixture was periodically agitated for 2.5 minutes and then the solution was drained through the frit. The resin was washed 6 times as follows: for each
20 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, 1.25 mL, 10 eq) followed by the addition of the chloroacetic anhydride (0.4M in DMF, 1.25 mL, 10 eq). The mixture was periodically agitated for 15 minutes, then the reaction
25 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 periodically agitated for 30 seconds before the solution was drained through the frit. To the reaction vessel was added NMM (0.8M in DMF, 1.25 mL, 10 eq) followed by the addition of the Chloroacetic anhydride (0.4M in DMF, 1.25 mL, 10 eq). The
30 mixture was periodically agitated for 15 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

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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
5 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 for 30 seconds before the solution was
10 drained through the frit. The resulting resin was then dried with a stream of nitrogen for 10 mins.

Global Deprotection Method A:

All manipulations were performed manually unless noted otherwise. The
15 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 Sieber 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 prepared using trifluoroacetic
20 acid:water:triisopropylsilane:dithiothreitol (92.5:2.5:2.5:2.5 v:v:v:w). The resin was removed from the reaction vessel and transferred to a 25 mL syringe equipped with a frit. To the syringe was added the “deprotection solution” (5.0 mL). The mixture was mixed in a shaker for 85 min. The solution was filtered through, concentrated and diluted in diethyl ether (30 mL). The precipitated solid was centrifuged for 3
25 minutes. The supernatant solution was decanted and the solid was resuspended in diethyl ether (25 mL). The suspension was centrifuged for 3 minutes. The supernatant was decanted and the remaining solid was suspended in diethyl ether (25 mL). The suspension was centrifuged for 3 minutes. The supernatant was decanted and the remaining solid was dried under high vacuum. The crude compound was
30 obtained as a white to off-white solid.

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Global Deprotection Method B:

All manipulations were performed manually unless noted otherwise. The procedure of “Global Deprotection Method B” describes an experiment performed on a 0.04 mmol scale, where the scale is determined by the amount of Sieber linker bound to the resin. The procedure can be scaled beyond 0.04 mmol scale by adjusting the described volumes by the multiple of the scale. A “deprotection solution” was prepared using trifluoroacetic acid:triisopropylsilane (96:4; v:v). The resin was removed from the reaction vessel and transferred to a 10 mL syringe equipped with a frit. To the syringe was added the “deprotection solution” (2.0-3.0 mL). The mixture was mixed in a shaker for 1 h or 1.5 h. The solution was filtered through, washed with deprotection solution (0.5 mL), concentrated and diluted in diethyl ether (30 mL). The precipitated solid was centrifuged for 3 minutes. The supernatant solution was decanted and the solid was resuspended diethyl ether (25 mL). The suspension was centrifuged for 3 minutes. The supernatant was decanted and the remaining solid was suspended diethyl ether (25 mL). The suspension was centrifuged for 3 minutes. The supernatant was decanted and the remaining solid was dried under high vacuum. The crude compound was obtained as a white to off-white solid.

Global Deprotection Method C:

All manipulations were performed manually unless noted. The procedure of “Global Deprotection Method C” describes an experiment performed on a 0.100 mmol scale, where the scale is determined by the amount of Sieber 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 prepared using trifluoroacetic acid:triisopropylsilane:dithiothreitol (95:2.5:2.5 v:v:w). The resin was removed from the reaction vessel and transferred to a Bio-Rad tube. To the Bio-Rad tube was added the “deprotection solution” (4.0 mL). The mixture was mixed in a shaker for 60 minutes. The solution was filtered through and diluted in diethyl ether (30 mL). The precipitated solid was centrifuged for 3 minutes. The supernatant solution was decanted and the solid was resuspended diethyl ether (25

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mL). The suspension was centrifuged for 3 minutes. The supernatant was decanted and the remaining solid was suspended diethyl ether (25 mL). The suspension was centrifuged for 3 minutes. The supernatant was decanted and the remaining solid was dried under high vacuum. The crude compound was obtained as a white to off-white solid.

Global Deprotection Method D:

All manipulations were performed manually unless noted otherwise. The procedure of “Global Deprotection Method B” describes an experiment performed on a 0.100 mmol scale, where the scale is determined by the amount of Sieber 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 prepared using trifluoroacetic acid:triisopropylsilane:dithiothreitol (94:3:3 v:v:w). The resin was removed from the reaction vessel and transferred to a 25 mL syringe equipped with a frit. To the syringe was added the “deprotection solution” (5.0 mL). The mixture was mixed in a shaker for 5 minutes. The solution was filtered through and diluted in diethyl ether (30 mL). The precipitated solid was centrifuged for 3 minutes. The supernatant solution was decanted and the solid was resuspended diethyl ether (25 mL). The suspension was centrifuged for 3 minutes. The supernatant was decanted and the remaining solid was suspended diethyl ether (25 mL). The suspension was centrifuged for 3 minutes. The supernatant was decanted and the remaining solid was dried under high vacuum. The crude compound was obtained as a white to off-white solid.

Global Deprotection Method E:

All manipulations were performed manually unless noted. The procedure of “Global Deprotection Method E” describes an experiment performed on a 0.100 mmol scale, where the scale is determined by the amount of Fmoc Gly-CITrt 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 prepared using trifluoroacetic acid:triisopropylsilane:dithiothreitol (95:2.5:2.5 v:v:w). The resin was removed from the reaction vessel and transferred

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to a Bio-Rad tube. To the Bio-Rad tube was added the “deprotection solution” (2.0 mL). The mixture was mixed in a shaker for 3 minutes. The solution was filtered, and collected in a centrifuge tube. To the Bio-Rad tube was added the “deprotection solution” (2.0 mL). The mixture was mixed in a shaker for 3 minutes. The solution was filtered, and collected in a centrifuge tube. To the Bio-Rad tube was added the “deprotection solution” (2.0 mL). The mixture was mixed in a shaker for 3 minutes. The solution was filtered, and collected in a centrifuge tube. The solution in the centrifuge tube was allowed to stand for 60 minutes. The collected solution was then diluted with diethyl ether (30 mL), and precipitate formed. The precipitated solid was centrifuged for 3 minutes. The supernatant solution was decanted and the solid was resuspended diethyl ether (25 mL). The suspension was centrifuged for 3 minutes. The supernatant was decanted and the remaining solid was suspended diethyl ether (25 mL). The suspension was centrifuged for 3 minutes. The supernatant was decanted and the remaining solid was dried under high vacuum. The crude compound was obtained as a white to off-white solid.

Global Deprotection Method F:

All manipulations were performed manually unless noted. The procedure of “Global Deprotection Method F” 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 prepared using trifluoroacetic acid:triisopropylsilane:dithiothreitol (95:2.5:2.5 v:v:w). The resin was removed from the reaction vessel and transferred to a 6 mls Bio-Rad tube. To the Bio-Rad was added the “deprotection solution” (4.0 mL). The mixture was mixed in a shaker for 90 minutes. The solution was filtered through and diluted in diethyl ether (30 mL). The precipitated solid was centrifuged for 3 minutes. The supernatant solution was decanted and the solid was resuspended diethyl ether (25 mL). The suspension was centrifuged for 3 minutes. The supernatant was decanted and the remaining solid was suspended diethyl ether (25 mL). The suspension was centrifuged for 3 minutes. The supernatant was decanted and the remaining solid was

dried under high vacuum. The crude compound was obtained as a white to off-white solid.

Cyclization Method A

All manipulations were performed manually unless noted otherwise. The procedure of "Cyclization Method A" describes an experiment performed on a 0.100 mmol scale, where the scale is determined by the amount of Sieber linker bound to the resin that was used to generate the compound. This scale is not based on a direct determination of the quantity of compound 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 compound solids were dissolved in a solution of acetonitrile:aqueous 8M Guanidine/50mM TRIS (1:3) (pH 8.6) (7 mL:18 mL or similar ratio), and the solution was then adjusted to pH = 8.5-9.0 using aq NaOH (1.0M), if necessary. The solution was then mixed using a shaker for 12 to 18 hours. The 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 compound.

Cyclization Method C:

All manipulations were performed manually unless noted. The procedure of "Cyclization Method C" describes an experiment performed on a 0.100 mmol scale, where the scale is determined by the amount of Sieber linker bound to the resin that was used to generate the compound. This scale is not based on a direct determination of the quantity of compound 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 compound solids were dissolved in a solution of acetonitrile:aqueous 0.1M ammonium bicarbonate buffer (11 mL:24 mL or similar ratio), and the solution was then carefully adjusted to pH = 8.5-9.0 using aq NaOH (1.0M). The solution was then mixed using a shaker for 12 to 18 hours. The 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 compound.

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Cyclization Method D:

All manipulations were performed manually unless noted. The procedure of “Cyclization Method D” describes an experiment performed on a 0.100 mmol scale, where the scale is determined by the amount of Sieber linker bound to the resin that was used to generate the compound. This scale is not based on a direct determination of the quantity of compound 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 compound solids were dissolved in a solution of acetonitrile:aqueous 0.1M ammonium bicarbonate buffer (11 mL:24 mL), and the solution was then carefully adjusted to pH = 8.5-9.0 using aq NaOH (1.0M). The solution was then mixed with stirring for 12 to 18 hours. The 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 compound.

Cyclization Method E:

All manipulations were performed manually unless noted. The procedure of “Cyclization Method E” describes an experiment performed on a 0.100 mmol scale, where the scale is determined by the amount of Sieber linker bound to the resin that was used to generate the compound. This scale is not based on a direct determination of the quantity of compound 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 compound solids were dissolved in a solution of aqueous 6M guanidine HCl buffer (15 mL), the solution was then mixed with stirring for 12 to 18 hours. The reaction solution was concentrated and 15 mL of DMSO was added to the residue affording a slurry which was filtered. This filtered solution was subjected to reverse-phase HPLC purification to afford the desired cyclic compound.

Manual coupling procedure A:

To Bio-Rad reaction vessel containing resin from the previous step was added piperidine:DMF (20:80 v/v, 5.0 mL). The mixture was periodically shaken for 5 minutes and then the solution was drained through the frit. The resin was washed

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successively five times as follows: for each wash, DMF (4.0 mL) was added through the top of the vessel and the resulting mixture was shaken for 60 seconds before the solution was drained through the frit. To the reaction vessel was added the amino acid (1.2-10 equivalents) typical (0.2M in DMF, 2.5 mL, 5 eq), then HATU (1.210
5 equivalents) typical (0.2M in DMF, 2.5 mL, 5 eq), and finally DIPEA (2.4- 20 equivalents) typical (0.8M in DMF, 1.25 mL, 10 eq). The mixture was shaken for 60 minutes to 18 hours, then the reaction solution was drained through the frit. The resin was washed successively four times as follows: for each wash, DMF (4.0 mL) was added through the top of the vessel and the resulting mixture was shaken for 60
10 seconds before the solution was drained through the frit.

N-methylation on-resin Method A. (Turner, R. A.; Hauksson, N. E.; Gipe, J. H.; Lokey, R. S. *Org. Lett.* **2013**, 15(19), 5012-5015):

All manipulations were performed manually unless noted. The procedure of
15 “N-methylation on-resin Method A” describes an experiment performed on a 0.100 mmol scale, where the scale is determined by the amount of Sieber linker bound to the resin that was used to generate the compound. This scale is not based on a direct determination of the quantity of compound used in the procedure. The procedure can be scaled beyond 0.100 mmol scale by adjusting the described volumes by the
20 multiple of the scale.

The resin was transferred into a 25 mL fritted syringe. To the resin was added piperidine:DMF (20:80 v/v, 5.0 mL). The mixture was shaken for 3 min. and then the solution was drained through the frit. The resin was washed 3 times with DMF (4.0 mL). To the reaction vessel was added piperidine:DMF (20:80 v/v, 4.0 mL).
25 The mixture was shaken for 3 min. and then the solution was drained through the frit. The resin was washed successively three times with DMF (4.0 mL) and three times with DCM (4.0 mL). The resin was suspended in DMF (2.0 mL) and ETHYL TRIFLUOROACETATE (0.119 mL, 1.00 mmol), 1,8-DIAZABICYCLO[5.4.0]UNDEC-7-ENE (0.181 mL, 1.20 mmol). The mixture was
30 placed on a shaker for 60 min.. The solution was drained through the frit. The resin was washed successively three times with DMF (4.0 mL) and three times with DCM (4.0 mL).

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The resin was washed three times with dry THF (2.0 mL) to remove any residual water. In an oven dried 4.0 mL vial was added THF (1.0 mL) and TRIPHENYLPHOSPHINE (131 mg, 0.500 mmol) on dry 4 Å molecular sieves (20 mg). The solution was transferred to the resin and diisopropyl azodicarboxylate (0.097 mL, 0.5 mmol) was added slowly. The resin was stirred for 15 min. The solution was drained through the frit and the resin was washed with three times with dry THF (2.0 mL) to remove any residual water. In an oven dried 4.0 mL vial was added THF (1.0 mL), TRIPHENYLPHOSPHINE (131 mg, 0.500 mmol) on dry 4 Å molecular sieves (20 mg). The solution was transferred to the resin and diisopropyl azodicarboxylate (0.097 mL, 0.5 mmol) was added slowly. The resin was stirred for 15 min.. The solution was drained through the frit. The resin was washed successively three times with DMF (4.0 mL) and three times with DCM (4.0 mL). The resin was suspended in Ethanol (1.0 mL) and THF (1.0 mL), and SODIUM BOROHYDRIDE (37.8 mg, 1.000 mmol) was added. The mixture was stirred for 30 min. and drained. The the resin was washed successively three times with DMF (4.0 mL) and three times with DCM (4.0 mL).

Lactam Formation A:

To crude compound obtained from "Cyclization Method D" is added anhydrous diethylformamide (7.5 mL) followed by 1.5 eq of HATU , 3 eq of *N*-Methylmorpholine. Reaction is stirred between 3 h to overnight at rt. Glacial Acetic Acid (3 drops) is added and the solvent is removed upon evaporation in a Genevac EZ2. Compound is solubilized in DMF (2 mL) filtered through a 0.2 um syringe filter and submitted to reverse phase purification.

Lactam formation B:

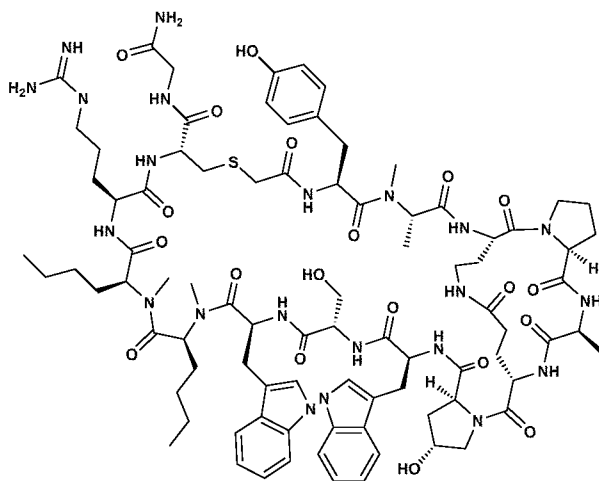
To the compound obtained from purification is added anhydrous diethylformamide (1 mL) followed by 1.5 eq of HATU as a freshly made stock in DMF (0.1M), and 3 eq of *N*-Methylmorpholine as a freshly prepared stock 0.3M in DMF. The reaction mixture is stirred overnight at rt, filtered through a 0.2 um filter, and submitted to reverse phase purification.

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2017227777*Microcleavage A:*

To a small < 10 mg sample of resin is added 2 drops of TIS and 1 mL of Trifluoroacetic acid, shake at rt. After 1 h, remove a small aliquot and dilute with 0.5 mL Acetonitrile, filter, and obtain analysis by LC-MS.

5 *General Synthetic Sequence:*

“General Synthetic Sequence” describes a general sequence of procedures that were used to afford the cyclic compounds described herein. The sequence is composed of the general methods described above. The sequence began with the resin-swelling procedure. Then a series of amino acids couplings were sequentially
10 performed using the single-coupling procedure if the N-terminus of the resin-bound compound was a primary amine or the double-coupling procedure if the N-terminus of the resin-bound compound was a secondary amine. Following the coupling of all amino acids in the sequence, additional procedures were performed in the order given: final wash, chloroacetic acid coupling, global deprotection, cyclization, and
15 finally lactam formation.

Preparation of Example 1400

Molecular Weight: 1828.13

Example 1400 was prepared following the "General Synthetic Sequence"
20 described above, composed of the following general procedures: “Prelude Method C: *Resin-swelling procedure*”, “Prelude Method C: *Single-coupling procedure*”, “Prelude Method C: *Secondary amine-coupling procedure*”, “Prelude Method C:

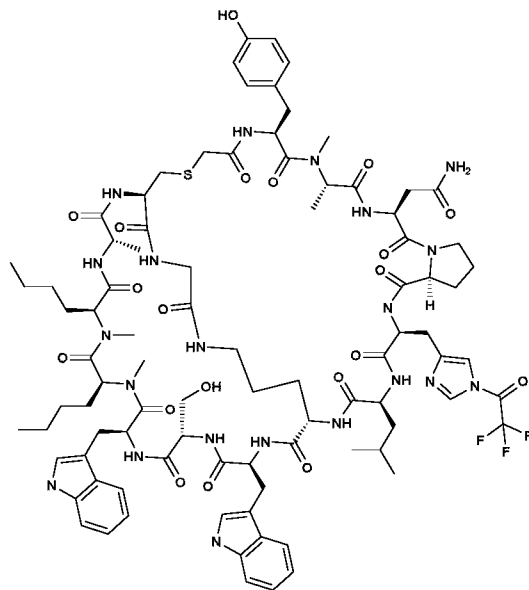
Final wash procedure”, *Chloroacetic acid coupling procedure B* “*Global Deprotection Method C*”, “*Cyclization Method D*”, and “*Lactam formation A*”

The crude material was purified via preparative LC/MS with the following conditions: Column: Waters XBridge C18, 19 x 250 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: 10-65% B over 25 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 0.5 mg, and its estimated purity by LCMS analysis was 99%.

Analysis LCMS Condition D: Retention time = 1.44 min; ESI-MS(+) m/z 914.7 (M+2H).

Analysis LCMS Condition E: Retention time = 1.46 min; ESI-MS(+) m/z 914.6 (M+2H).

Preparation of Example 1401



Molecular Weight: 1905.13

Example 1401 was prepared following the “General Synthetic Sequence” described above, composed of the following general procedures: “Prelude Method C: *Resin-swelling procedure*”, “Prelude Method C: *Single-coupling procedure*”, “Prelude Method C: *Secondary amine-coupling procedure*”, “Prelude Method C:

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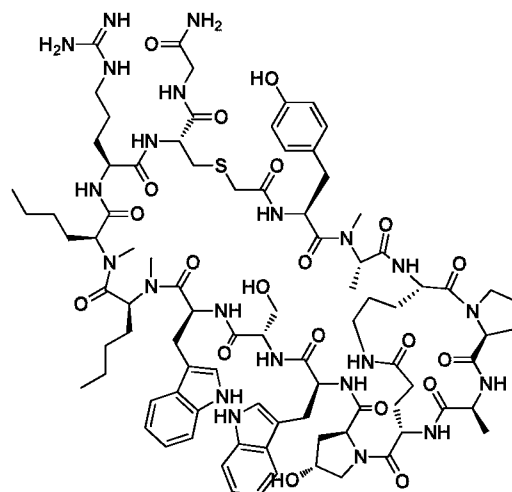
Final wash procedure”, Chloroacetic acid coupling procedure B “Global Deprotection Method C”, “Cyclization Method D”

The crude material was purified via preparative LC/MS with the following conditions: Column: Waters XBridge C18, 19 x 250 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: 10-60% B over 25 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 250 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: 15-60% B over 25 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 3.7 mg.

“Lactam formation B” was followed.

The crude material was purified via preparative LC/MS with the following conditions: Column: Waters XBridge C18, 19 x 250 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: 15-65% B over 25 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 1.2 mg, and its estimated purity by LCMS analysis was 91%. *Analysis LCMS Condition D:* Retention time = 1.54 min; ESI-MS(+) m/z 954.3 (M+2H). *Analysis LCMS Condition E:* Retention time = 1.52 min; ESI-MS(+) m/z 954.1 (M+2H).

Preparation of Example 1402



Molecular Weight: 1842.13

Example 1402 was prepared following the “General Synthetic Sequence” described above, composed of the following general procedures: “Prelude Method C: *Resin-swelling procedure*”, “Prelude Method C: *Single-coupling procedure*”,

- 5 “Prelude Method C: *Secondary amine-coupling procedure*”, “Prelude Method C: *Final wash procedure*”, *Chloroacetic acid coupling procedure B* “*Global Deprotection Method C*”, “*Cyclization Method D*”, and “*Lactam formation A*”. The crude material was purified via preparative LC/MS with the following conditions: Column: Waters XBridge C18, 19 x 250 mm, 5- μ m particles; Mobile Phase A: 5:95
- 10 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B: 95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 10-65% B over 25 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 4.6 mg, and its estimated purity by LCMS analysis was 99%.
- 15 *Analysis LCMS Condition D*: Retention time = 1.44 min; ESI-MS(+) m/z 921.7 (M+2H).
- Analysis LCMS Condition E*: Retention time = 1.46 min; ESI-MS(+) m/z 921.7 (M+2H).

20

Preparation of Example 1403



5
10

15

Analysis LCMS Condition D: Retention time = 1.39 min; ESI-MS(+) m/z 961.3 (M+2H).

5 *Analysis LCMS Condition E*: Retention time = 1.41 min; ESI-MS(+) m/z 961.2 (M+2H).

[illegible]

Example 1404 was prepared following the “General Synthetic Sequence” described above, composed of the following general procedures: “Prelude Method C: *Resin-swelling procedure*”, “Prelude Method C: *Single-coupling procedure*”, “Prelude Method C: *Secondary amine-coupling procedure*”, “Prelude Method C: *Final wash procedure*”, *Chloroacetic acid coupling procedure B* “*Global Deprotection Method C*”, “*Cyclization Method D*”. The crude material was purified via preparative LC/MS with the following conditions: Column: Waters XBridge C18, 19 x 250 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: 10-65% B over 25 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 250

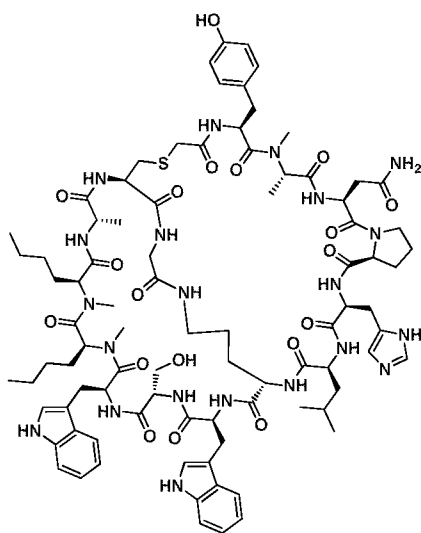
mm, 5- μ m particles; Mobile Phase A: 5:95 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B: 95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 15-60% B over 25 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of the product was 12.8 mg.

“*Lactam formation B*” was followed. The crude material was purified via preparative LC/MS with the following conditions: Column: Waters XBridge C18, 19 x 250 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: 10-65% B over 25 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 1.1 mg, and its estimated purity by LCMS analysis was 92%.

Analysis LCMS Condition D: Retention time = 1.49 min; ESI-MS(+) m/z 961.2 (M+2H).

Analysis LCMS Condition E: Retention time = 1.51 min; ESI-MS(+) m/z 961.3 (M+2H).

Preparation of Example 1405



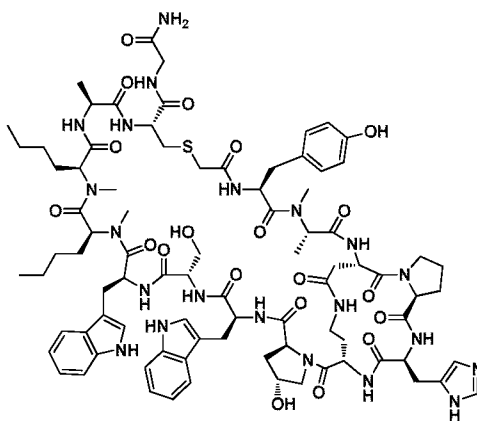
Molecular Weight: 1809.10

Example 1405 was prepared following the “General Synthetic Sequence” described above, composed of the following general procedures: “Prelude Method C:

Resin-swelling procedure”, “Prelude Method C: *Single-coupling procedure*”,
 “Prelude Method C: *Secondary amine-coupling procedure*”, “Prelude Method C:
Final wash procedure”, *Chloroacetic acid coupling procedure B* “*Global
 Deprotection Method C*”, “*Cyclization Method D*”, and “*Lactam formation A*”

- 5 The crude material was purified via preparative LC/MS with the following
 conditions: Column: XBridge C18, 19 x 250 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: 15-65% B over 25
 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 the product was 2.4 mg, and its estimated purity by LCMS analysis was 94%.
Analysis LCMS Condition D: Retention time = 1.55 min; ESI-MS(+) m/z 905.4
 (M+2H).
Analysis LCMS Condition E: Retention time = 1.55 min; ESI-MS(+) m/z 905.6
 15 (M+2H).

Preparation of Example 1500



Molecular Weight: 1795.03

- Example 1500 was prepared following the “General Synthetic Sequence”
 20 described above, composed of the following general procedures: “Prelude Method C:
Resin-swelling procedure”, “Prelude Method C: *Single-coupling procedure*”,
 “Prelude Method C: *Secondary amine-coupling procedure*”, “Prelude Method C:
Final wash procedure”, *Chloroacetic acid coupling procedure B* “*Global
 Deprotection Method C*”, “*Cyclization Method D*”, and “*Lactam formation A*”. The

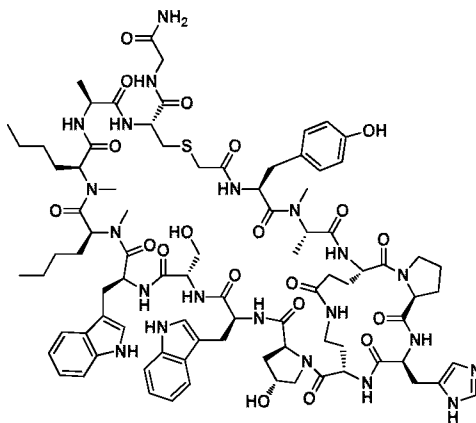
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crude material was purified via preparative LC/MS with the following conditions:
 Column: Waters XBridge C18, 19 x 250 mm, 5- μ m particles; Mobile Phase A: 5:95
 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B: 95:5
 acetonitrile: water with 10-mM ammonium acetate; Gradient: 10-65% B over 25
 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
 material was further purified via preparative LC/MS with the following conditions:
 Column: XBridge C18, 19 x 250 mm, 5- μ m particles; Mobile Phase A: 5:95
 acetonitrile: water with 0.1% trifluoroacetic acid; Mobile Phase B: 95:5 acetonitrile:
 10 water with 0.1% trifluoroacetic acid; Gradient: 20-60% B over 25 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
 1.3 mg, and its estimated purity by LCMS analysis was 99%.

Analysis LCMS Condition D: Retention time = 1.50 min; ESI-MS(+) m/z 898.5
 15 (M+2H).

Analysis LCMS Condition E: Retention time = 1.44 min; ESI-MS(+) m/z 898.2
 (M+2H).

Preparation of Example 1501



Molecular Weight: 1809.05

20

Example 1501 was prepared following the “General Synthetic Sequence”
 described above, composed of the following general procedures: “Prelude Method C:
Resin-swelling procedure”, “Prelude Method C: *Single-coupling procedure*”,
 “Prelude Method C: *Secondary amine-coupling procedure*”, “Prelude Method C:

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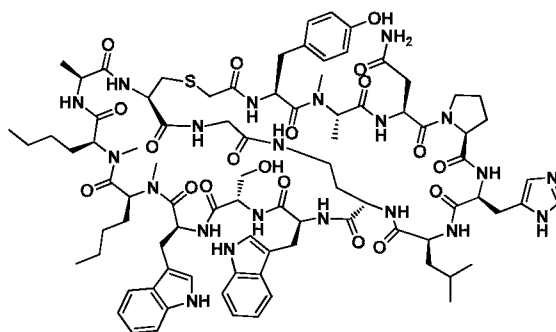
Final wash procedure”, *Chloroacetic acid coupling procedure B* “*Global Deprotection Method C*”, “*Cyclization Method D*”, and “*Lactam formation A*”. The crude material was purified via preparative LC/MS with the following conditions: Column: Waters XBridge C18, 19 x 250 mm, 5- μ m particles; Mobile Phase A: 5:95 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B: 95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 10-65% B over 25 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 250 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: 15-60% B over 25 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 4.8 mg, and its estimated purity by LCMS analysis was 99%.

Analysis LCMS Condition D: Retention time = 1.45 min; ESI-MS(+) m/z 905.3 (M+2H).

Analysis LCMS Condition E: Retention time = 1.41 min; ESI-MS(+) m/z 905.4 (M+2H).

ESI-HRMS(+) m/z : Calculated: 904.9325 (M+2H); Found: 904.9305 (M+2H)

Preparation of Example 1502



Molecular Weight: 1795.07

Example 1502 was prepared following the “General Synthetic Sequence” described above, composed of the following general procedures: “Prelude Method C: *Resin-swelling procedure*”, “Prelude Method C: *Single-coupling procedure*”,

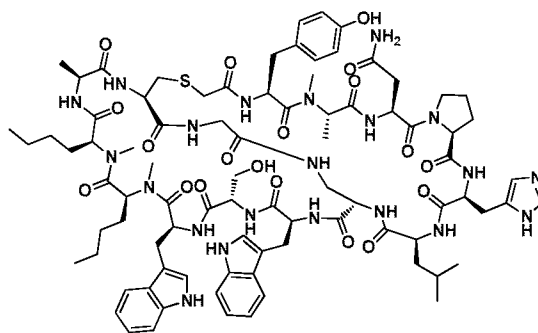
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“Prelude Method C: *Secondary amine-coupling procedure*”, “Prelude Method C: *Final wash procedure*”, *Chloroacetic acid coupling procedure B* “*Global Deprotection Method E*”, “*Cyclization Method D*”, and “*Lactam formation A*”. The crude material was purified via preparative LC/MS with the following conditions:

- 5 Column: Waters XBridge C18, 19 x 250 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: 10-65% B over 25 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
- 10 purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 250 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: 20-65% B over 25 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined
- 15 and dried via centrifugal evaporation. The material was further purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 250 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: 20-65% B over 25 minutes, then a 5-minute hold at
- 20 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of the product was 9.8 mg, and its estimated purity by LCMS analysis was 97%.

Analysis LCMS Condition D: Retention time = 1.60 min; ESI-MS(+) m/z 898.5 (M+2H).

- 25 *Analysis LCMS Condition E*: Retention time = 1.56 min; ESI-MS(+) m/z 898.4 (M+2H).

Preparation of Example 1503

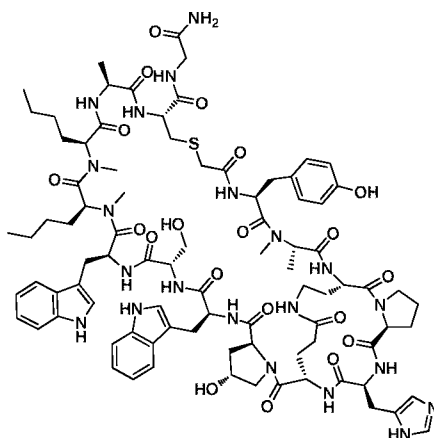
Molecular Weight: 1781.04

Example 1503 was prepared following the “General Synthetic Sequence” described above, composed of the following general procedures: “Prelude Method C:

- 5 *Resin-swelling procedure*”, “Prelude Method C: *Single-coupling procedure*”, “Prelude Method C: *Secondary amine-coupling procedure*”, “Prelude Method C: *Final wash procedure*”, *Chloroacetic acid coupling procedure B* “*Global Deprotection Method E*”, “*Cyclization Method D*”, and “*Lactam formation A*”. The crude material was purified via preparative LC/MS with the following conditions:
- 10 Column: Waters XBridge C18, 19 x 250 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: 15-65% B over 25 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
- 15 purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 250 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: 25-65% B over 25 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined
- 20 and dried via centrifugal evaporation. The yield of the product was 3.7 mg, and its estimated purity by LCMS analysis was 96%.

Analysis LCMS Condition D: Retention time = 1.63 min; ESI-MS(+) m/z 891.7 (M+2H).

- 25 *Analysis LCMS Condition E*: Retention time = 1.60 min; ESI-MS(+) m/z 891.2 (M+2H).

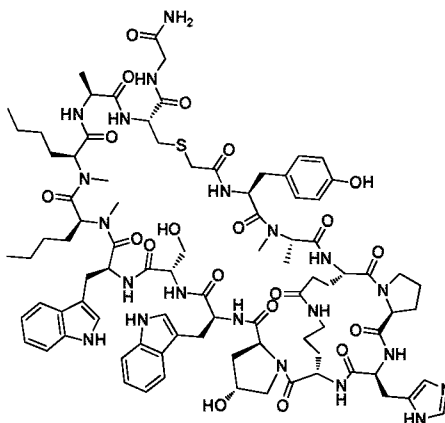
Preparation of Example 1504

Molecular Weight: 1809.05

- Example 1504 was prepared following the “General Synthetic Sequence” described above, composed of the following general procedures: “Prelude Method C: Resin-swelling procedure”, “Prelude Method C: Single-coupling procedure”, “Prelude Method C: Secondary amine-coupling procedure”, “Prelude Method C: Final wash procedure”, Chloroacetic acid coupling procedure B “Global Deprotection Method F”, “Cyclization Method D”, and “Lactam formation A”. The crude material was purified via preparative LC/MS with the following conditions:
- Column: Waters XBridge C18, 19 x 250 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: 10-60% B over 25 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 250 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: 10-65% B over 25 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 2.4 mg, and its estimated purity by LCMS analysis was 100%.
- Analysis LCMS Condition D:* Retention time = 1.43 min; ESI-MS(+) m/z 905.4 (M+2H).

Analysis LCMS Condition E: Retention time = 1.40 min; ESI-MS(+) m/z 905.3 (M+2H).

Preparation of Example 1505



Molecular Weight: 1823.08

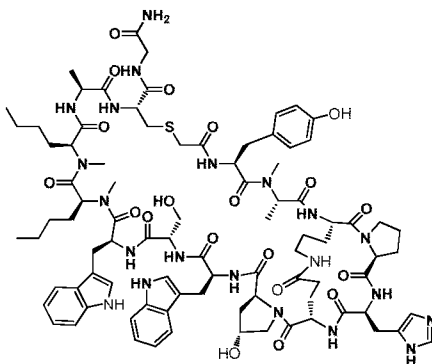
5 Example 1505 was prepared following the “General Synthetic Sequence” described above, composed of the following general procedures: “Prelude Method C: *Resin-swelling procedure*”, “Prelude Method C: *Single-coupling procedure*”, “Prelude Method C: *Secondary amine-coupling procedure*”, “Prelude Method C: *Final wash procedure*”, *Chloroacetic acid coupling procedure B* “*Global*
10 *Deprotection Method F*”, “*Cyclization Method D*”, and “*Lactam formation A*”. The crude material was purified via preparative LC/MS with the following conditions: Column: Waters XBridge C18, 19 x 250 mm, 5- μ m particles; Mobile Phase A: 5:95 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B: 95:5
15 acetonitrile: water with 10-mM ammonium acetate; Gradient: 10-65% B over 25 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 250 mm, 5- μ m particles; Mobile Phase A: 5:95
20 acetonitrile: water with 0.1% trifluoroacetic acid; Mobile Phase B: 95:5 acetonitrile: water with 0.1% trifluoroacetic acid; Gradient: 15-60% B over 25 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 3.7 mg, and its estimated purity by LCMS analysis was 100%.

Analysis LCMS Condition D: Retention time = 1.50 min; ESI-MS(+) m/z 912.2 (M+2H).

Analysis LCMS Condition E: Retention time = 1.42 min; ESI-MS(+) m/z 912.4 (M+2H).

5

Preparation of Example 1506



Molecular Weight: 1823.08

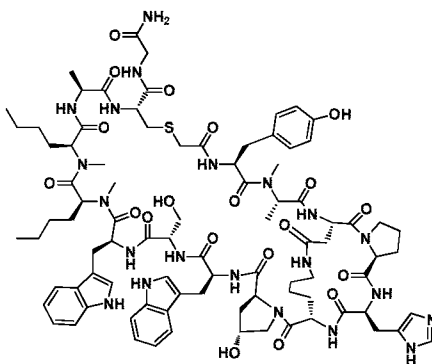
Example 1506 was prepared following the “General Synthetic Sequence” described above, composed of the following general procedures: “Prelude Method C: Resin-swelling procedure”, “Prelude Method C: Single-coupling procedure”, “Prelude Method C: Secondary amine-coupling procedure”, “Prelude Method C: Final wash procedure”, Chloroacetic acid coupling procedure B “Global Deprotection Method E”, “Cyclization Method D”, and “Lactam formation 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: 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 yield of the product was 1.2 mg, and its estimated purity by LCMS analysis was 96%.

Analysis LCMS Condition H: retention time = 1.49 min.; ESI-MS(+) m/z 912.6 (M+2H).

Analysis LCMS Condition I: retention time = 2.60 min.; ESI-MS(+) m/z 912.7 (M+2H).

ESI-HRMS(+) m/z : Calculated: 911.9403(M+2H); Found: 911.9391 (M+2H).

Preparation of Example 1507

Example 1507 was prepared following the “General Synthetic Sequence”

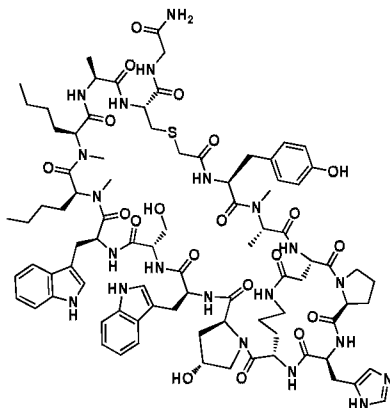
- 5 described above, composed of the following general procedures: “Prelude Method C: *Resin-swelling procedure*”, “Prelude Method C: *Single-coupling procedure*”, “Prelude Method C: *Secondary amine-coupling procedure*”, “Prelude Method C: *Final wash procedure*”, *Chloroacetic acid coupling procedure B* “*Global Deprotection Method E*”, “*Cyclization Method D*”, and “*Lactam formation A*”. The
- 10 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: 40-80% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired
- 15 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: 15-55% B over 30 minutes, then a 5-minute hold
- 20 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 4.5 mg, and its estimated purity by LCMS analysis was 98%.

Analysis LCMS Condition H: retention time = 1.54 min.; ESI-MS(+) m/z 913.2 (M+2H).

- 25 *Analysis LCMS Condition I*: retention time = 2.67 min.; ESI-MS(+) m/z 912.9 (M+2H).

ESI-HRMS(+) m/z : Calculated: 911.9385 (M+2H); Found: 911.9403 (M+2H).

Preparation of Example 1508



Molecular Weight: 1809.05

Example 1508 was prepared following the “General Synthetic Sequence” described above, composed of the following general procedures: “Prelude Method C: Resin-swelling procedure”, “Prelude Method C: Single-coupling procedure”, “Prelude Method C: Secondary amine-coupling procedure”, “Prelude Method C: Final wash procedure”, Chloroacetic acid coupling procedure B “Global Deprotection Method E”, “Cyclization Method D”, and “Lactam formation 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 0.1% trifluoroacetic acid; Mobile Phase B: 95:5 acetonitrile: water with 0.1% trifluoroacetic acid; 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 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 yield of the product was 6.7 mg, and its estimated purity by LCMS analysis was 100%.

Analysis LCMS Condition H: retention time = 1.55 min.; ESI-MS(-) m/z 904.7 (M+2H).

Analysis LCMS Condition I: retention time = 3.11 min.; ESI-MS(+) m/z 905.1 (M+2H).

ESI-HRMS(+) m/z : Calculated: 904.9302 (M+2H); Found: 904.9325 (M+2H)

5 *The following procedures were used for Examples 5001-5008:*

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 “ m/z ” unit designation. Compounds with exact masses greater than 1000 were often detected as double-charged or triple-charged ions.

Analysis Condition A:

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:

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.

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2017227777*Analysis Condition C:*

Column: Phenomenex Luna C18 2.0 X 30 mm 3.0 μ m particles; Mobile Phase A: acetonitrile:water (10:90) with 0.1% TFA; Mobile Phase B: acetonitrile:water (90:10) with 0.1% TFA; Temperature: 40 °C; Gradient: 0%B, 0-100% B over 2 minutes, then
5 a 1.0-minute hold at 100% B; Flow: 0.8 mL/min; Detection: UV at 220 nm.

Analysis Condition D:

Column: Waters Aquity 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;
10 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.

Analysis Condition E:

Column: Waters Aquity 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;
15 Temperature: 40 °C; Gradient: 0%B, 0-100% B over 3 minutes, then a 0.5-minute hold at 100% B; Flow: 0.8 mL/min; Detection: UV at 220 nm.

General Procedures:

20

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
25 exceeded 0.100 mmol, a 40 mL polypropylene tube fitted with a bottom frit was 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
30 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

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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

- 5 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); 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; 15 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 20 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 compound synthesis sequences began with a resin-swelling procedure, described below as “Resin-swelling procedure”. Coupling of amino acids to a 25 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 compound is described by the “Chloroacetyl chloride coupling procedure” detailed below.

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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 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 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 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 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 piperidine:DMF (20:80 v/v, 2.0 mL). The mixture was periodically agitated for 3

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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 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, 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, 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 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:

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 was washed successively six times as follows: for each wash, DMF (2.0 mL) was

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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 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 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 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” 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 this solution was delivered to the reaction.

Chloroacetyl chloride coupling procedure:

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

5 *Global Deprotection Method A:*

10 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 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 15 (1000 RPM for 1 minute, then 500 RPM for 1-2h). The mixture was filtered through 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 20 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 25 and discarded to afford the crude compound as a white to off-white solid.

Cyclization Method A:

30 All manipulations were performed manually unless noted. The procedure of “Cyclization 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 that was used to generate the compound. This scale is not based on a direct determination of the quantity of compound used in the procedure. The procedure can be scaled

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beyond 0.100 mmol scale by adjusting the described volumes by the multiple of the scale. The crude compound solids were dissolved in MeCN:aq. 0.1M NH₄OAc (1:1) to a total volume of 60 mL, and the solution was carefully then adjusted to pH = 8.5-9.0 using aq NaOH (1.0M). The solution was then stirred for 12-18h. The reaction solution was concentrated and the residue was then dissolved in DMSO:MeOH. This solution was subjected to reverse-phase HPLC purification to afford the desired cyclic compound.

Cyclization Method B:

All manipulations were performed manually unless noted. The procedure of "Cyclization 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 that was used to generate the compound. This scale is not based on a direct determination of the quantity of compound 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 compound solids were dissolved in MeCN:aq. 0.1M NH₄OAc (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 12-18h. The reaction solution was concentrated and the residue was then dissolved in DMSO:MeOH. This solution was subjected to reverse-phase HPLC purification to afford the desired cyclic compound.

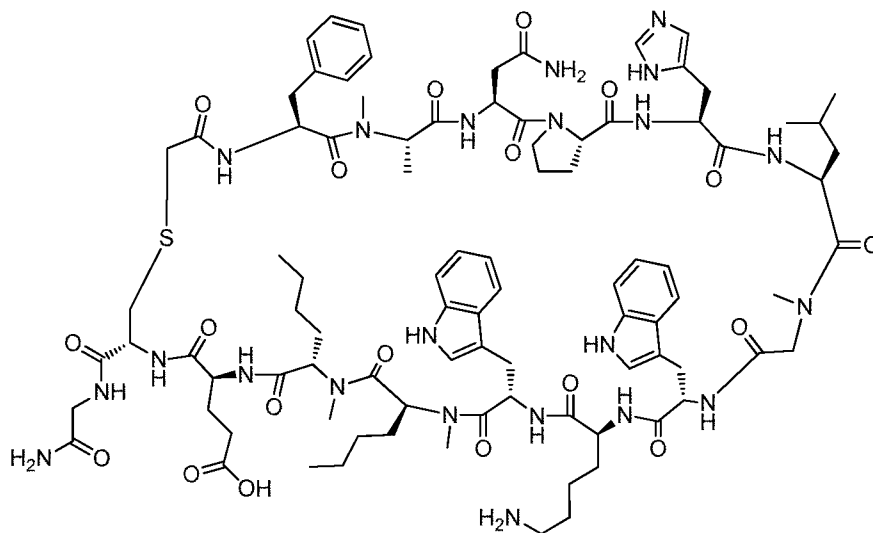
General Synthetic Sequence A:

"General Synthetic Sequence A" describes a general sequence of procedures that were used to afford the cyclic compounds 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 compound was a primary

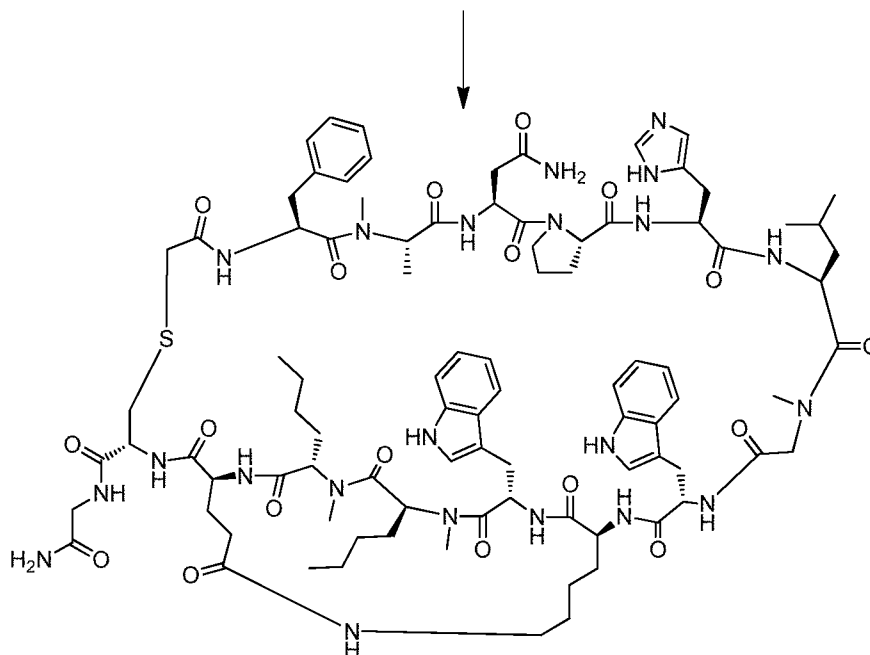
amine or “Prelude Method A: Double-coupling procedure” if the N-terminus of the resin-bound compound was a secondary amine. “Prelude Method A: Chloroacetyl chloride coupling procedure” was followed; then “Global Deprotection Method A” was followed; then “Cyclization Method A” was followed.

5

Preparation of Example 5001



Intermediate 5001



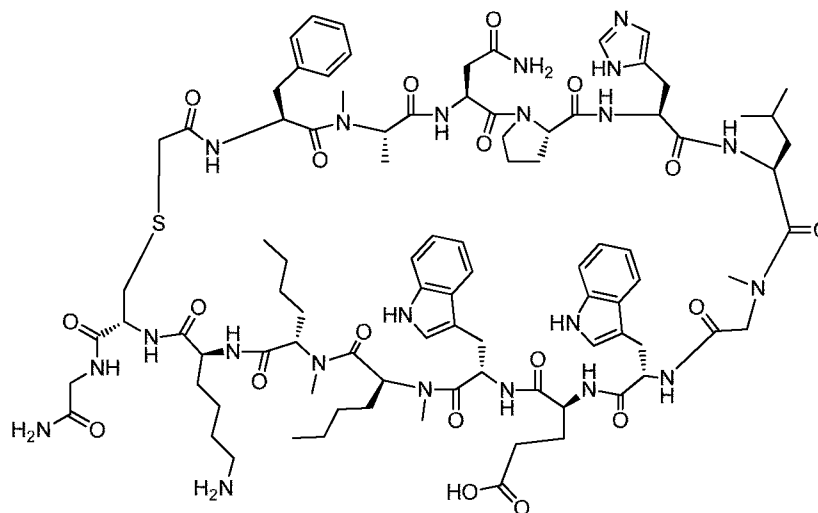
Example 5001

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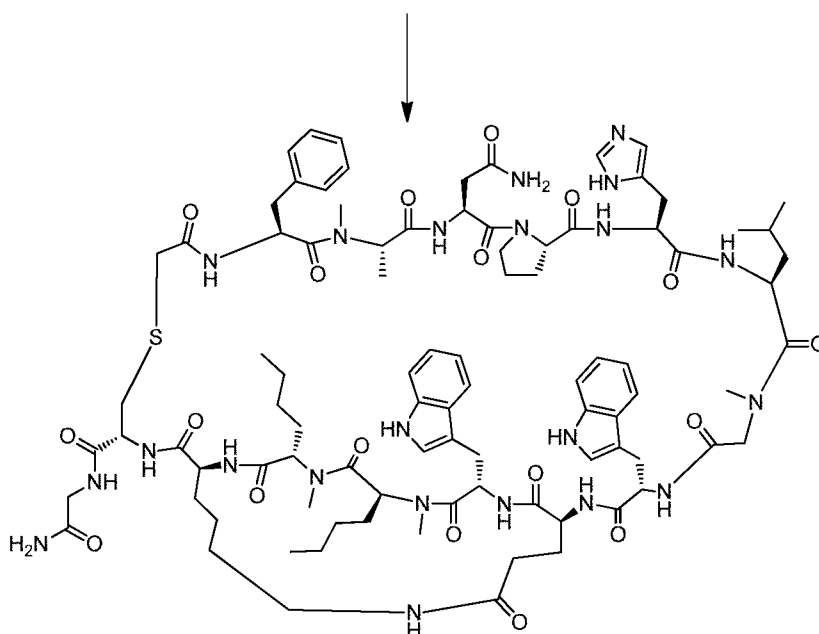
Intermediate 5001 was prepared following "General Synthetic Sequence A" on a 0.200 mmol scale. The crude material was purified via preparative HPLC with the following conditions: Column: Luna C18, 30 x 100 mm, 5- μ m particles; Mobile Phase A: 5:95 acetonitrile: water with 0.1% TFA; Mobile Phase B: 95:5 acetonitrile: water with 0.1% TFA; Gradient: 0-60% B over 10 minutes, then a 5-minute hold at 100% B; Flow: 42 mL/min. The desired product eluted at 9.32 min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of the product was 53.8 mg. Analysis condition D: Retention time = 1.106 min; ESI-MS(+) m/z 934.05 (M+2H).

Example 5001 was prepared as follows: To a 2 mL vial charged Intermediate 5001 (53.8 mg, 0.029 mmol) in DMF (3 mL) was added DIPEA (0.101 mL, 0.577 mmol) and HATU (43.8 mg, 0.115 mmol). The solution was stirred at room temperature for 1h. 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: 50-90% B over 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 11.2 mg, and its estimated purity by LCMS analysis was 99%. Analysis condition A: Retention time = 1.96 min; ESI-MS(+) m/z 924.6 (M+2H) Analysis condition B: Retention time = 3.13 min; ESI-MS(+) m/z 924.7 (M+2H).

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Preparation of Example 5002

Intermediate 5002



Example 5002

Intermediate 5002 was prepared following "General Synthetic Sequence A" on a 0.200 mmol scale. The crude material was purified via preparative HPLC with the following conditions: Column: Luna C18, 30 x 100 mm, 5- μ m particles; Mobile Phase A: 5:95 acetonitrile: water with 0.1% TFA; Mobile Phase B: 95:5 acetonitrile: water with 0.1% TFA; Gradient: 10-60% B over 10 minutes, then a 5-minute hold at 100% B; Flow: 42 mL/min. Fractions containing the desired product were combined

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and dried via centrifugal evaporation. The yield of the product was 50.0 mg.

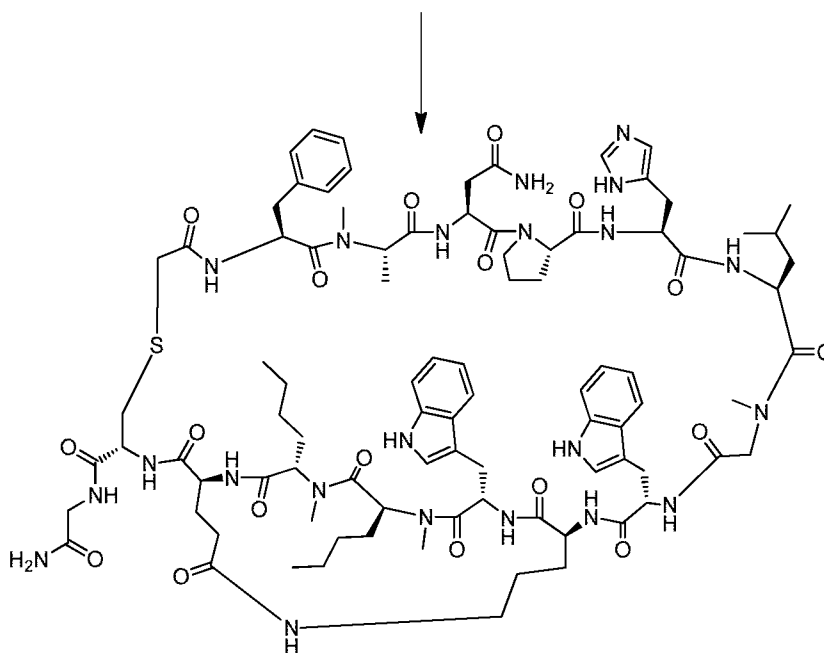
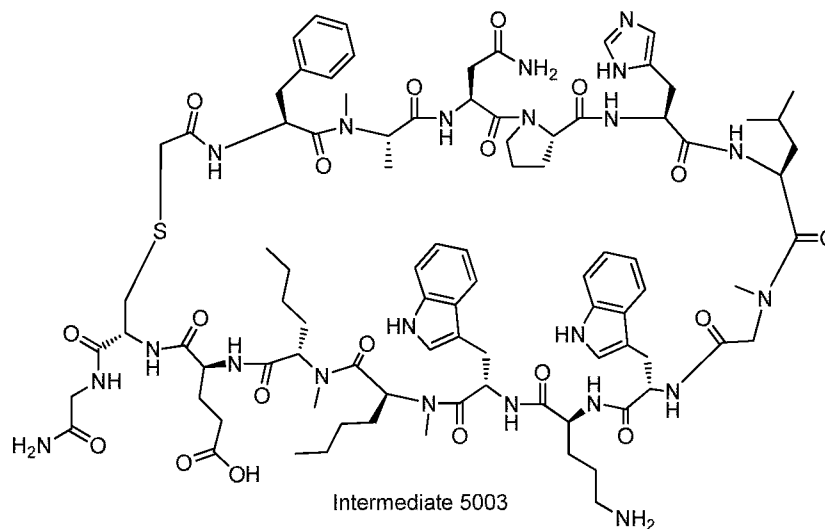
Analysis condition C: Retention time = 1.272 min; ESI-MS(+) m/z 933.75 (M+2H).

Example 5002 was prepared as follows: To a 7 mL vial charged Intermediate 5002 (50 mg, 0.024 mmol) in DMF (3 mL) was added DIPEA (0.083 mL, 0.478 mmol) and HATU (36.3 mg, 0.096 mmol). The solution was stirred at room temperature for 30 minutes. 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: 30-70% B over 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 14.6 mg, and its estimated purity by LCMS analysis was 100%.

Analysis condition A: Retention time = 1.82 min; ESI-MS(+) m/z 924.8 (M+2H)

Analysis condition B: Retention time = 3.01 min; ESI-MS(+) m/z 923.3 (M-2H).

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Preparation of Example 5003

Example 5003

Intermediate 5003 was prepared following "General Synthetic Sequence A" on a 0.200 mmol scale. The crude material was purified via preparative HPLC with the following conditions: Column: Luna C18, 30 x 100 mm, 5- μ m particles; Mobile Phase A: 5:95 acetonitrile: water with 0.1% TFA; Mobile Phase B: 95:5 acetonitrile: water with 0.1% TFA; Gradient: 10-60% B over 10 minutes, then a 5-minute hold at 100% B; Flow: 42 mL/min. Fractions containing the desired product were combined

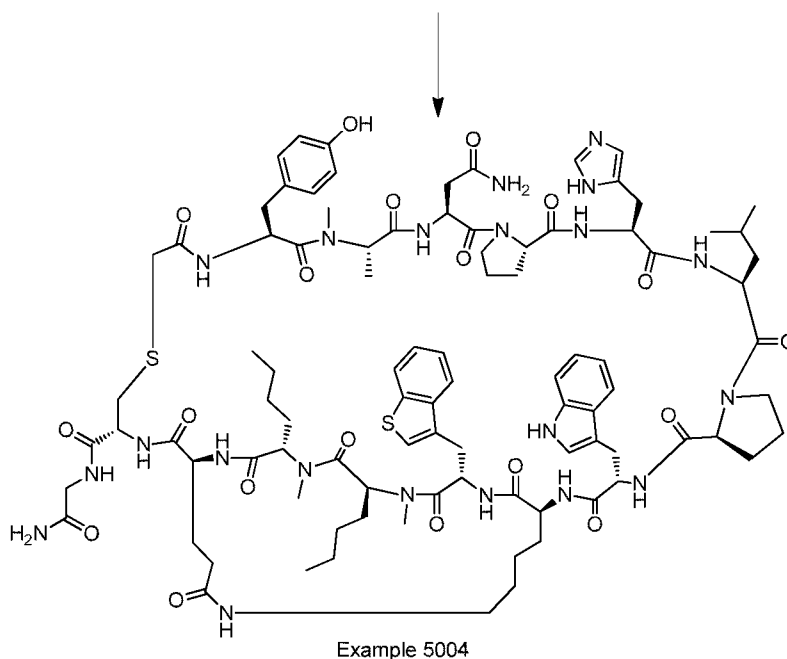
and dried via centrifugal evaporation. The yield of the product was 50.0 mg.

Analysis condition C: Retention time = 1.300 min; ESI-MS(+) m/z 926.75 (M+2H).

Example 5003 was prepared as follows: To a 7 mL vial charged Intermediate 5003 (50 mg, 0.024 mmol) in DMF (3 mL) was added DIPEA (0.084 mL, 0.481

- 5 mmol) and HATU (36.6 mg, 0.096 mmol). The solution was stirred at room temperature for 30 minutes. 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: 25-65% B over 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 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: 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 12.5 mg, and its estimated purity by LCMS analysis was 98%. Analysis condition A: Retention time = 1.94 min; ESI-MS(+) m/z 917.5 (M+2H)
- 10 20 Analysis condition B: Retention time = 3.03 min; ESI-MS(+) m/z 917.4 (M+2H)

Intermediate 5004



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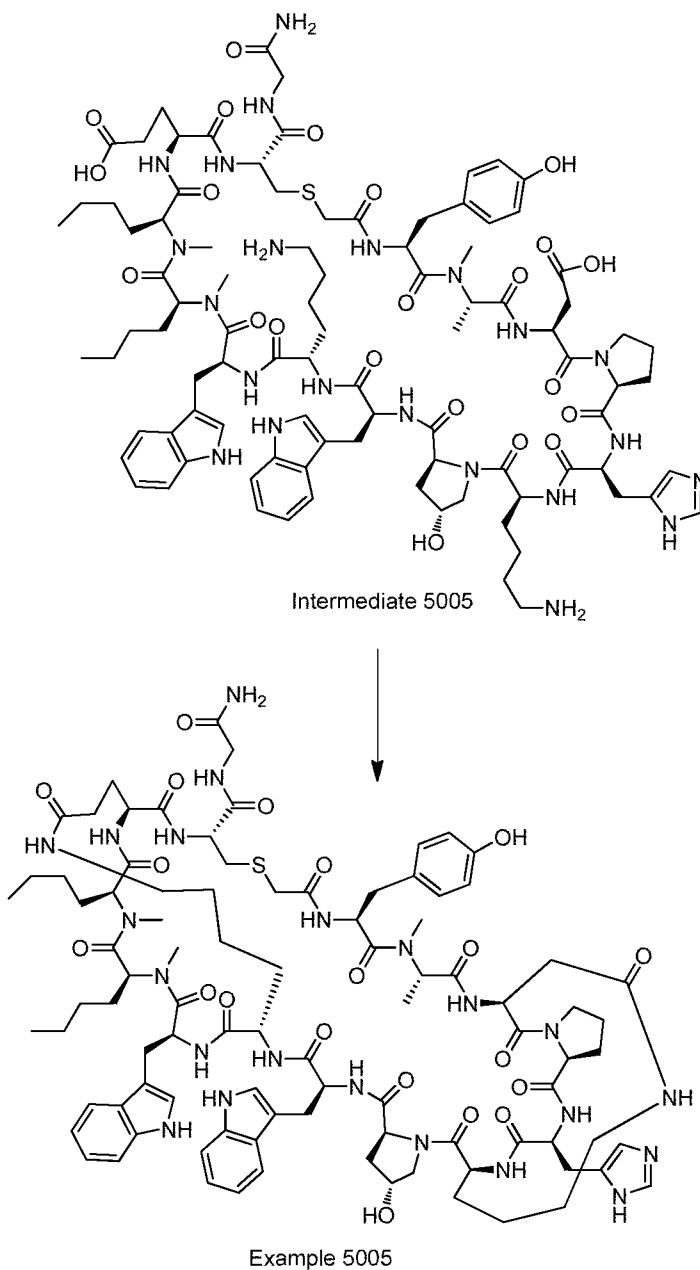
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Example 5004 was prepared as follows: Intermediate 5004 (35.5 mg, 0.018 mmol) was dissolved in DMF (2.0 mL). To the solution was added DIPEA (0.075 mL, 0.43 mmol), then HATU (28 mg, 0.074 mmol). The solution was stirred at room temperature for 45 minutes. 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: 35-75% B over 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 4.9 mg, and its estimated purity by LCMS analysis was 95%.

Analysis condition A: Retention time = 2.14 min; ESI-MS(+) m/z 954.1 (M+2H)

Analysis condition B: Retention time = 2.93 min; ESI-MS(+) m/z 952.4 (M-2H).

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Preparation of Example 5005

Intermediate 5005 was prepared following "General Synthetic Sequence A" on a 0.600 mmol scale, using "Cyclization Method B" instead of "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 15

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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

- 5 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 resulting material was triturated with aq. HCl, then dried in vacuo. The yield of the product was 112.8
- 10 mg, and its estimated purity by LCMS analysis was 93%.

Analysis condition A: Retention time = 1.37 min; ESI-MS(+) m/z 971.2 (M+2H)

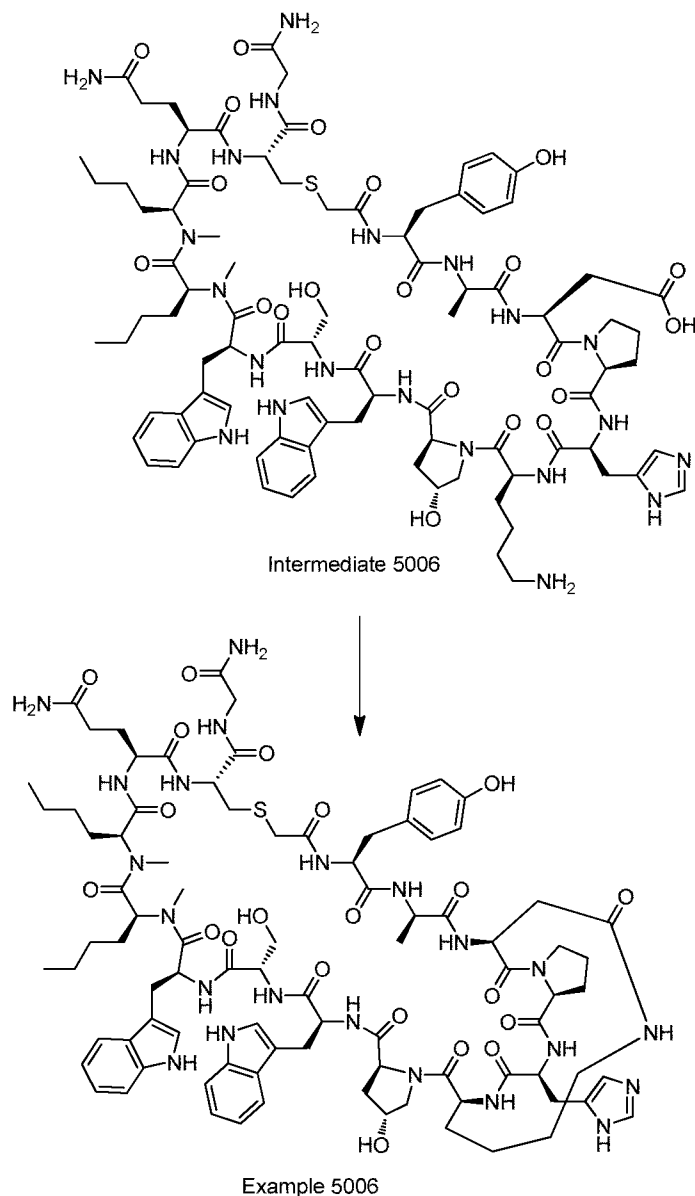
Analysis condition B: Retention time = 2.58 min; ESI-MS(+) m/z 971.3 (M+2H)

- Example 5005 was prepared as follows: To a 15 mL vial equipped with a stir bar and charged with Intermediate 5005 (26.0 mg, 0.013 mmol) was added DMF
- 15 (0.50 mL), then DIPEA (0.023 mL, 0.129 mmol), then HATU (12.28 mg, 0.032 mmol). The solution was stirred at room temperature for 15 minutes. To the reaction was added ethanolamine (0.1 mL). 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;
- 20 Mobile Phase B: 95:5 methanol: water with 10-mM ammonium acetate; Gradient: 45-85% B over 30 minutes, then a 7-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 100%.

- 25 Analysis condition A: Retention time = 1.58 min; ESI-MS(+) m/z 952.8 (M+2H)

Analysis condition B: Retention time = 2.73 min; ESI-MS(+) m/z 953.2 (M+2H).

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Preparation of Example 5006

Intermediate 5006 was prepared following "General Synthetic Sequence A" on a 0.400 mmol scale, using "Cyclization Method B" instead of "Cyclization

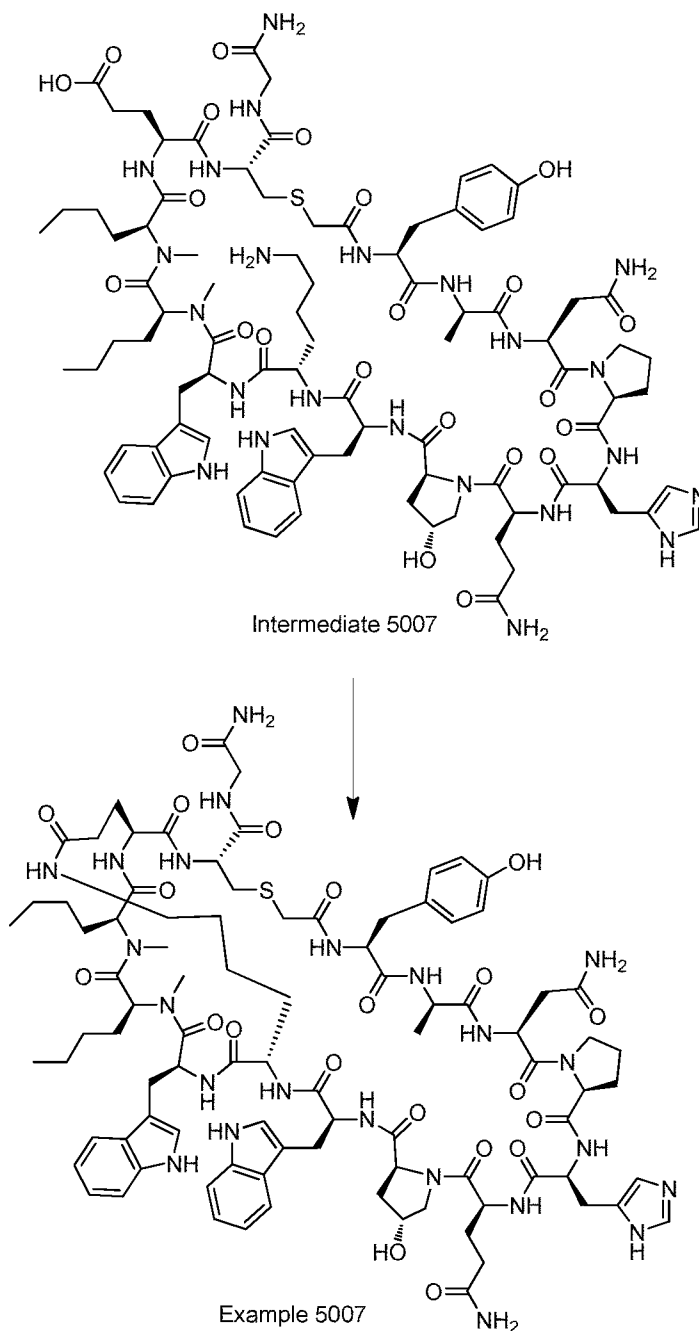
5 Method A". The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A: 5:95 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B: 95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 10-50%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

10 the desired product were combined and dried via centrifugal evaporation. The

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- 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: 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 yield of the product was 54.7 mg, and its estimated purity by LCMS analysis was 100%. Analysis condition A: Retention time = 1.43 min; ESI-MS(+) m/z 941.3 (M-2H) Analysis condition B: Retention time = 2.62 min; ESI-MS(+) m/z 940.8 (M-2H)
- 10 ESI-HRMS(+) m/z: Calculated: 942.4485 Found: 942.4475.
- Example 5006 was prepared as follows: To a 1 dram vial equipped with a stir bar and charged with Intermediate 5006 (40 mg mg, 0.020 mmol) was added NMP (1.00 mL), then DIPEA (0.036 mL, 0.20 mmol), then HATU (19 mg, 0.051 mmol). The solution was stirred at room temperature for 4h. To the reaction solution was
- 15 added MeNH₃Cl (5 mg) and HATU (10 mg). The reaction was stirred at room temperature for 30 minutes. To the solution was added AcOH (3 drops). 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
- 20 ammonium acetate; Gradient: 45-100% 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
- 25 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 5.2 mg, and its estimated purity by LCMS analysis was 100%.
- 30 Analysis condition A: Retention time = 1.497 min; ESI-MS(+) m/z 933.45 (M+2H) Analysis condition B: Retention time = 2.139 min; ESI-MS(+) m/z 933.70 (M+2H) ESI-HRMS(+) m/z: Calculated: 933.4432 Found: 933.4416.

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Preparation of Example 5007

Intermediate 5007 was prepared following "General Synthetic Sequence A" on a 0.400 mmol scale, using "Cyclization Method B" instead of "Cyclization

5 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:

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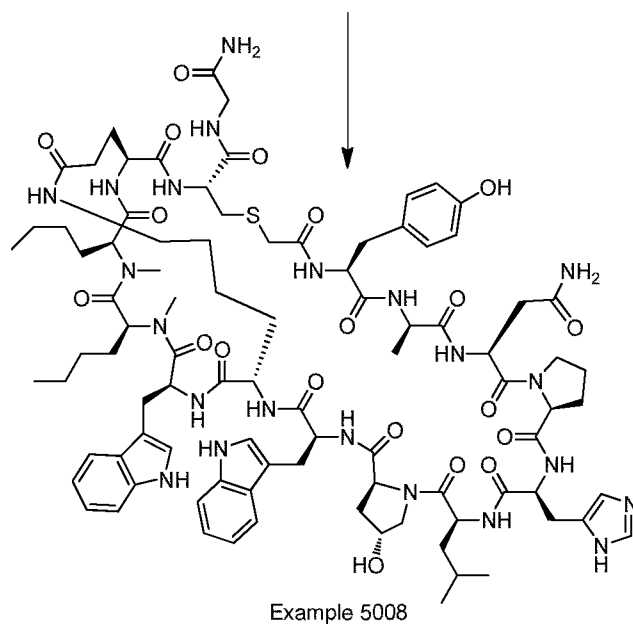
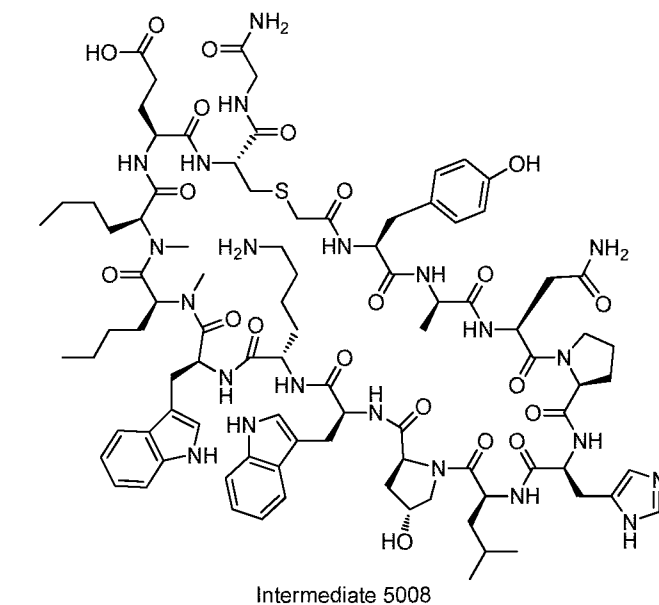
95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 10-50% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of the product was 75.1 mg, and its estimated purity by LCMS analysis was 100%.

- 5 Analysis condition A: Retention time = 1.408 min; ESI-MS(+) m/z 961.40 (M-2H)
Analysis condition B: Retention time = 2.912 min; ESI-MS(+) m/z 963.45 (M+2H)
ESI-HRMS(+) m/z: Calculated: 962.9618 Found: 962.9592

- Example 5007 was prepared as follows: To a 1 dram vial equipped with a stir bar and charged Intermediate 5007 (49 mg, 0.025 mmol) was added NMP (1.00 mL),
10 then DIPEA (0.043 mL, 0.25 mmol), then HATU (23 mg, 0.062 mmol). The solution was stirred at room temperature for 4h. To the reaction solution was added MeNH₃Cl (5 mg) and HATU (10 mg). The reaction was stirred at room temperature for 1h. To the solution was added AcOH (3 drops). The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x
15 200 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 yield of the product was 3.3 mg, and its
20 estimated purity by LCMS analysis was 97%.

Analysis condition A: Retention time = 1.55 min; ESI-MS(+) m/z 952.3 (M-2H)
Analysis condition B: Retention time = 2.68 min; ESI-MS(+) m/z 954.3 (M+2H)
ESI-HRMS(+) m/z: Calculated: 953.9565 Found: 953.9542.

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Preparation of Example 5008

Intermediate 5008 was prepared following "General Synthetic Sequence A" on a 0.400 mmol scale, using "Cyclization Method B" instead of "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

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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 122.4 mg, and its estimated purity by LCMS analysis was 97%.

Analysis condition A: Retention time = 1.55 min; ESI-MS(+) m/z 957.5 (M+2H)

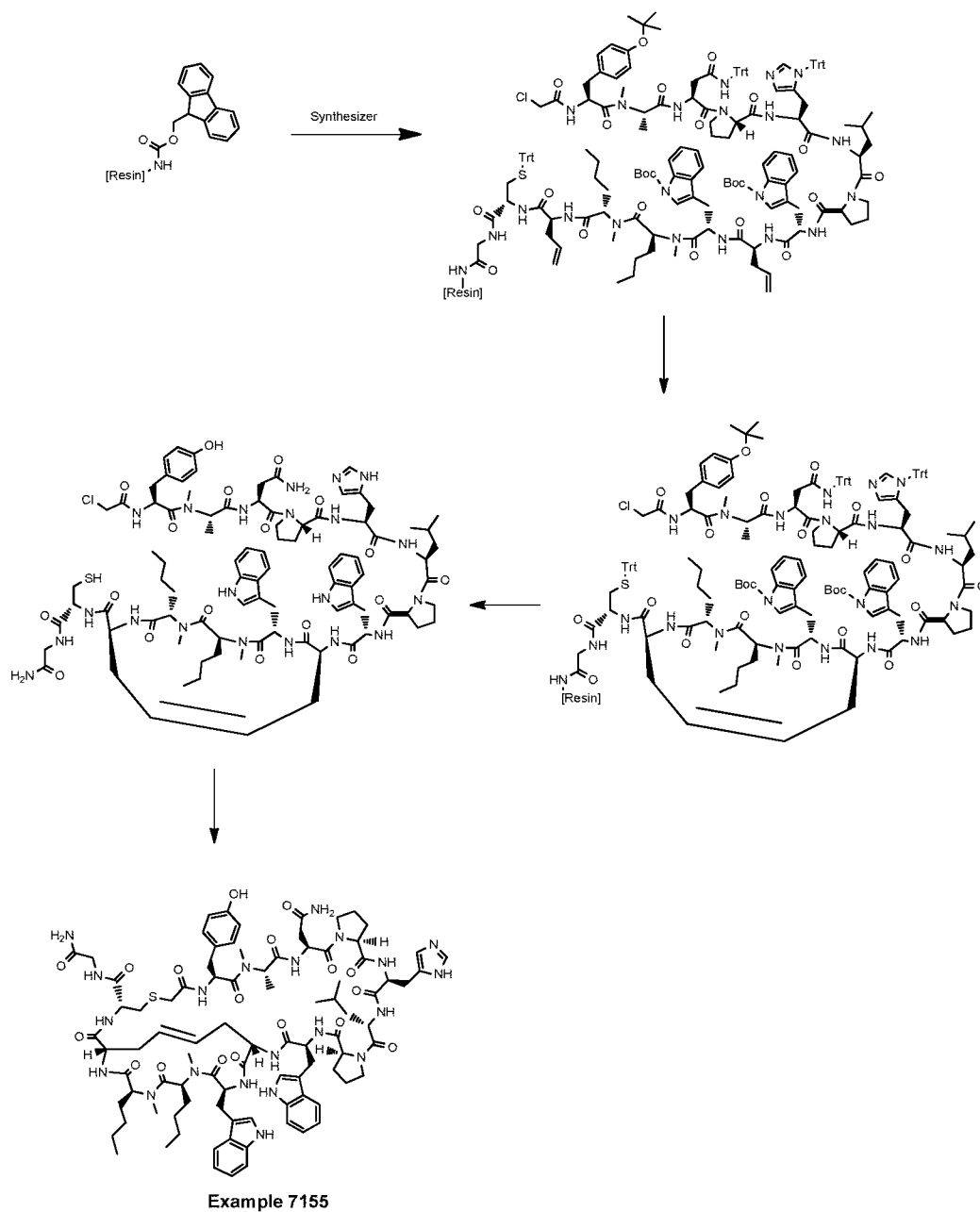
- 5 Analysis condition B: Retention time = 1.82 min; ESI-MS(+) m/z 954.5 (M-2H)
ESI-HRMS(+) m/z: Calculated: 955.9577 Found: 955.9543

- Example 5008 was prepared as follows: To a 1 dram vial equipped with a stir bar and charged Intermediate 5008 (46 mg, 0.023 mmol) was added NMP (1.00 mL), then DIPEA (0.041 mL, 0.23 mmol), then HATU (22 mg, 0.058 mmol). The solution
10 was stirred at room temperature for 4h. To the reaction solution was added MeNH₃Cl (5 mg) and HATU (10 mg). The solution was stirred at room temperature for 1h, then to the solution was added AcOH (3 drops). The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 200 mm, 5-μm particles; Mobile Phase A: 5:95 acetonitrile: water with 10-
15 mM ammonium acetate; Mobile Phase B: 95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 20-60% 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
20 mm, 5-μm 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: 50-100% 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 1.7 mg, and its estimated
25 purity by LCMS analysis was 100%.

Analysis condition E: Retention time = 1.833 min; ESI-MS(+) m/z 948.15 (M+2H)

Analysis condition B: Retention time = 2.545 min; ESI-MS(+) m/z 946.55 (M+2H).

Preparation of Example 7155



Example 7155 was prepared on a 100 $\mu\text{mol} \times 4$ scale by following the general synthetic sequence described for the preparation of Example 5001, composed of the following general procedures: “Prelude Method A: Resin-swelling procedure”, “Prelude Method A: Single-coupling procedure”, “Prelude Method A: Double-coupling procedure”, “Prelude Method A: Chloroacetyl chloride coupling procedure”.

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To the resulting resin bound compound (100 μ mol), in a microwave vial, was added 1 eq (100 μ m, 63mgs) of Hoveyda-Grubb's second generation catalyst and 17mL of dichloroethane. The vial was sealed, and the mixture was de-gassed/flushed with nitrogen and then subjected to microwave heating at 120 $^{\circ}$ C for 1 hour. The reaction was repeated 4 times, the resulting resin was combined in a solid phase synthesis vessel, filtered, washed with DMF (4 x 20mL), then with (DCM 5 x 20mL) and dried under vacuum overnight. The material was divided into four 100 μ mol batches.

The "Global Deprotection Method A" and "Cyclization Method A" were then employed.

Global Deprotection Method A: The procedure of "Global Deprotection Method A" describes an experiment performed on a 100 μ mol x 4 scale, where the scale is determined by the amount of Rink linker bound to the resin. A "deprotection solution" was prepared by combining in a 40 mL glass jar trifluoroacetic acid (22 mL), phenol (1.325 g), water (1.25 mL) and triisopropylsilane (0.5 mL). To the resin contained in a solid phase synthesis vessel vial was added the "deprotection solution" (8.0 mL). The mixture was mixed on an orbital shaker (175RPM for 2h). The mixture was filtered, the solids washed with additional "de-protection solution" (4.0 mL) and collected into a 40 mL screw capped vial. To 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 3 minutes at 2000 RPM, the solution decanted away from the solids and discarded. The process was repeated three times, the solids were then allowed to sit on the bench and air dry for 1-2hrs before carrying on affording the crude compound as an off-white solid.

Cyclization Method A: The procedure of "Cyclization Method A" describes an experiment performed on a 100 μ mol x 4 scale. The crude compound solids were dissolved in MeCN:aq. 0.1M NH₄OAc (60mL:60mL), and the solution was then carefully adjusted to pH = 9.0 using aq NaOH (1.0M). The vials were capped and the solution was then mixed at 175RPM on an orbital shaker overnight (~18h). The reaction solution was concentrated and the residue was then dissolved in MeOH.

The crude compound was purified via preparative LC/MS with the following conditions: Column: XBridge C18, 5 μ m, 19 x 200 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; 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 yield of the product was 3.0 mg, and its estimated purity by LCMS analysis was 84%.

Analysis condition A: Retention time = 1.63 min; ESI-MS(+) m/z = 909.5 (M + 2H)

Analysis condition B: Retention time = 2.70 min; ESI-MS(+) m/z = 909.5 (M + 2H).

The following analytical LC/MS conditions were used to determine the final purity.

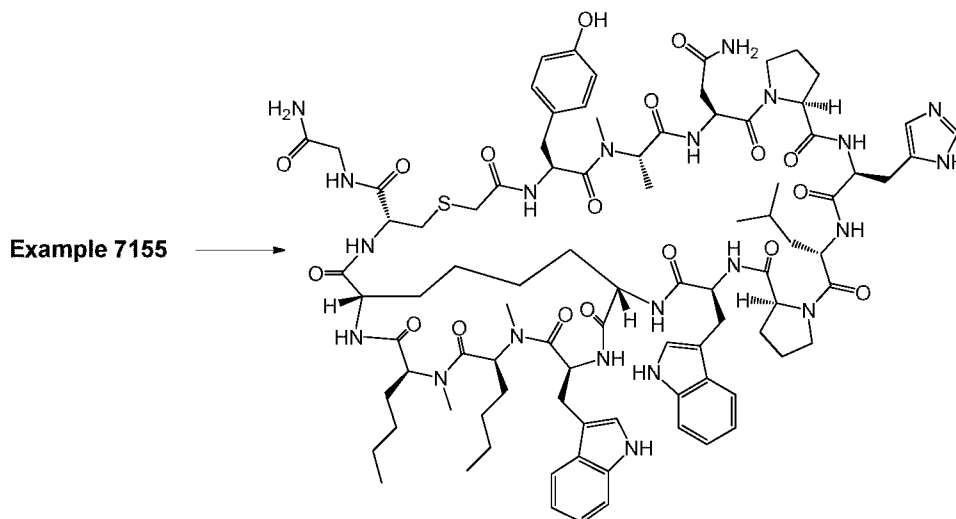
10 Analysis condition A: Column: Waters BEH C18, 2.0 x 50 mm, 1.7- μ m particles; Mobile Phase A: 5:95 acetonitrile:water with 10 mM ammonium acetate; Mobile Phase B: 95:5 acetonitrile:water with 10 mM ammonium acetate; Temperature: 50°C; Gradient: 0%B, 0-100% B over 3 minutes, then a 0.5-minute hold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

15 Analysis condition B: 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.

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Preparation of Example 7156



To a round-bottom flask was added 3.0mgs of Example 7155 (1.65 μ mol) and 2mL of methanol. The flask was sealed and the solution was de-gassed and flushed with nitrogen. To the solution was then added 1eq of 10% Pd/C, the flask was fitted with a balloon of hydrogen and the mixture stirred overnight at room temperature. The resulting product was filtered and purified via preparative LC/MS with the following conditions: Column: XBridge C18, 5 μ m, 19 x 200 mm, 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 of the product was 1.7 mg, and its estimated purity by LCMS analysis was 100%.

Analysis condition A: Retention time = 1.69 min; ESI-MS(+) m/z = 910.5 (M + 2H)

Analysis condition B: Retention time = 2.70 min; ESI-MS(+) m/z = 910.5 (M + 2H).

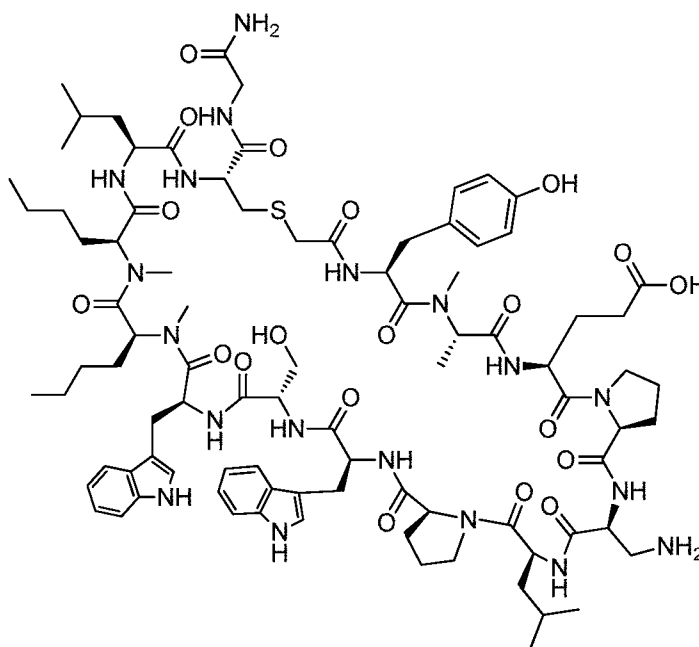
The following analytical LC/MS conditions were used to determine the final purity.

Analysis condition A: 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: Column: Waters BEH C18, 2.0 x 50 mm, 1.7-μm 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.

Preparation of Intermediate 10500



Intermediate 10500

Intermediate 10500 was prepared following “General Synthetic Sequence A” but using “Cyclization Method B” instead of “Cyclization Method A. The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 200 mm, 5-μm 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: 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: XBridge C18, 19 x 200

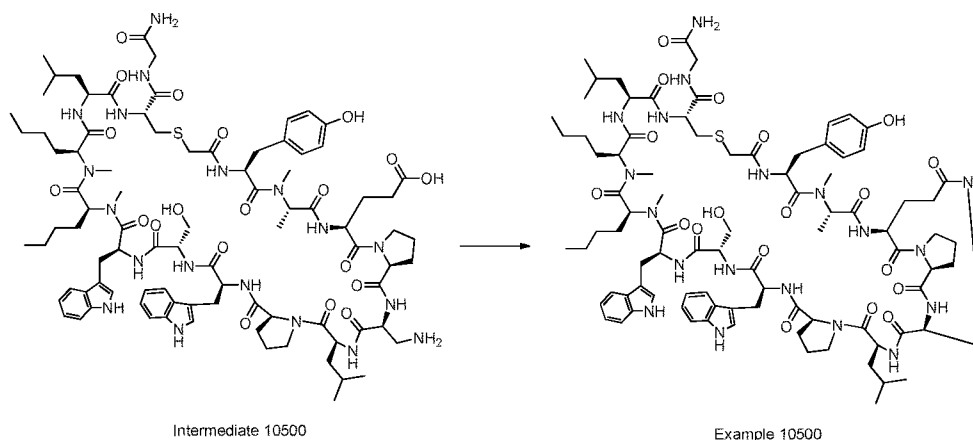
mm, 5- μ m particles; Mobile Phase A: 5:95 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B: 95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 10-50% B over 40 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of the product was 25.8 mg, and its estimated purity by LCMS analysis was 99%.

Analysis condition A: Retention time = 1.66 min; ESI-MS(+) m/z 908.4 (M+2H).

Analysis condition B: Retention time = 2.81 min; ESI-MS(+) m/z 908.4 (M+2H).

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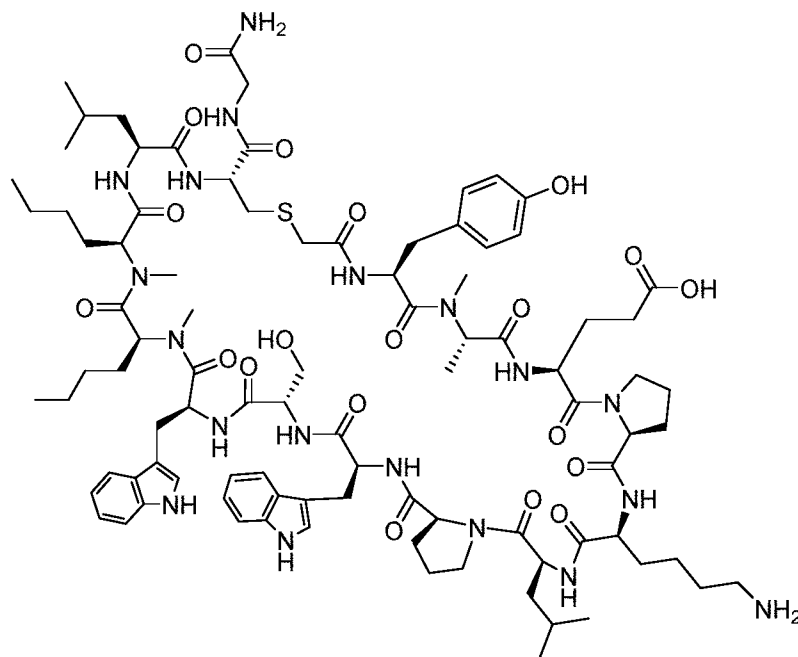
Preparation of Example 10500



Intermediate 10500 (15 mg, 8.26 μ mol) was dissolved in DMF (1 mL) followed by the addition of Hunig'sBase (7.22 μ l, 0.041 mmol) then HATU (4.71 mg, 0.012 mmol). The reaction was stirred for 1 h. The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 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: 30-70% 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 1.2 mg, and its estimated purity by LCMS analysis was 99%.

Analysis condition A: Retention time = 1.85 min; ESI-MS(+) m/z 899.5 (M+2H).

Analysis condition B: Retention time = 2.98 min; ESI-MS(+) m/z 899.7 (M+2H).

Preparation of Intermediate 10501

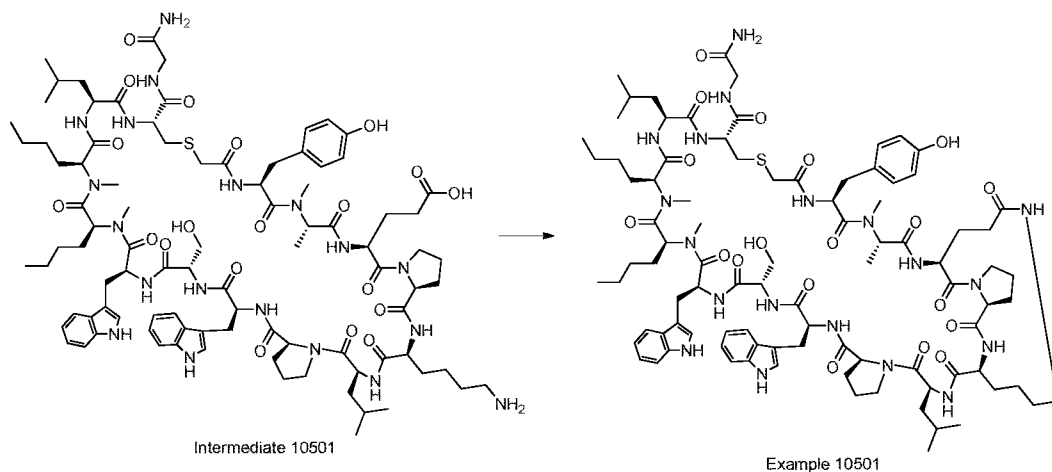
Intermediate 10501

Intermediate 10501 was prepared following “General Synthetic Sequence A”.

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

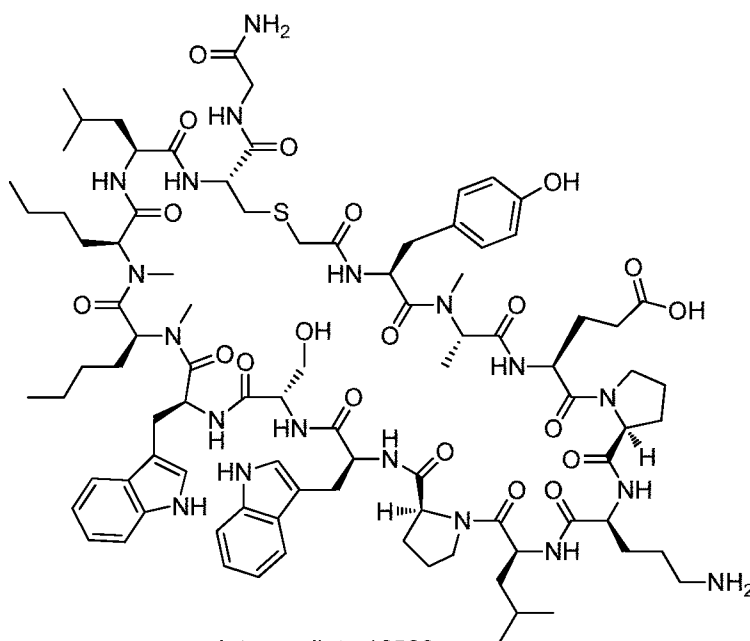
- 5 conditions: Column: XBridge C18, 19 x mm, 5- μ m particles; Mobile Phase A: 5:95 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B: 95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 15-55% B over 30 minutes, then a 5-minute hold at 100% B; Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation. The yield of
- 10 the product was 34.0 mg, and its estimated purity by LCMS analysis was 100%. Analysis condition A: Retention time = 1.64 min; ESI-MS(+) m/z 929.4 (M+2H). Analysis condition B: Retention time = 2.82 min; ESI-MS(+) m/z 929.8 (M+2H).

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Preparation of Example 10501

Example 10501 was prepared following the method used to to prepare Example 10500. 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: 25-65% 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 6.5 mg, and its estimated purity by LCMS analysis was 100%.

Analysis condition A: Retention time = 1.75 min; ESI-MS(+) m/z 920.6 (M+2H).
 Analysis condition B: Retention time = 2.88 min; ESI-MS(+) m/z 920.6 (M+2H).

Preparation of Intermediate 10502

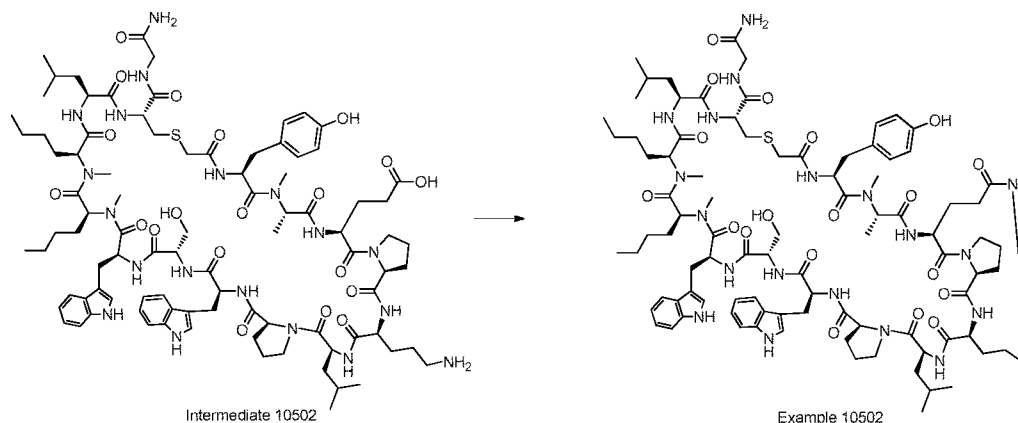
Intermediate 10502

Intermediate 10502 was prepared following “General Synthetic Sequence A”.

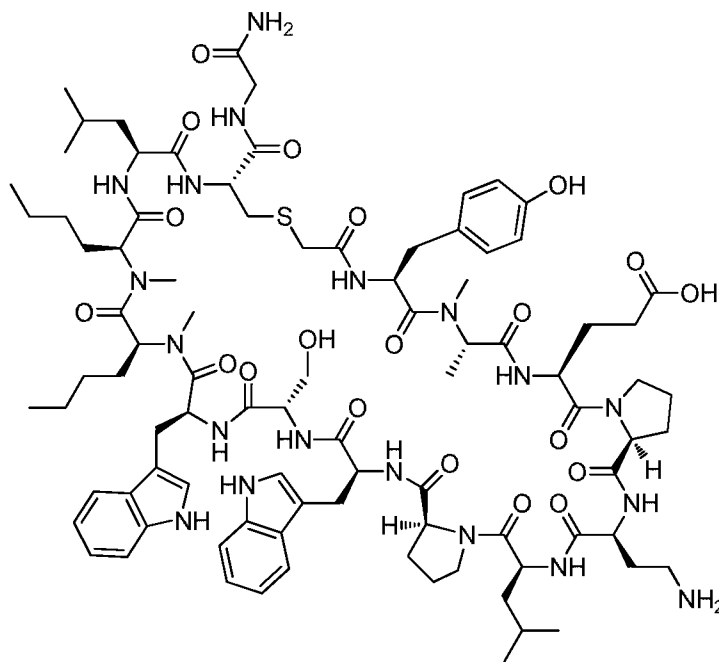
The crude material was purified via preparative LC/MS with the following

- 5 conditions: Column: XBridge C18, 19 x 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 yield of
 - 10 the product was 34.0 mg, and its estimated purity by LCMS analysis was 100%.
- Analysis condition A: Retention time = 1.68 min; ESI-MS(+) m/z 922.5 (M+2H).
 Analysis condition B: Retention time = 2.83 min; ESI-MS(+) m/z 922.8 (M+2H).

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Preparation of Example 10502

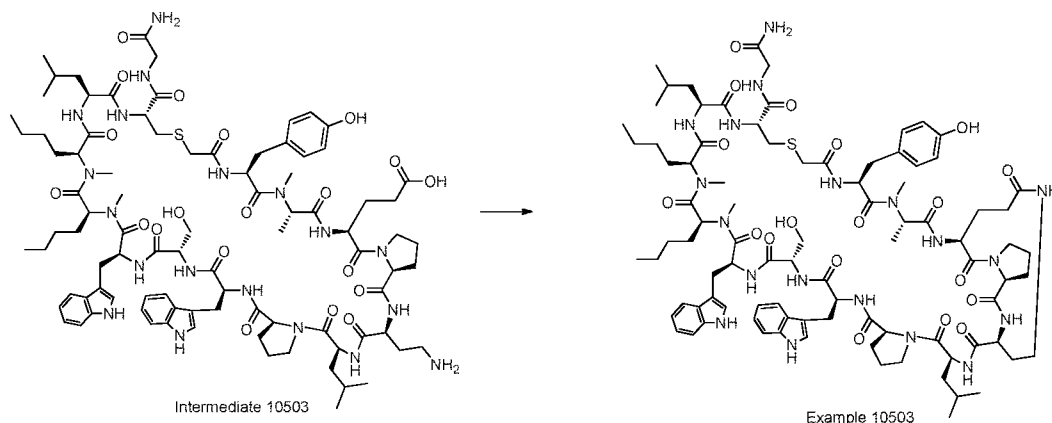
Example 10502 was prepared following the method used to to prepare Example 10500. The crude material was purified via preparative LC/MS with the following conditions: Column: XBridge C18, 19 x 200 mm, 5- μ m particles; Mobile Phase A: 5:95 acetonitrile: water with 10-mM ammonium acetate; Mobile Phase B: 95:5 acetonitrile: water with 10-mM ammonium acetate; Gradient: 25-65% 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 3.1 mg, and its estimated purity by LCMS analysis was 98%. Analysis condition A: Retention time = 1.77 min; ESI-MS(+) m/z 913.4 (M+2H). Analysis condition B: Retention time = 2.88 min; ESI-MS(+) m/z 913.7 (M+2H).

Preparation of Intermediate 10503

Intermediate 10503

Intermediate 10503 was prepared following “General Synthetic Sequence 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 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
- 10 desired product were combined and dried via centrifugal evaporation. The yield of the product was 23.4 mg, and its estimated purity by LCMS analysis was 100%.
 Analysis condition A: Retention time = 1.65 min; ESI-MS(+) m/z 915.8 (M+2H)
 Analysis condition B: Retention time = 2.84 min; ESI-MS(+) m/z 915.8 (M+2H)

Preparation of Example 10503

Example 10503 was prepared following the method used to to prepare Example 10500. 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: 25-65% 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 1.8 mg, and its estimated purity by LCMS analysis was 100%. Analysis condition A: Retention time = 1.78 min; ESI-MS(+) m/z 906.8 (M+2H). Analysis condition B: Retention time = 2.92 min; ESI-MS(+) m/z 906.7 (M+2H).

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

The ability of the macrocyclic compounds 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

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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 µl of assay buffer, followed by addition of PD-1-Ig (20 nM final) in 1 µl of assay buffer and further incubation for 15m. PD-L1 fusion proteins from either human, cynomologous 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 µl 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 compound inhibitors were pre-incubated with PD-L1-His (10 nM final) for 60 minutes in 4 µl of assay buffer followed by addition of biotinylated Compound No. 71 (0.5 nM final) in 1 µl of assay buffer. Binding was allowed to equilibrate for 30 minutes followed by addition of europium cryptate labeled Streptavidin (2.5 pM final) and APC-labeled anti-His (20 nM final) in 5 µl 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

1	LDSPDRPWNP	PTFSPALLVV	TEGDNATFTC	SFSNTSESFV	LNWYRMSPSN
51	QTDKLAAPFE	DRSQPGQDCR	FRVTQLPNGR	DFHMSVVRAR	RNDSGTYLCG
101	AISLAPKAQI	KESLRAELRV	TERRAEVPTA	HPSPSPRPAG	QFQGSPGGGG
151	GREPKSSDKT	HTSPFPAPAE	LLGGSSVFLF	PPKPKDTLMI	SRTPEVTCVV
201	VDVSHEDPEV	KFNWYVDGVE	VHNAKTKPRE	EQYNSTYRVV	SVLTVLHQDW
251	LNGKEYKCKV	SNKALPAPIE	KTISKAKGQP	REPQVYTLPP	SRDELTKNQV
301	SLTCLVKGFY	PSDIAVEWES	NGQPENNYKT	TPPVLDSDGS	FFLYSKLTVD
351	KSRWQQGNVF	SCSVMEALH	NHYTQKSLSL	SPGK	

(SEQ ID NO:1)

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

hPDL1(19-239)-TVMV-His

1	FTVTVPKDLY	VVEYGSNMTI	ECKFPVEKQL	DLAALIVYWE	MEDKNIIQFV
51	HGEEDLKVOH	SSYRQARLL	KDQLSLGNAA	LQITDVKLQD	AGVYRCMISY
101	GGADYKRITV	KVNAPYNKIN	QRILVVDPT	SEHELTCQAE	GYPKAEVIWT
151	SSDHQVLSGK	TTTNSKREE	KLFNVTSTLR	INTTTNEIFY	CTFRRLDPEE
201	NHTAELVIPE	LPLAHPNER	TGSSETVRFQ	GHHHHHH	

(SEQ ID NO:2)

10 The results are shown in Table 1. As shown, the macrocyclic compounds 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 = 0.10-1 μ M; B = 0.01-0.099 μ M; C = 0.003 – 0.0099 μ M.

Table 1

<i>Example Number</i>	<i>HTRF IC₅₀ (μM)</i>
5001	C
5002	B
5003	B
5004	C
1400	B
1401	B
1402	B
10500	A

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<i>Example Number</i>	<i>HTRF IC₅₀ (μM)</i>
10501	A
10502	A
10503	A
5005	B
7155	B
1403	0.007
1404	C
1405	B
1500	B
1501	B
1502	--
1503	0.993
1504	B
1505	B
1506	B
1507	B
1508	B
5006	B
5007	0.014
5008	C
7156	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.

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

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

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

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

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

R^a and R^j are each independently selected from hydrogen and methyl;

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

R¹, R⁸, and R¹⁰ are independently selected from a natural amino acid side chain and an unnatural amino acid side chain;

R³ and R⁶ are independently selected from a natural amino acid side chain and an unnatural amino acid side chain, or, R³ and R⁶ together form a bridge containing between 2 and 7 carbon atoms, optionally one double bond, and optionally one -C(O)NH- or -NHC(O)- group;

R⁹ and R¹³ are independently selected from a natural amino acid side chain and an unnatural amino acid side chain, or, R⁹ and R¹³ together form a bridge containing between 2 and 7 carbon atoms, optionally one double bond, and optionally one -C(O)NH- or -NHC(O)- group;

R⁷ is selected from a natural amino acid side chain and an unnatural amino acid side chain; or R⁷ and R¹⁶ together form a bridge containing between 2 and 7 carbon atoms, optionally one double bond, and optionally one -C(O)NH- or -NHC(O)- group; or R⁷ and R^g can form a ring as described below;

provided that at least one of R^3 and R^6 ; R^9 and R^{13} , and R^7 and R^{16} form a bridge;

R^5 is a natural amino acid side chain or an unnatural amino acid side chain, or, R^3 and R^5 together form a bridge containing between 2 and 7 carbon atoms, optionally one double bond, and optionally one $-C(O)NH-$ or $-NHC(O)-$ group; or R^5 and R^e can form a ring as described below;

R^2 , R^4 , R^{11} , and R^{12} are independently selected from a natural amino acid side chain and an unnatural amino acid side chain or form a ring with the corresponding vicinal R group as described below;

R^b is methyl or, R^b and R^2 , 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 hydrogen or methyl, or, R^d and R^4 , 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;

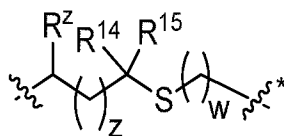
R^e is hydrogen or methyl, or, R^e and R^5 , together with the atoms to which they are attached, form a ring selected from azetidine, pyrrolidine, morpholine, piperidine, piperazine, and tetrahydrothiazole; wherein each ring is optionally substituted with one to four groups independently selected from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

R^g is hydrogen or methyl or R^g and R^7 , 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, 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;

R^k is hydrogen or methyl, or, R^k and R^{11} , 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; 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.

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



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

z is 0;

w is 1;

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

R^Z is $-C(O)NHR^{16}$.

4. A compound of claim 3 wherein

R^1 is phenyl C_1 - C_3 alkyl wherein the phenyl is optionally substituted with hydroxy;

R^2 is C_1 - C_7 alkyl;

R^3 is $-CH_2C(O)NH_2$ or R^3 and R^6 together form a bridge containing between 2 and 7 carbon atoms, optionally one double bond, and optionally one $-C(O)NH-$ or $-NHC(O)-$ group, or R^3 and R^5 together form a bridge containing between 2 and 7 carbon atoms, optionally one double bond, and optionally one $-C(O)NH-$ or $-NHC(O)-$ group,

R^4 and R^d , together with the atoms to which they are attached, form a pyrrolidine ring;

R^5 is selected from C_1 - C_7 alkyl and $-CH_2$ (imidazolyl) optionally substituted with $CF_3C(O)-$, or R^3 and R^5 together form a bridge containing between 2 and 7 carbon atoms, optionally one double bond, and optionally one $-C(O)NH-$ or $-NHC(O)-$ group;

R^6 is selected from C_1 - C_7 alkyl and $-(CH_2)_2C(O)NH_2$, or R^3 and R^6 together form a bridge containing between 2 and 7 carbon atoms, optionally one double bond, and optionally one $-C(O)NH-$ or $-NHC(O)-$ group;

R^7 is hydrogen or R^7 and R^g , together with the atoms to which they are attached, form a pyrrolidine ring optionally substituted with a hydroxy group or R^7 and R^{16} together form a bridge containing between 2 and 7 carbon atoms, optionally one double bond, and optionally one $-C(O)NH-$ or $-NHC(O)-$ group;

R^8 is $-(CH_2)$ indolyl;

R^9 is selected from hydroxymethyl and $-CH_2CH_2CO_2H$ or R^9 and R^{13} together form a bridge containing between 2 and 7 carbon atoms, optionally one double bond, and optionally one $-C(O)NH-$ or $-NHC(O)-$ group;

R^{10} is selected from $-(CH_2)$ indolyl and $-(CH_2)$ benzothienyl, each optionally substituted with $-CH_2CO_2H$;

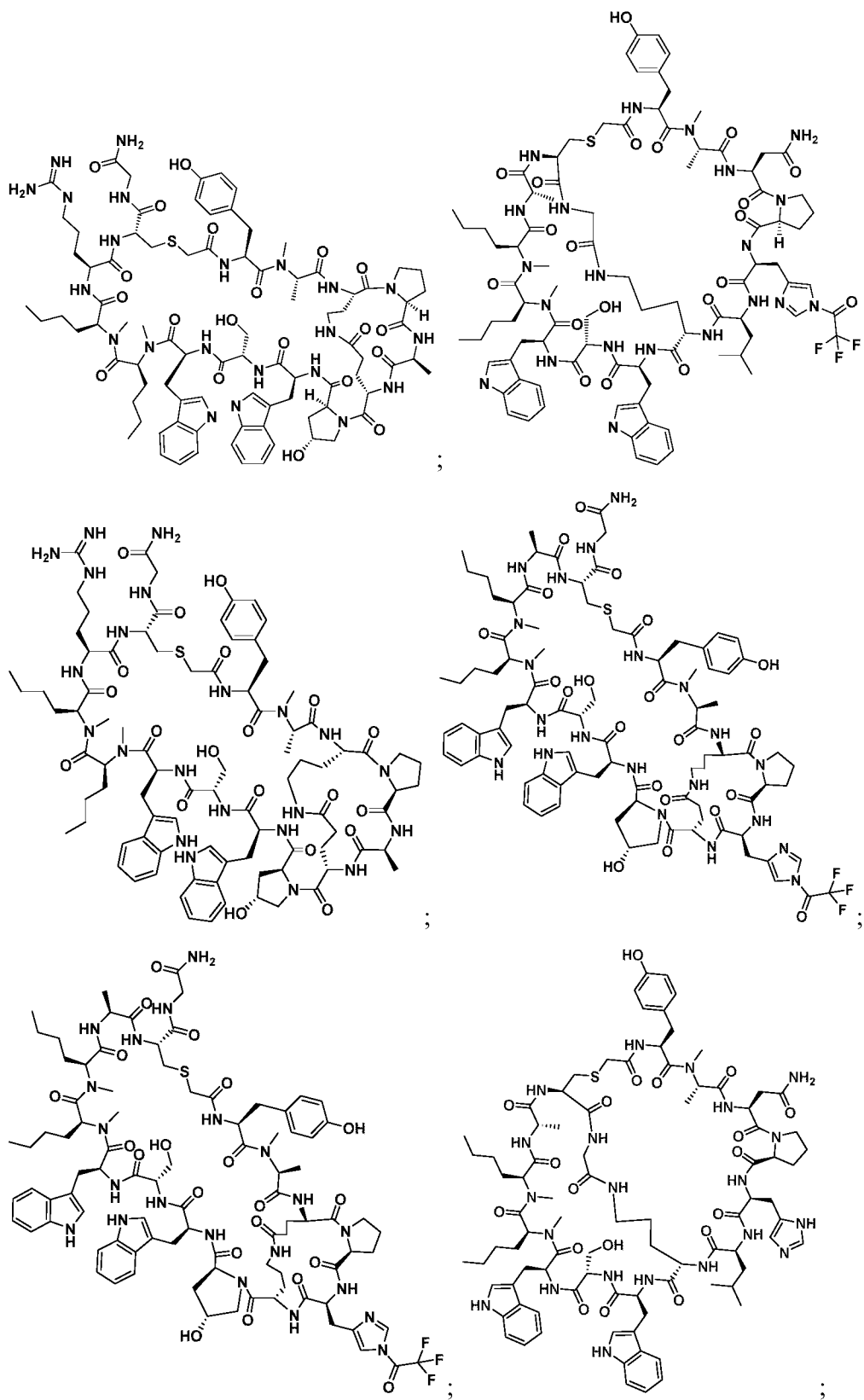
R^{11} is C_1 - C_7 alkyl;

R^{12} is C_1 - C_7 alkyl; and

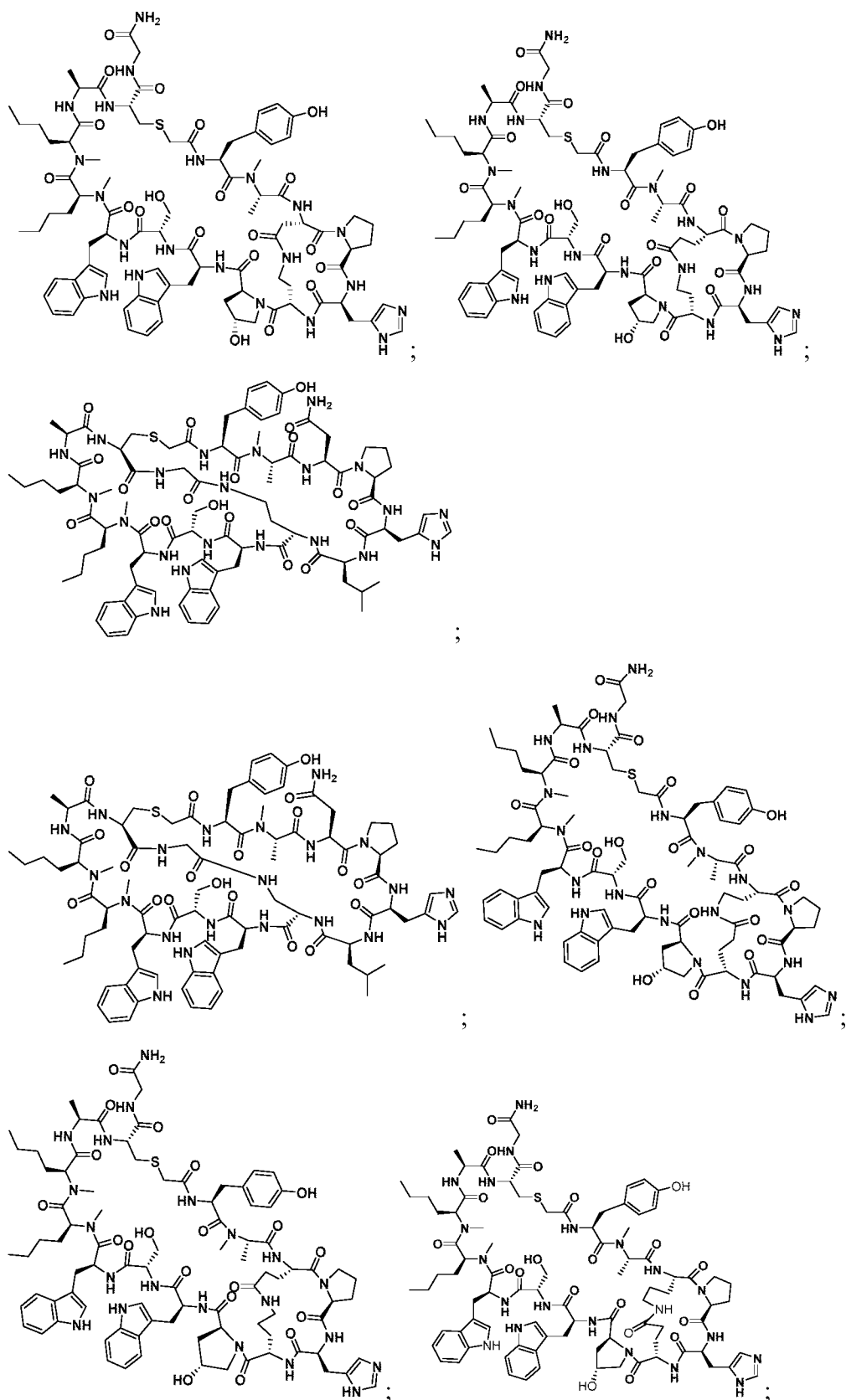
R^{13} is selected from C_1 - C_7 alkyl and $-(CH_2)_3NHC(NH)NH_2$ or R^9 and R^{13} together form a bridge containing between 2 and 7 carbon atoms, optionally one double bond, and optionally one $-C(O)NH-$ or $-NHC(O)-$ group.

5. A compound selected from:

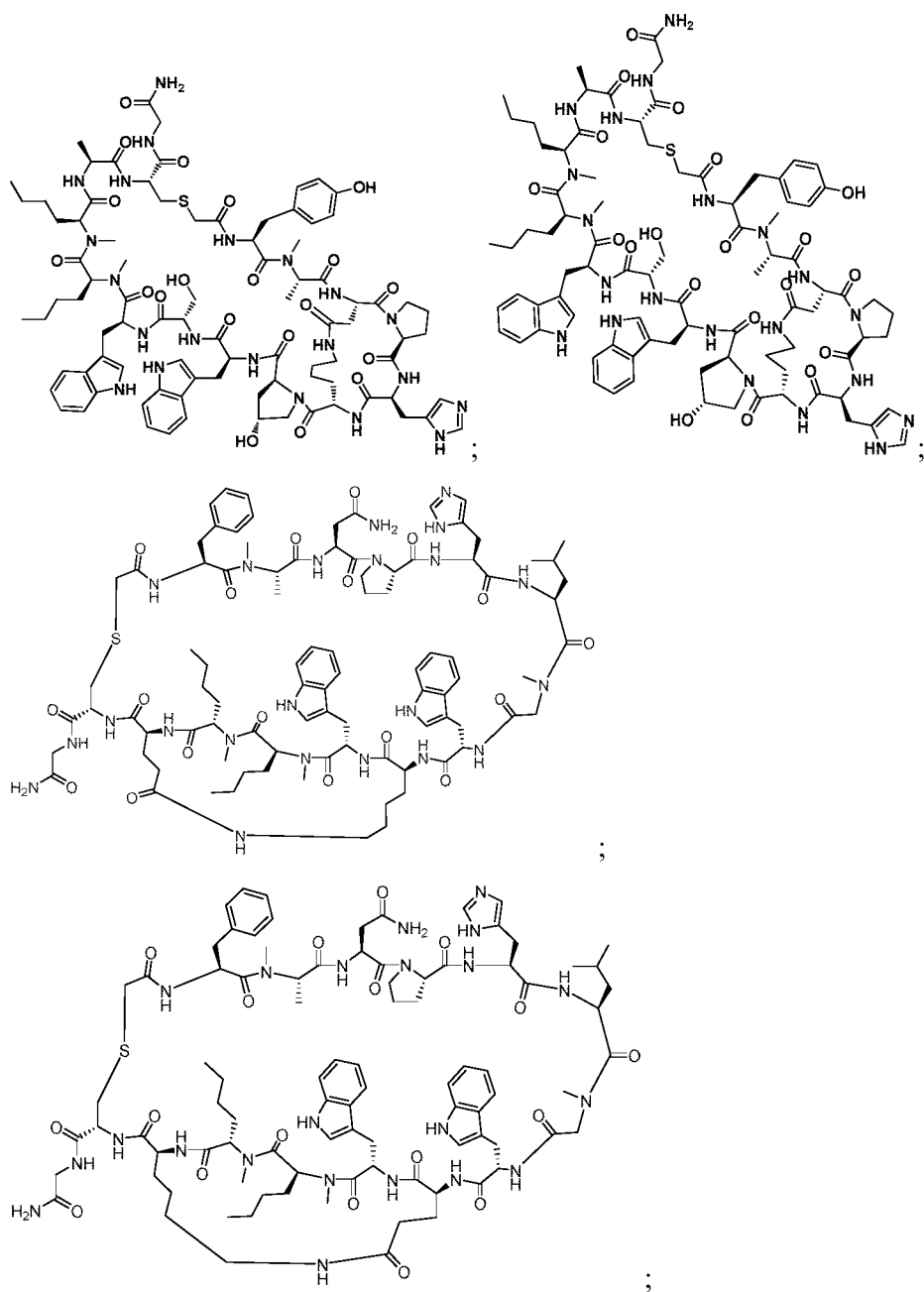
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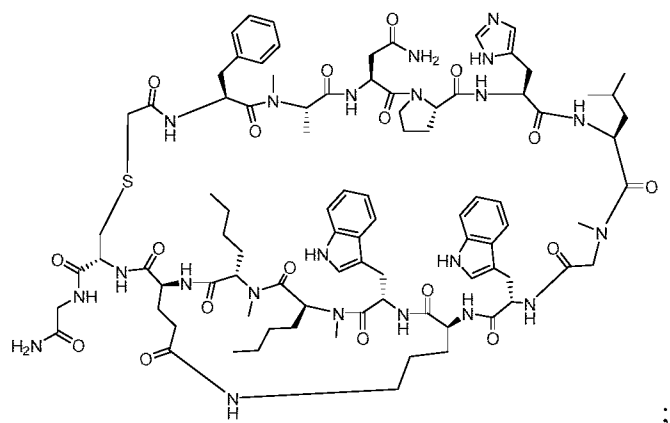
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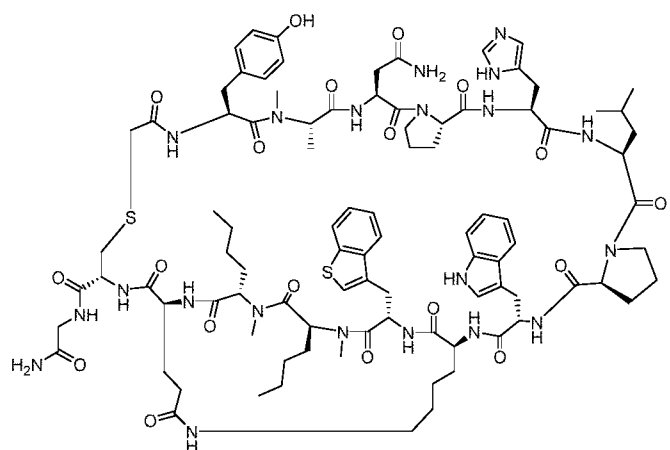
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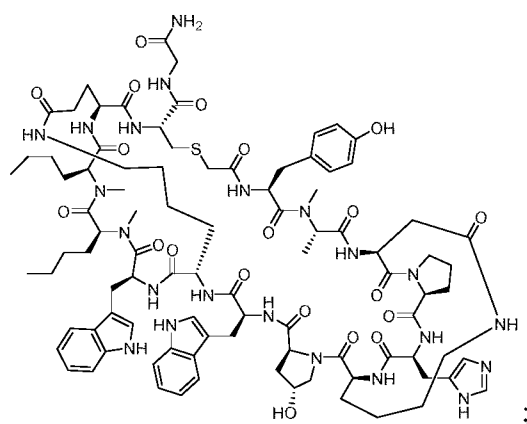
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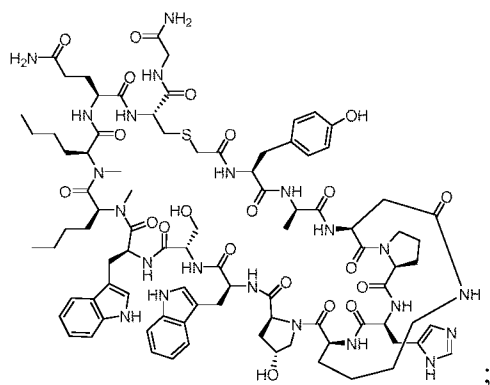
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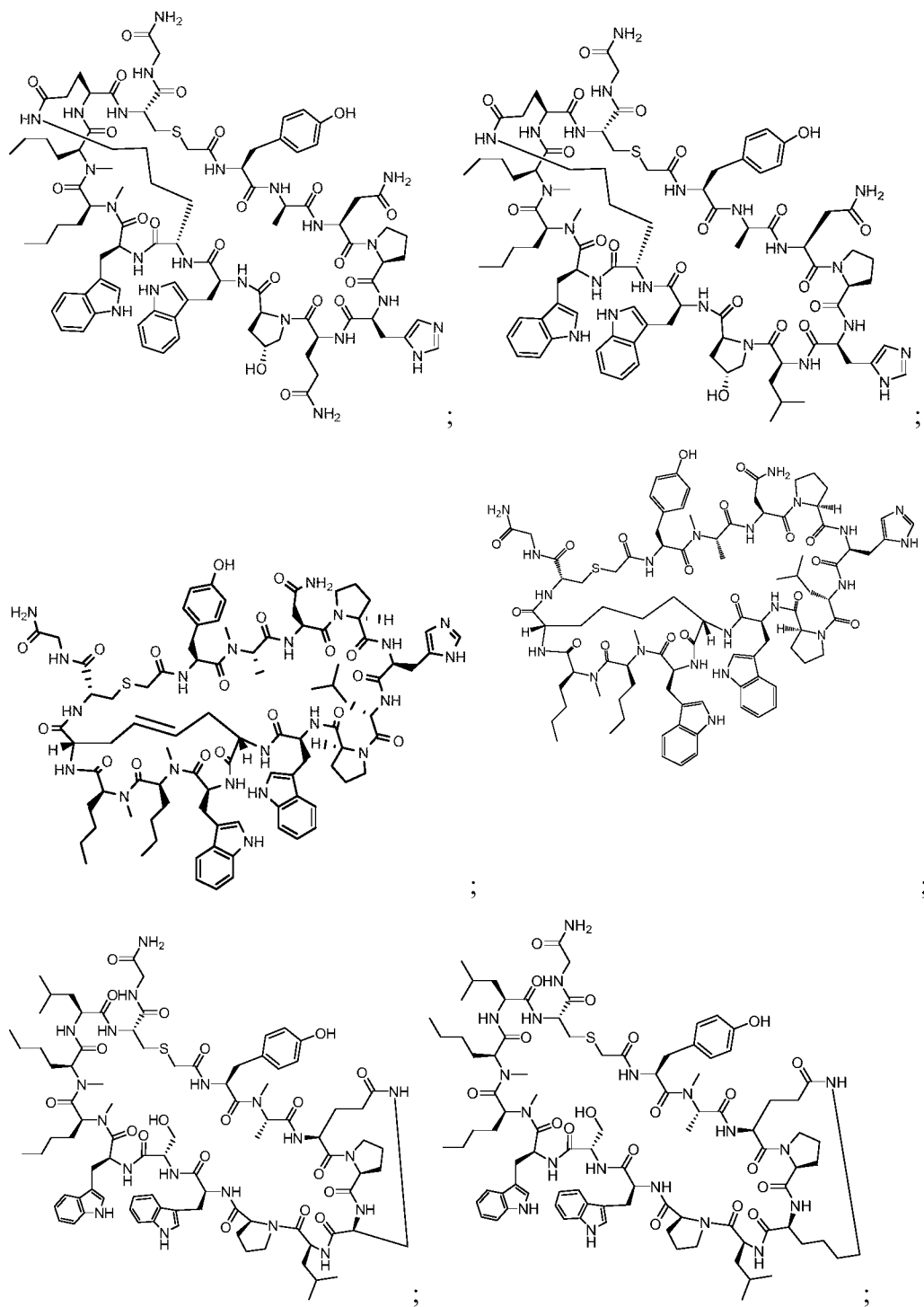


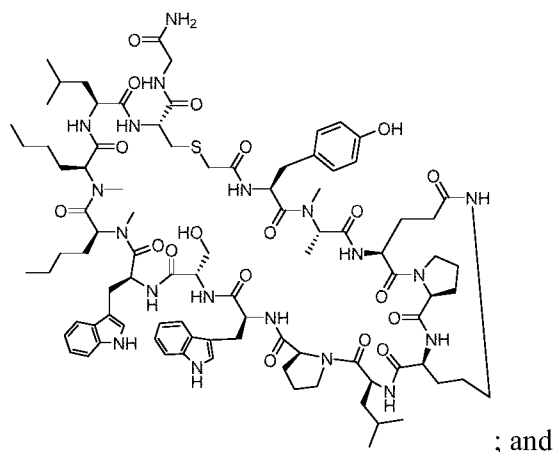
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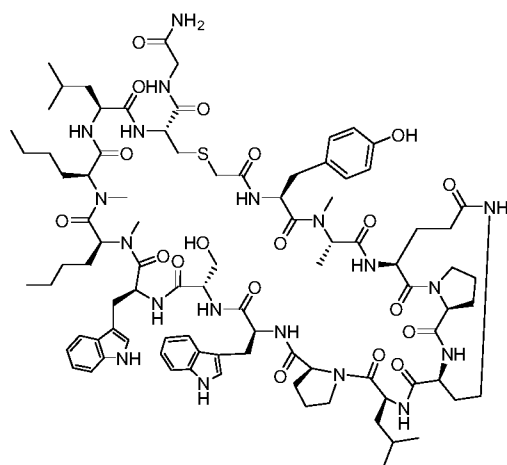
;

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; and



; or a pharmaceutically acceptable salt

thereof.

6. A method of enhancing, stimulating, and/or increasing an immune response mediated by the interaction of PD-L1 with PD-1 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 mediated by the interaction of PD-L1 with PD-1 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 mediated by the interaction of PD-L1 with PD-1 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 mediated by the interaction of PD-L1 with PD-1 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.

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			180					185					190		
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Pro	Glu	Leu	Pro	Leu	Ala	His	Pro	Pro	Asn	Glu	Arg	Thr	Gly	Ser	Ser
	210					215					220				
Glu	Thr	Val	Arg	Phe	Gln	Gly	His	His	His	His	His	His	His	His	His
225					230					235					