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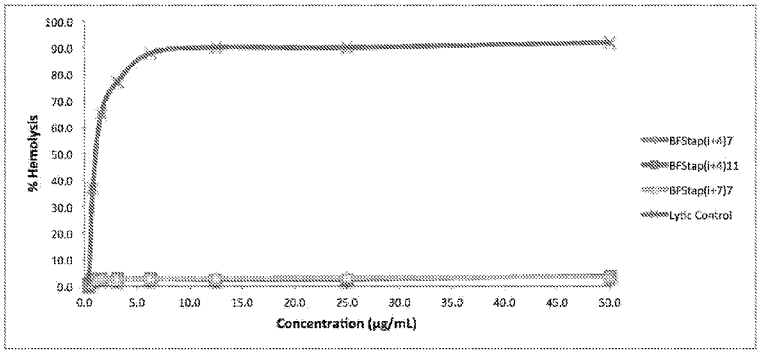


FIG. 8

(57) Abstract: Structurally stabilized, e.g., stapled, peptides with the ability to translocate through microbial cell membranes to the interior of microbial cells and exert a biological activity there are provided, as are methods of designing, making and using such peptides.

STAPLED INTRACELLULAR-TARGETING ANTIMICROBIAL PEPTIDES TO TREAT INFECTION

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of United States Provisional Application No. 62/301,426, filed February 29, 2016, the content of which is incorporated herein by reference in its entirety.

TECHNICAL FIELD

This disclosure relates to structural stabilization of intracellular-targeting antimicrobial peptides and methods for using such peptides to treat bacterial and other pathogenic infections.

BACKGROUND OF THE INVENTION

The rise of antibiotic superbugs is a major threat to healthcare systems around the globe. In the United States alone, the Centers for Disease Control and Prevention (CDC) estimates that healthcare costs due to antibiotic-resistant infections amount to $20 billion per year [1]. With the antibiotic pipeline drying up over the past decade, there are very few drug candidates to restock our antibiotic arsenal to address resistant infections [2-4]. In addition, due to the regulatory hurdles and low financial incentive for pharmaceutical and biotechnology companies to invest in antibiotic research, the burden of antibiotic discovery has fallen to academic research centers and hospitals [5-6]. These events have led to new government initiatives, such as the U.S. Generating Antibiotic Incentives Act and the Innovative Medicines Initiative New Drugs for Bad Bugs, which would introduce larger financial incentives for companies to develop novel agents, and would fast-track the drug approval process of potential antibiotic candidates [2]. Furthermore, the Obama administration recently allotted $1.2 billion dollars to investment in increased surveillance for antibiotic resistance outbreaks and antibiotic drug discovery.

Though such initiatives are important steps in the right direction, the need for innovation in antibiotic discovery technologies is crucial for restocking the drug pipeline with new candidates. Traditional drug discovery technologies have served us well this far; however, as bacterial resistance has evolved over time, these technologies have become increasingly obsolete. Alternative treatment options, such as bacteriophage therapy and vaccinations, are

currently being heavily pursued and could help alleviate the burden of antibiotic resistance and even reduce its prevalence [7-10]. Nonetheless, much development and research needs to be done before such therapeutics could reach the bedside of patients who need novel antibiotics urgently. While other treatment options are being researched and advanced, antibiotics are the most effective and reliable therapeutic modality available for microbial infections. Thus, their continued development is of crucial importance, as is the need for new reservoirs of natural compounds from which candidates can be developed. One potential reservoir is a cohort of compounds called antimicrobial peptides (AMPs).

AMPs are an evolutionarily conserved class of proteins that form an essential line of defense (in particular, as a key component of the humoral immune response) against microbial invasion [2]. These peptides are produced by many disparate organisms and have been found to exhibit a wide spectrum of activity against bacteria, fungi, protozoa, and even viruses.

AMPs can be divided into four main structural groups: stabilized β-sheet peptides with two to four disulfide bridges; loop peptides with a single disulfide bridge; α-helical peptides; and extended structures rich in arginine, glycine, proline, tryptophan, and histidine [2-4]. Typically 12 to 50 amino acids in length, these peptides are usually cationic with amphipathic character. These biophysical properties allow them to interact with bacterial membranes resulting in either disruption of membrane integrity or translocation and disruption of intracellular processes [3-5]. Other properties of AMPs include immune system modulation through various mechanisms such as increasing the production of cytokines, activating immune cells, and expediting wound healing [6,7].

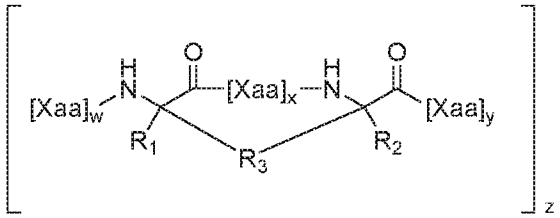
While much focus has been levied onto certain α-helical AMPs due to their membrane lytic properties and the vast amount of structural and mechanistic data available, other families of AMPs have remained much less studied and characterized. These less-characterized AMPs include a group of AMPs known as intracellular-targeting antimicrobial peptides (I-TAMPs). Due to their ability to translocate across membranes, this class of AMPs can target various microbial processes inside the microbial cell, including inhibition of DNA synthesis, protein synthesis, chaperone-assisted protein folding, enzymatic activity, and/or cell wall synthesis [3,8]. I-TAMPs have been discovered in fish, mollusks, insects, amphibians and mammals [3,9]. However, it has been difficult to characterize these AMPs properly, since at high concentrations, most AMPs become lytic and current model membrane systems are too simplistic [8].

Nevertheless, there are certain intracellular-targeting antimicrobial peptides that have been well-studied, and consensus has been reached with regard to their mode of action. One such peptide is buforin II, a histone-derived AMP (H-DAMP), which was isolated from the stomach tissue of the Asian toad, *Bufo bufo garagriozans* [13]. Unlike other amphibian AMPs like magainin II, buforin II does not disrupt the integrity of bacterial membranes. Instead, buforin II translocates into the bacterial cytosol, where it is able to bind to DNA and inhibit transcription [10]. Nevertheless, the generally poor stability and low potency of I-TAMPs, including buforin II, have reduced interest in further development of buforin II and/or other I-TAMPs as active pharmaceutical agents and have led to their being generally relegated to use as research reagents.

SUMMARY

This document describes the installation of all-hydrocarbon staple(s) into I-TAMP sequences, yielding compounds with enhanced antimicrobial activity yet little to no off-target toxicity. Hydrocarbon stapling of peptides recapitulates the α-helical secondary structure of a critical subcomponent of I-TAMPs and thereby enhances their import into bacteria and, consequently, their biological activity inside the bacterial cell [14,15]. In addition to improved α-helical stabilization, hydrocarbon stapling enhances proteolytic resistance, thereby conferring improved pharmacokinetic properties relative to natural peptides, which are often susceptible to rapid degradation *in vivo* [16]. Moreover, the stapled I-TAMPs of this document can, in some embodiments, have no or minimal lytic or cell growth-inhibiting activity against target microbial cells. This disclosure shows that applying hydrocarbon stapling to discrete sequences within I-TAMPs results in structural stabilization, enhanced antimicrobial activity, and significantly lowered hemolytic activity.

More specifically, the document provides an internally cross-linked (ICL) intra-microbial cell targeting anti-microbial peptide (I-TAMP) having the Formula (I),



Formula (I)

or a pharmaceutically acceptable salt thereof,

wherein;

each Xaa is independently an amino acid;

each R_1 and R_2 is independently H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heteroarylalkyl, or heterocyclylalkyl, any of which is substituted or unsubstituted;

each R_3 is independently alkylene, alkenylene, or alkynylene, any of which is substituted or unsubstituted;

each R_4 is independently $-NH_3$ or $-OH$, wherein each $-NH_3$ is optionally coupled with another chemical entity;

each x is independently 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10;

each w and y is independently 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20; and

z is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10,

wherein the peptide exhibits an intracellular antimicrobial effect against at least one microbe.

In some embodiments:

each R_1 and R_2 are independently H or a C_1 to C_{10} alkyl, alkenyl, alkynyl, or;

each R_3 is alkylene, alkenylene or alkynylene (e.g., a C_8 or C_{11} alkenylene) substituted with 1–6 R_4;

each R_4 is, independently $-NH_3$ or $-OH$, wherein each $-NH_3$ is optionally coupled with a chemical entity such as benzylic acid derivatives, enzyme inhibitors, a carbohydrate group, a hydroxyl group, a phosphate group, a farnesyl group, an isofarnesyl group, a fatty acid group, or a linker for conjugation or functionalization;

x is 2, 3, or 6, R_3 replacing, relative to the corresponding parent non-internally cross-linked AMP, the side chains of at least one pair of amino acids;

w and y are independently an integer from 0–20;

z is an integer from 1–10 (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10); and

each Xaa is independently an amino acid (e.g., one of the 20 naturally occurring amino acids or any non-naturally occurring amino acid),

provided that: when x is 2, R_3 is C_8 alkylene, alkenylene, or alkynylene; when x is 3, R_3 is C_8 alkylene, alkenylene, or alkynylene; and when x is 6, R_3 is C_{11} alkylene, alkenylene, or alkynylene and provided that the sum of x, w, and y is at least 10, and

such that the ICL I-TAMP contains at least 10 contiguous amino acids of any one of SEQ ID NOs: 1–13 or a variant thereof having 1, 2, 3, 4, or 5 amino acid substitutions, or another polypeptide sequence described herein except that: (a) within the 10 contiguous amino acids the side chains of at least one pair (e.g., one or two pairs) of amino acids separated by 2, 3, or 6 amino acids are replaced, relative to the corresponding parent non-internally cross-linked AMP, by the linking group, R_3, which connects the alpha carbons of the pair of amino acids as depicted in Formula I; and (b) the alpha carbon of the first of the pair of amino acids is substituted with R_1 as depicted in Formula I and the alpha carbon of the second of the pair of amino acids is substituted with R_2 as depicted in Formula I. R_3 can be substituted with two R_4 and the R_4 can be –OH. Alternatively, R_3 can be an optionally substituted $–NH_3$ and the other is –OH.

Moreover, the document additionally provides an internally cross-linked (ICL) intra-microbial cell targeting anti-microbial peptide (I-TAMP) containing amino acids, the side chains of at least one pair (e.g., one or two pairs) of amino acids separated by 2, 3, or 6 amino acids being replaced, relative to the corresponding parent non-internally cross-linked AMP, by the linking group, R_3, which connects the alpha carbons of the pair of amino acids such that:

each R_3 is independently alkylene, alkenylene, or alkynylene (e.g., a C_6, C_7, or C_{11} alkenylene) optionally substituted with 1–6 R_4;

each R_4 is independently $–NH_3$ or –OH, wherein each $–NH_3$ is optionally substituted; and each R_1 and R_2 is independently C_1 to C_{10} alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heteroarylalkyl, or heterocyclylalkyl, any of which is substituted or unsubstituted.

In some embodiments, the ICL I-TAMP contains at least 10 contiguous amino acids of any of SEQ ID NOs: 1–13 or a variant thereof having 1, 2, 3, 4, or 5 amino acid substitutions, or another polypeptide sequence described herein except that: (a) within the 10 contiguous amino acids the side chains of at least one pair (e.g., one or two pairs) of amino acids separated by 2, 3, or 6 amino acids are replaced, relative to the corresponding parent non-internally cross-linked AMP, by the linking group, R_3, which connects the alpha carbons of the pair of amino acids and

the H of the alpha carbon of each pair of amino acids having their side chains replaced by linking group R_3 is optionally, independently replaced by a C_1 to C_{10} alkyl, alkenyl, or alkynyl. This peptide can contain at least 10 contiguous amino acids of SEQ ID NO: 7.

Either of the above-described ICL I-TAMPs can contain the sequence of any one of SEQ ID NOs: 14–60. They can, for example, contain the sequence of BFStap(i+4)2, BFStap(i+4)3, BFStap(i+4)4, BFStap(i+4)6, BFStap(i+4)7, BFStap(i+4)8, BFStap(i+4)11, BFStap(i+7)1, BFStap(i+7)2, BFStap(i+7)3, BFStap(i+7)4, BFStap(i+7)5, BFStap(i+7)6, BFStap(i+7)7, BFStap(i+7)8, or BFStap(i+7)9. In certain instances, the above-described ICL I-TAMPs can contain the sequence of any one of SEQ ID NOs: 43, 47, or 55.

The document also features a method of treating or preventing a microbial infection, the method including administering an effective amount of any of the ICL I-TAMPs described above to a subject having, or at risk of having, an infection with a microbial organism. The subject can be an animal or plant. The animal can be a mammal, e.g., a human. The microbial organism can be a bacterial organism, e.g., a gram-positive bacterial organism or a Gram-negative bacterial organism. The method can further include administering an effective amount of at least one antibiotic. The antibiotic can act synergistically with the ICL I-TAMP to inhibit or prevent infection with the microbial organism. The ICL I-TAMP and the antibiotic can act synergistically to overcome or prevent resistance to the antibiotic.

Another aspect of the document is a composition containing one or more of the any of the ICL I-TAMPs described above. The composition can further contain a medical or hygienic device. The one or more ICL I-TAMPs can be coated onto or impregnated into the medical or hygienic device. The composition can also contain one or more antibiotics.

Also provided by the document is a method of inhibiting the growth of, or killing, a microbial organism that involves contacting the microbial organism with one or more of any of the above-described ICL I-TAMPS. The microbial organism can be an extracellular microbial organism or an intracellular microbial organism. The contacting can occur in a subject comprising the microbial organism. Alternatively, the method can be an *in vitro* method. It is understood that the method can be implemented using any of the features described in the document (e.g., those described above for a method of treating or preventing a microbial infection).

Another feature of the document is a method of making any of the above-described ICL I-TAMPs, the method involving: synthesizing the ICL I-TAMP such that the ICL I-TAMP comprises an α-helical region comprising a first surface hydrophobic patch, the replacement with the linking groups maintaining or resulting in, relative to the corresponding parent non-internally crosslinked I-TAMP, discontinuity between the first hydrophobic patch and one or more additional surface hydrophobic patches on internally cross-linked peptide. The method can further include adding to the linking group a hydrolyzing modification, e.g., dihydroxylation.

Yet another feature of the document is a method of designing the any of the above-described ICL I-TAMPs, involving:

-creating one or more panels of ICL I-TAMPs, each panel containing a plurality of panel member ICL I-TAMPs in each of which: (a) the side chains of at least one pair of amino acids separated by 2, 3, or 6 amino acids are replaced by the linking group, R_3, which connects the alpha carbons of the pair of amino acids; and (b) in each member of each panel, the pair of amino acids is at different positions as compared to the other members of the relevant panel; and

-testing each member of all panels for (i) the presence of discontinuity between a first surface hydrophobic patch in an α-helical region of the relevant member and one or more additional surface hydrophobic patches on the α-helical region of the member; and (ii) the ability of each member of each panel for its ability to translocate into a microbial cell and lyse or inhibit the growth of a mammalian cell. The method can further involve manufacturing one or members of all the panels that have a relatively high ability to translocate into a microbial cell and/or a relatively low ability to lyse or inhibit the growth of a mammalian cell.

As used herein, the terms "about" and "approximately" are defined as being within plus or minus 10% of a given value or state, preferably within plus or minus 5% of said value or state.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Methods and materials are described herein for use in the present invention; other, suitable methods and materials known in the art can also be used. The materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, sequences, database entries, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

Other features and advantages of the invention will be apparent from the following detailed description and figures, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 is a chart showing the sequences of buforin II and exemplary stapled analogues. Buforin II sequence aligned with the sequences of stapled derivatives demonstrate the position of the *i, i+4* staple, which is represented by the pair: **X,X**, and the *i, i+7* staple, which is represented by the pair: **8,X**. Other residues that were mutated are boxed. The sequences listed from top to bottom correspond to SEQ ID NOs.: 37 to 60 and 7. "**8**" is (R)-2-(7'-octenyl)alanine; and "**X**" is (S)-2-(4'-pentenyl)alanine.

FIGURE 2 shows the circular dichroism (CD) spectra of buforin II and *i, i+4* stapled analogues in the absence and presence of trifluoroethanol (TFE). **FIGURE 2 PANEL A** is a graph of a CD spectrum of buforin II (F10W) and *i, i+4* stapled analogues in buffer solution. **FIGURE 2 PANEL B** is a graph of a CD spectrum of buforin II (F10W) and *i, i+4* stapled analogues in TFE:buffer (1:1) mixture.

FIGURE 3 shows the circular dichroism spectra of buforin II and *i, i+7* stapled analogues in the absence and presence of trifluoroethanol (TFE). **FIGURE 3 PANEL A** is a graph of a CD spectrum of buforin II (F10W) and *i, i+7* stapled analogues in buffer solution. **FIGURE 3 PANEL B** is a graph of a CD spectrum of buforin II (F10W) and *i, i+7* stapled analogues in TFE:buffer (1:1) mixture.

FIGURE 4 PANEL A shows the chemical structures of the unnatural amino acids used to generate various kinds of staples. **FIGURE 4 PANEL B** illustrates peptides with staples of various lengths. **FIGURE 4 PANEL C** illustrates a staple walk along a peptide sequence.

FIGURE 5 is a schematic showing representations of various kinds of double and triple stapling strategies along with exemplary staple walks.

FIGURE 6 is a schematic showing exemplary staple walks using various lengths of branched double staple moieties.

FIGURE 7 is a schematic showing exemplary chemical alterations that are employed to generate stapled antimicrobial peptide derivatives.

FIGURE 8 is a graph showing the hemolysis of stapled buforin II analogues. Stapled buforin II analogs were incubated with 1% v/v human red blood cells (RBCs) in phosphate buffer for 1 hour at 37 °C and then the supernatant was collected and hemoglobin release was measured using UV absorbance at 570 nm. As opposed to a stapled lytic control peptide, the buforin II analogs displayed hemolysis levels below 5% at all the concentrations tested up to 50 μg/mL.

DETAILED DESCRIPTION

I-TAMP sequences possess the unique property of microbial cell translocation to achieve their anti-microbial activity inside the microbial cell. This disclosure informs the development of structurally-stabilized I-TAMPs to optimize/maximize their antimicrobial potency and proteolytic stability for therapeutic development. It will be appreciated that such structurally stabilized I-TAMPs should have no or minimal lytic or cell growth-inhibiting activity against target microbial cells. Such drug repurposing is another critically important avenue for transforming FDA-approved agents, such as methotrexate, into selective antimicrobials. Thus, this invention addresses the previous limitations in developing I-TAMPs as antimicrobial agents by chemically-stabilizing I-TAMP structures and also adapting them for bacterial delivery of therapeutic cargos.

As a proof of concept, we applied hydrocarbon stapling to the sequence of buforin II. By installing hydrocarbon staple(s) at specific locations within the buforin II sequence, we achieved dramatic increases in potency, including, e.g., a 200-fold increase in antimicrobial activity compared to the unmodified (parent) buforin II sequence. These enhancements in potency were accompanied by structural stabilization as determined by circular dichroism spectroscopy. Moreover, these increases in activity were not necessarily accompanied by increases in non-specific hemolytic activity against human red blood cells. For example, in certain examples, hemolytic activity was identified only at concentrations 10 times greater than the minimum

inhibitory concentration (MIC) for bacterial treatment, highlighting a significant, wide therapeutic window.

Importantly, the mechanism of action of buforin II involving bacterial membrane transit, enables the development of chimeric compounds that link buforin II to antibiotics, which are otherwise incapable of accessing the interior of Gram-negative bacteria. Moreover, the specific stapling strategy discovered for I-TAMPs reinforces both the bacterial uptake capacity of this AMP and the stability of the peptide for *in vivo* applications.

The invention further provides novel opportunities to harness the potential of intracellular AMPs to enable new therapeutics for overcoming treatment resistance. Since I-TAMPs like buforin II act on bacterial intracellular targets by translocating across their membranes, stapled I-TAMPs provide a new delivery platform for selective targeting of bacteria, enabling repurposing of previously ineffective antibiotics due to bacterial membrane impenetrance. An exemplary class of cargo is Gram-positive antimicrobials, e.g., beta-lactam drugs, which are otherwise blocked by the outer membrane of Gram-negative bacteria. Additional classes of cargo include, e.g., antibiotics that are otherwise toxic for human use but could be selectively targeted to bacteria, and cancer therapeutics that have bacterial cross-targets, e.g., methotrexate [19].

I-TAMPs from Fish

The principles and methods of the invention apply to all I-TAMPs that function as antimicrobials in fish (including, e.g., in skin and/or intestinal secretions) and stapled analogs thereof. As examples of this application, the following sequences and exemplary stapled analogs are listed below. In these sequences and all the others listed below, "X" indicates the position of staples. Some of the sequences contain an "-NH_2 at the C-terminus; this indicates amidation of the C-terminal residue.

Pleurocidin (From winter flounder)

GWGSFFKKAAHVGKHVGKAALTHYL-NH_2 (SEQ ID NO: 1)

GW**X**SFF**X**KAAH**X**GKH**X**GKAALTHYL-NH_2 (SEQ ID NO: 14)

GWGSFFKK**X**AHV**X**KHVGK**X**ALT**X**YL-NH_2 (SEQ ID NO: 15)

Hipposin (From Atlantic halibut)

SGRGKTGGKARAKAKTRSSRAGLQFPVGRVHRLLRKGNYAHRVGAGAPVYL (SEQ ID NO: 2)

SGRXKTGGKAXAKAKTRSSRAGLQFPVGRVHRLLRKGNYAHRVGAGAPVYL (SEQ ID NO: 16)

SGRGKTGGKXRAKXKTRSSRAGLQFPXGRVXRLLRKGNYAHRVGAGAPVYL (SEQ ID NO: 17)

Himanturin (From the Round Whip Ray, *H. pastinacoides)*

KAKSRSSRAGLQFPVGRVHRLLRKGNYAERVGAGAPVYL (SEQ ID NO: 3)

KAKSRXSRAXLQFPVGRVHRLLRKGNYAXRVGAGAXVYL (SEQ ID NO: 18)

KAKSRSSRAGLXFPVXRVHRLLRKGNYXERVGAGXPVYL (SEQ ID NO: 19)

Rainbow Trout H2A (From *Oncorhynchus mykiss*)

SGRGKTGGKARAKAKTRSSRAGLQFPVGRVHRLLRKGNYAERVGAGAPVYL (SEQ ID NO: 4)

SGRGKTGGKARAKAKTRSSXAGLQFPXGRVHRLLRXGNYAERXGAGAPVYL (SEQ ID NO: 20)

SGRGKTGGKXRAKAKTXSSRAGLQFPVGRVHRLXRKGNYAXRVGAGAPVYL (SEQ ID NO: 21)

I-TAMPs from Mollusks

The principles and methods of the invention apply to all I-TAMPs that function as antimicrobials in mollusks (including, e.g., in skin and/or intestinal secretions) and the stapled analogs thereof. As examples of this application, the following sequences and exemplary stapled analogs are listed below:

Abhisin (From *Haliotis discus)*

MSGRGKGGKTKAKAKSRSSRAGLQFPVGRIHRLLRKGNYA (SEQ ID NO: 5)

MSGRGKGXKTKAKAXSRSSRAGLQFPVGRIHRLLRKGNYA (SEQ ID NO: 22)

MSGRGKGGKTKAKAKSRSSRAGLQFPXGRIHRLXRKGNYA (SEQ ID NO: 23)

Scallop AMP (From *Chlamys farreri)*

MSGRGKGGKVKGKAKSRSSRAGLQFPVGRIHRLLRKGNYA (SEQ ID NO: 6)

XSGRGKGXKVKGKAKSRSSRAGLQFPVGRIHRLLRKGNYA (SEQ ID NO: 24)

MSGRGKGGKVKGKXKSRSSRXGLQFPVGRIHRLLRKGNYA (SEQ ID NO: 25)

I-TAMPs from Amphibians

The principles and methods of the invention apply to all I-TAMPs that function as antimicrobials in amphibians (including, e.g., in secretions) and stapled analogs thereof. As examples of this application, the following sequences and exemplary stapled analogs are listed below:

Buforin (Stomach Secretion from Asian Toad *Bufo garagrizans* – Example below is Buforin II)

TRSSRAGLQFPVGRVHRLLRK (SEQ ID NO: 7)

TRSSRAGLQFPXGRXHRLLRK (SEQ ID NO: 26)

TRSSRAGLQFPVGRXHRLXRK (SEQ ID NO: 27)

TRSSRAGLQFPXGRVHRLXRK (SEQ ID NO: 28)

I-TAMPs from Mammals

The principles and methods of the invention apply to all I-TAMPs that function as antimicrobials in mammals (including, e.g., in secretions) and stapled analogs thereof. As examples of this application, the following sequences and exemplary stapled analogs are listed below:

Human Histone H1 truncated analogues

KLNKKAASGE (SEQ ID NO: 8)

KLNKKAASGEAKPKA (SEQ ID NO: 9)

KAKSPKKAKA (SEQ ID NO: 10)

KXNKKXASGEAKPKA (SEQ ID NO: 29)

KAXSPKKAKX (SEQ ID NO: 30)

Histatin 5 (From Homo sapiens)

DSHAKRKKGYKRKFHEKHHSHRGY (SEQ ID NO: 11)
DSHAXRKKGYKRXFHEKHHSHRGY (SEQ ID NO: 31)
DSXAKRXKGYKRKXHEKHHSXRGY (SEQ ID NO: 32)

I-TAMPs from Insects

The principles and methods of the invention apply to all I-TAMPs that function as antimicrobials in Insects (including, e.g., in skin and/or intestinal secretions) and stapled analogs thereof. As examples of this application, the following sequences and exemplary stapled analogs are listed below:

Drosocin (From *Drosophila)*
GKPRPYSPRP(T*)SHPRPIRV (SEQ ID NO: 12)
T*: Threonine is glycosylated
XKPRPYSXRP(T*)SHPRPIRV (SEQ ID NO: 33)
GKPRPYXPRP(T*)SHXRPIRV (SEQ ID NO: 34)

Apidaecin IB (From honey bees)
GNNRPVYIPQPRPPHPRL (SEQ ID NO: 13)
GNNRPXYIPQPRXPHPRL (SEQ ID NO: 35)
GNNXPVYXPQPRPPXPRX (SEQ ID NO: 36)

The present disclosure provides structurally stabilized peptides related to anti-bacterial peptides (AMP) (referred to at times as stabilized α-helices of AMP or stabilized AMP or STAMP) comprising at least two modified amino acids joined by an internal (intramolecular) cross-link (or staple), wherein the at least two amino acids are separated by 2, 3, or 6 amino acids. Stabilized peptides herein include stapled peptides, including peptides having two staples and/or stitched peptides. The structurally stabilized peptide of this disclosure are generally derived from I-TAMPs.

For example, in some embodiments, the compound exhibits at least a 1.25, 1.5, 1.75 or 2-fold increase in α-helicity as determined by circular dichroism compared to a corresponding uncrosslinked peptide. In some embodiments, the compound can exhibit about 10%, about 15%,

about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, or about 100% helicity.

Amino acids are the building blocks of the peptides herein. The term "amino acid" refers to a molecule containing both an amino group and a carboxyl group as well as a side chain. Amino acids suitable for inclusion in the peptides disclosed herein include, without limitation, natural alpha-amino acids such as D- and L-isomers of the 20 common naturally occurring alpha-amino acids found in peptides (e.g., Ala (A), Arg (R), Asn (N), Cys (C), Asp (D), Gln (Q), Glu (E), Gly (G), His (H), Ile (I), Leu (L), Lys (K), Met (M), Phe (F), Pro (P), Ser (S), Thr (T), Trp (W), Tyr (Y), and Val (V), unnatural alpha-amino acids (including, but not limited to α,α-disubstituted and N-alkylated amino acids), natural beta-amino acids (e.g., beta-alanine), and unnatural beta-amino acids. Amino acids used in the construction of peptides of the present invention can be prepared by organic synthesis, or obtained by other routes, such as, for example, degradation of or isolation from a natural source.

There are many known unnatural amino acids, any of which may be included in the peptides of the present invention. Some non-limiting examples of unnatural amino acids are 4-hydroxyproline, desmosine, gamma-aminobutyric acid, beta-cyanoalanine, norvaline, 4-(E)-butenyl-4(R)-methyl-N-methyl-L-threonine, N-methyl-L-leucine, 1-amino-cyclopropanecarboxylic acid, 1-amino-2-phenyl-cyclopropanecarboxylic acid, 1-amino-cyclobutanecarboxylic acid, 4-amino-cyclopentenecarboxylic acid, 3-amino-cyclohexanecarboxylic acid, 4-piperidylacetic acid, 4-amino-l-methylpyrrole-2-carboxylic acid, 2,4-diaminobutyric acid, 2,3-diaminopropionic acid, 2,4-diaminobutyric acid, 2-aminoheptanedioic acid, 4-(aminomethyl)benzoic acid, 4-aminobenzoic acid, ortho-, meta- and /para-substituted phenylalanines (e.g., substituted with -C(=O)C_6H_5; -CF_3; -CN; -halo; -NO2; CH_3), disubstituted phenylalanines, substituted tyrosines (e.g., further substituted with -C=O)C_6H_5; -CF_3; -CN; -halo; -NO_2; CH_3), and statine. Additionally, amino acids can be derivatized to include amino acid residues that are hydroxylated, phosphorylated, sulfonated, acylated, and glycosylated, to name a few.

A "peptide" or "polypeptide" comprises a polymer of amino acid residues linked together by peptide (amide) bonds. The terms, as used herein, refer to proteins, polypeptides, and peptides of any size, structure, or function. Typically, a peptide or polypeptide will be at least three amino

acids long. A peptide or polypeptide may refer to an individual protein or a collection of proteins. In some instances, peptides can include only natural amino acids, although non-natural amino acids (i.e., compounds that do not occur in nature but that can be incorporated into a polypeptide chain) and/or amino acid analogs as are known in the art may alternatively be employed. Also, one or more of the amino acids in a peptide or polypeptide may be modified, for example, by the addition of a chemical entity such as a carbohydrate group, a hydroxyl group, a phosphate group, a farnesyl group, an isofarnesyl group, a fatty acid group, a linker for conjugation, functionalization, or other modification, etc. A peptide or polypeptide may also be a single molecule or may be a multi- molecular complex, such as a protein. A peptide or polypeptide may be just a fragment of a naturally occurring protein or peptide. A peptide or polypeptide may be naturally occurring, recombinant, or synthetic, or any combination thereof. "Dipeptide" refers to two covalently linked amino acids.

In some instances, a peptide has or can be induced to have alpha helical secondary structure.

In some instances, a peptide is a modified peptide that includes 1, 2, 3, 4, or 5 amino acid substitutions (e.g., 1, 2, 3, 4, or 5 amino acids are replaced with A or 1, 2, 3, 4, or 5 amino acids are conservatively substituted).

In some instances, stabilized peptides can be produced from I-TAMPs having at least 80% (e.g., 80%, 85%, 90%, 95%, 98%, 99%, 99.5%, or 100%) identity to one of SEQ ID NOs: 1–13 or I-TAMPs that include one of SEQ ID NOs: 1–13 with one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, preferably 1-2, 1-3, 1-4, or 1-5) conservative amino acid substitutions. In some instances, stabilized peptides can have at least 80% (e.g., 80%, 85%, 90%, 95%, 98%, 99%, 99.5%, or 100%) identity to one of SEQ ID NOs: 14-36 or can include one of SEQ ID NOs: 1–13 with one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, for example, 1-2, 1-3, 1-4, or 1-5) conservative amino acid substitutions. In some cases, the stabilized peptide has the sequence of one of SEQ ID NOs: 1–13 with one or two staples (e.g., one staple between two amino acids separated by 2 or 3 (or 6) amino acids or two staples each between two amino acids that are separated by 2 or 3 (or 6) amino acids). In addition, 1, 2, 3, 4, or 5 of the amino acids (whose side chains are not replaced with a staple) in this stabilized peptide can be replaced by a conservative substitution or can be replaced by A. In

some cases, the stabilized peptide has the sequence of one of SEQ ID NOs: 37-60. In another case, the stabilized peptide has the sequence of one of SEQ ID NOs: 43, 47, or 55.

In some instances, a "conservative amino acid substitution" can include substitutions in which one amino acid residue is replaced with another amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine).

Methods for determining percent identity between amino acid sequences are known in the art. For example, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). The length of a reference sequence aligned for comparison purposes can be at least 30%, at least 40%, at least 50%, at least 60%, or at least 70%, 80%, 90%, or 100% of the length of the reference sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The determination of percent identity between two amino acid sequences can be accomplished using, for example, the BLAST 2.0 program. Sequence comparison is performed using an ungapped alignment and using the default parameters (Blossom 62 matrix, gap existence cost of 11, per residue gapped cost of 1, and a lambda ratio of 0.85). The mathematical algorithm used in BLAST programs is described in Altschul et al. (Nucleic Acids Res. 25:3389-3402, 1997).

As disclosed above, peptides herein include at least two modified amino acids that together form an internal (intramolecular) cross-link (or staple), wherein the at least two modified amino acids are separated by: (A) two amino acids (i.e., *i*, *i*+*3*), (B) three amino acid (i.e., *i*, *i*+*4*), or (C) six amino acids (i.e., *i*, *i*+*7*).

In the case of a cross-link between *i* and *i* + *3* the cross-link can be, for example, a C_7 alkylene or alkenylene. In the case of a cross-link between *i* and *i* + *4* the cross-link can be, for example, a C_8 alkylene or alkenylene. In the case of a cross-link between *i* and *i* + *7* the cross-link can be, for example, a C_{11}, C_{12}, or C_{13} alkylene or alkenylene. When the cross-link is an alkenylene, there can one or more double bonds.

In the case of a cross-link between *i* and *i* + *3* the cross-link can be, for example, a C_6, C_7, or C_8 alkylene or alkenylene (e.g., a C_6 alkenylene having one double bond). In the case of a cross-link between *i* and *i* + *4* the cross-link can be, for example, a C_8 alkylene or alkenylene. In the case of a cross-link between *i* and *i* + *7* the cross-link can be, for example, a C_{11}, C_{12}, or C_{13} alkylene or alkenylene (e.g., a C_{11} alkenylene having one double bond). When the cross-link is alkenylene, there can be one or more double bonds. The cross-link can be optionally substituted with 1-5 substituents selected from –OH and $–NH_3$.

"Peptide stapling" is a term coined from a synthetic methodology wherein two olefin-containing side-chains (e.g., cross-linkable side chains) present in a polypeptide chain are covalently joined (e.g., "stapled together") using a ring-closing metathesis (RCM) reaction to form a cross-linked ring (Blackwell et al., J. Org. Chem., 66: 5291-5302, 2001; Angew et al., Chem. Int. Ed. 37:3281, 1994). As used herein, the term "peptide stapling" includes the joining of two (e.g., at least one pair of) double bond-containing side-chains, triple bond-containing side-chains, or double bond-containing and triple bond-containing side chain, which may be present in a polypeptide chain, using any number of reaction conditions and/or catalysts to facilitate such a reaction, to provide a singly "stapled" polypeptide. The term "multiply stapled" polypeptides refers to those polypeptides containing more than one individual staple, and may contain two, three, or more independent staples of various spacings and compositions. Additionally, the term "peptide stitching," as used herein, refers to multiple and tandem "stapling" events in a single polypeptide chain to provide a "stitched" (e.g., tandem or multiply stapled) polypeptide, in which two staples, for example, are linked to a common residue. Peptide stitching is disclosed, e.g., in WO 2008121767 and in WO 2010/068684, which are both hereby incorporated by reference in their entireties. In some instances, staples, as used herein, can retain the unsaturated bond or can be reduced (e.g., as mentioned below in the stitching paragraph description).

While many peptide staples have all-hydrocarbon cross-links, other type of cross-links or staples can be used. For example, triazole-containing (e.g, 1, 4 triazole or 1, 5 triazole) crosslinks

can be used (see, e.g., Kawamoto et al. 2012 Journal of Medicinal Chemistry 55:1137; WO 2010/060112).

Stapling of a peptide using all-hydrocarbon cross-link has been shown to help maintain its native conformation and/or secondary structure, particularly under physiologically relevant conditions (see, e.g., Schafmiester et al., J. Am. Chem. Soc., 122:5891-5892, 2000; Walensky et al., Science, 305:1466-1470, 2004).

Stapling the polypeptide herein by an all-hydrocarbon crosslink predisposed to have an alpha-helical secondary structure can improve stability and various pharmacokinetic properties.

Stabilized peptides herein include at least two internally cross-linked or stapled amino acids, wherein the at least two amino acids are separated, for example, by two (i.e., *i, i+3*), three (i.e., *i, i+4*), or six (i.e., *i, i+7*) amino acids. While at least two amino acids are required to support an internal cross-link (e.g., a staple), additional pairs of internally cross-linked amino acids can be included in a peptide, e.g., to support additional internal cross-links (e.g., staples). For example, peptides can include 1, 2, 3, 4, 5, or more staples.

FIGURE 4 PANEL A shows example chemical structures of unnatural amino acids that can be used to generate various crosslinked compounds. **FIGURE 4 PANEL B** illustrates peptides with hydrocarbon crosslinks between i and i+3, i and i+4, and i and i+7 residues. **FIGURE 4 PANEL C** illustrates a staple walk along a peptide sequence. **FIGURE 5** illustrates various peptide sequences with double and triple stapling strategies, and exemplary staple walks.

Alternatively or in addition, peptides can include three internally cross-linked or stitched amino acids, e.g., yielding two staples arising from a common origin. A peptide stitch includes at least three internally cross-linked amino acids, wherein the middle of the three amino acids (referred to here as the core or central amino acid) forms an internal cross-link (between alpha carbons) with each of the two flanking modified amino acids. The alpha carbon of the core amino acid has side chains that are internal cross-links to the alpha carbons of other amino acids in the peptide, which can be saturated or not saturated. Amino acids cross-linked to the core amino acid can be separated from the core amino acid in either direction by 2, 3, or 6 amino acids (e.g., *i, i-3, i, i-4, i, i-7, i, i+3, i, i+4, i, i+7*, where "*i*" is the core amino acid). The number of amino acids on either side of the core (e.g., between the core amino acid and an amino acid cross-linked to the core) can be the same or different.

In some embodiments, peptides herein can include a combination of at least one (e.g., 1, 2, 3, 4, or 5) staple and at least one (e.g., 1, 2, 3, 4, or 5) stitch. **FIGURE 6** illustrates example staple walks using various lengths of branched stitched moieties.

Selection of amino acids for modification (e.g., to support an internal cross-link) can also be facilitated by staple scanning. The term "staple scan" refers to the synthesis of a library of stapled peptides whereby the location of the *i* and *i+3*; *i* and *i+4*; and *i* and *i+7* single and multiple staple, or stitches, are positioned sequentially down the length of the peptide sequence, sampling all possible positions, to identify desired, effective, suitable, or optimal properties and activities for the stapled or stitched constructs. Examples of staple scanning methods are illustrated in the figures. Suitable tethers are described herein and in, e.g., US2005/0250680, PCT/US2008/058575, WO 2009/108261, and WO 2010/148335.

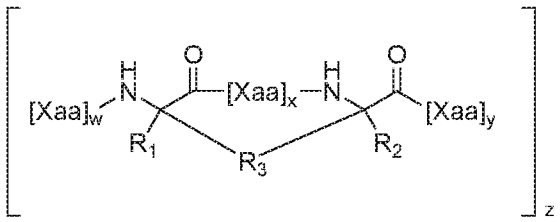
Amino acid side chains suitable for use in the peptides disclosed herein are known in the art. For example, suitable amino acid side chains include methyl (as the alpha- amino acid side chain for alanine is methyl), 4-hydroxyphenylmethyl (as the alpha-amino acid side chain for tyrosine is 4-hydroxyphenylmethyl) and thiomethyl (as the alpha-amino acid side chain for cysteine is thiomethyl), etc. A "terminally unsaturated amino acid side chain" refers to an amino acid side chain bearing a terminal unsaturated moiety, such as a substituted or unsubstituted, double bond (e.g., olefinic) or a triple bond (e.g., acetylenic), that participates in crosslinking reaction with other terminal unsaturated moieties in the polypeptide chain. In certain embodiments, a "terminally unsaturated amino acid side chain" is a terminal olefinic amino acid side chain. In certain embodiments, a "terminally unsaturated amino acid side chain" is a terminal acetylenic amino acid side chain. In certain embodiments, the terminal moiety of a "terminally unsaturated amino acid side chain" is not further substituted.

As noted above, an internal tether or cross-link can extend across the length of one helical turn (i.e., about 3.4 amino acids (i.e., *i, i+3,* or *i, i+4*) or two helical turns (i.e., about 7 amino acids (i.e., *i, i+7*). Accordingly, amino acids positioned at *i* and *i+3*; *i* and *i+4*; or *i* and *i+7* are ideal candidates for chemical modification and cross-linking. Thus, for example, where a peptide has the sequence …Xaa_1, Xaa_2, Xaa_3, Xaa_4, Xaa_5, Xaa_6, Xaa_7, Xaa_8, Xaa_9… (wherein, "…" indicates the optional presence of additional amino acids), cross-links between Xaa_1 and Xaa_4, or between Xaa_1 and Xaa_5, or between Xaa_1 and Xaa_8 are useful as are cross-links between Xaa_2 and Xaa_5, or between Xaa_2 and Xaa_6, or between Xaa_2 and Xaa_9, etc.

Polypeptides can include more than one crosslink within the polypeptide sequence to either further stabilize the sequence or facilitate the stabilization of longer polypeptide stretches. If the polypeptides are too long to be readily synthesized in one part, independently synthesized, cross-linked peptides can be conjoined by a technique called native chemical ligation (see, e.g., Bang, et al., J. Am. Chem. Soc. 126:1377). Alternately, large peptides are routinely synthesized using a convergent approach whereby fully protected fragments are specifically and sequentially reacted to form the full length desired product, after final deprotection, such as in the industrial synthesis of Fuzeon. **FIGURE** 7 is a schematic showing example chemical alterations that are employed to generate stapled antimicrobial peptide derivatives.

Compounds

The invention features a modified polypeptide of Formula (I),



Formula (I)

or a pharmaceutically acceptable salt thereof,

wherein;

each Xaa is independently an amino acid;

each R_1 and R_2 are independently H or a C_1 to C_{10} alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heteroarylalkyl, or heterocyclylalkyl;

each R_3 is independently alkylene, alkenylene or alkynylene (e.g., a C_6, C_7, or C_{11} alkenylene) substituted with 1-6 R_4;

each R_4 is independently $-NH_3$ or -OH, wherein each $-NH_3$ is optionally substituted;

wherein each R_3 replaces, relative to the corresponding parent (i.e., unmodified) non-internally cross-linked AMP, the side chains of at least one pair (e.g., one or two pairs) of amino acids separated by 2, 3, or 6 amino acids (i.e., x = 2, 3, or 6).

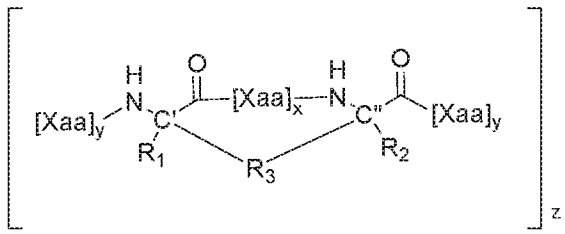
As used above, and elsewhere in the present document, a "corresponding parent (i.e., unmodified) non-internally cross-linked AMP" can be a wild-type AMP, or any of the variants of

a wild-type AMP disclosed in the present document, except that such a variant would not include an internal cross-link as described herein.

In the case of Formula I, the following embodiments are among those disclosed.

In cases where x = 2 (i.e., *i* + *3* linkage), R_3 can be, for example, a C_7 alkylene or alkenylene. Where it is an alkenylene there can one or more double bonds. In cases where x = 6 (i.e., *i* + *4* linkage), R_3 can be, for example, a C_{11}, C_{12}, or C_{13} alkylene or alkenylene. Where it is an alkenylene, there can one or more double bonds. In cases where x = 3 (i.e., *i* + *4* linkage), R_3 can be, for example, a C_8 alkylene or alkenylene. Where it is an alkenylene, there can one or more double bonds.

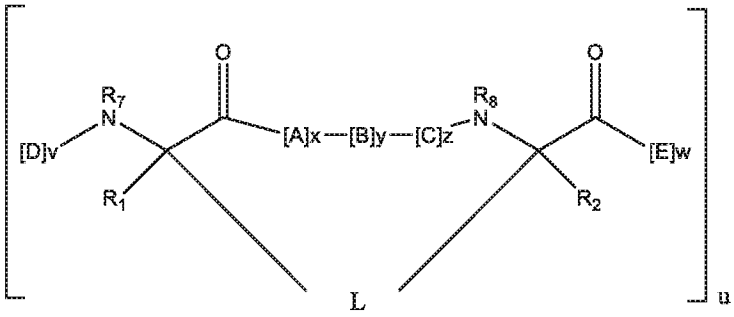
In certain instances, the two alpha, alpha disubstituted stereocenters (alpha carbons) are both in the R configuration or S configuration (e.g., *i, i+4* cross-link), or one stereocenter is R and the other is S (e.g., *i, i+7* cross-link). Thus, where Formula I is depicted as



the C' and C'' disubstituted stereocenters can both be in the R configuration or they can both be in the S configuration, for example when x is 3. When x is 6, the C' disubstituted stereocenter is in the R configuration and the C" disubstituted stereocenter is in the S configuration or the C' disubstituted stereocenter is in the S configuration and the C" disubstituted stereocenter is in the R configuration. An R_3 double bond may be in the E or Z stereochemical configuration. Similar configurations are possible for the carbons in Formula II corresponding to C' and C" in the formula depicted immediately above.

In some instances, the polypeptide includes an amino acid sequence which, in addition to the amino acids side chains that are replaced by a cross-link, have 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 amino acid changes (e.g., conservative amino acid changes) in any of SEQ ID NOs: 1–13.

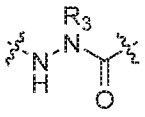
In some embodiments, a compound has the Formula (II):



Formula (II)

wherein:

- each A, C, D, and E is independently a natural or non-natural amino acid;
- each B is independently a natural or non-natural amino acid, amino acid analog,



, [-NH-L_4-CO-], [-NH-L_4-SO_2-], or [-NH-L_4-];

- each R_1 is independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, any of which is unsubstituted or substituted, or together with R_1 and the atom to which both R_1 and L are bound forms a ring;
- each R_2 is independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, any of which is unsubstituted or substituted, or together with R_2 and the atom to which both R_2 and L are bound forms a ring;
- each R_3 is independently hydrogen, alkyl, alkenyl, alkynyl, arylalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, cycloalkylalkyl, aryl, or heteroaryl, any of which is unsubstituted or substituted;
- each L is independently a macrocycle-forming linker;
- each L_4 is independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, heteroarylene, or [-R_4-K-R_4-]$_n$, any of which is unsubstituted or substituted;
- each R_4 is independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene, any of which is unsubstituted or substituted;
- each K is independently O, S, SO, SO_2, CO, CO_2, $CONR_3$, OSO_2NR_3, NR_{3q}, $CONR_{3q}$, $OCONR_{3q}$, or OSO_2NR_{3q}, wherein each R_{3q} is independently a point of attachment to

R_1 or R_2;

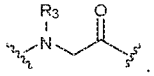
- each n is independently 1, 2, 3, 4, or 5;
- each R_7 is independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, aryl, or heteroaryl, any of which is unsubstituted or substituted, or part of a cyclic structure with a D residue;
- each R_8 is independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, aryl, or heteroaryl, any of which is unsubstituted or substituted, or part of a cyclic structure with an E residue;
- each v and w is independently an integer from 0-1000, from 1-1000, or 3-1000;
- each x, y and z is independently 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10; and
- u is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

In some embodiments, each v and w is independently 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20. In some embodiments, each w is independently an integer from 3-1000, for example 3-500, 3-200, 3-100, 3-50, 3-30, 3-20, or 3-10. In some embodiments, w is an integer from 3-10, for example 3-6, 3-8, 6-8, or 6-10. In some embodiments, w is 3. In other embodiments, w is 6. In some embodiments, each v is independently an integer from 1-1000, for example 1-500, 1-200, 1-100, 1-50, 1-30, 1-20, or 1-10. In some embodiments, v is 2.

In one example, at least one of R_1 and R_2 is alkyl that is unsubstituted or substituted with halo-. In another example, both R_1 and R_2 are independently alkyl that is unsubstituted or substituted with halo–. In some embodiments, at least one of R_1 and R_2 is methyl. In other embodiments, R_1 and R_2 are methyl.

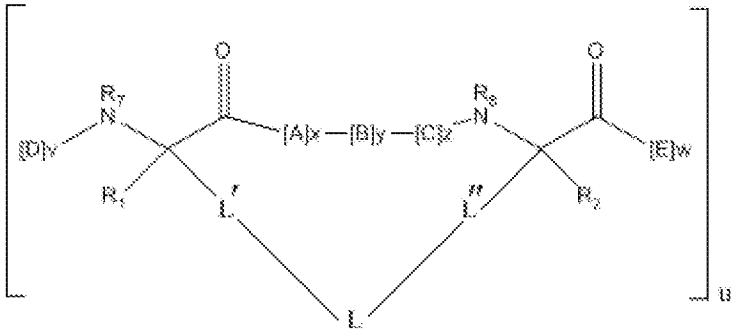
In some embodiments, x+y+z is at least 2 or at least 3. In other embodiments, x+y+z is 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10. In some embodiments, the sum of x+y+z is 3 or 6. In some embodiments, the sum of x+y+z is 3. In other embodiments, the sum of x+y+z is 6.

Each occurrence of A, B, C, D, or E in a macrocycle or macrocycle precursor is independently selected. For example, a sequence represented by the formula $[A]_x$, when x is 3, encompasses embodiments where the amino acids are not identical, e.g. Gln–Asp–Ala, as well as embodiments where the amino acids are identical, e.g. Gln–Gln–Gln. This applies for any value of x, y, or z in the indicated ranges. Similarly, when u is greater than 1, each compound can encompass compounds that are the same or different. For example, a compound can comprise compounds comprising different linker lengths or chemical compositions.

In some embodiments, the compound comprises a secondary structure that is an α-helix where R_8 is –H, allowing for intrahelical hydrogen bonding. In some embodiments, at least one of A, B, C, D, or E is an α,α-disubstituted amino acid. In one example, B is an α,α-disubstituted amino acid. For instance, at least one of A, B, C, D, or E is 2-aminoisobutyric acid. In other embodiments, at least one of A, B, C, D, or E is  .

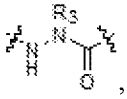
In other embodiments, the length of the macrocycle-forming linker L as measured from a first Cα to a second Cα is selected to stabilize a desired secondary peptide structure, such as an α-helix formed by residues of the compound including, but not necessarily limited to, those between the first Cα to a second Cα.

In some embodiments, a compound of Formula (II) has the Formula (IIa):



Formula (IIa)

wherein:

- each A, C, D, and E is independently a natural or non-natural amino acid;
- each B is independently a natural or non-natural amino acid, amino acid analog,  , [-NH-L_4-CO-], [-NH-L_4-SO_2-], or [-NH-L_4-];
- each L is independently a macrocycle-forming linker;
- each L' is independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene, any of which is unsubstituted or substituted, or a bond, or together with R_1 and the atom to which both R_1 and L' are bound forms a ring;
- each L'' is independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene, any of which is unsubstituted or

substituted, or a bond, or together with R_2 and the atom to which both R_2 and L'' are bound forms a ring;

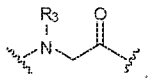
- each R_1 is independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, any of which is unsubstituted or substituted, or together with L' and the atom to which both R_1 and L' are bound forms a ring;
- each R_2 is independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, any of which is unsubstituted or substituted, or together with L'' and the atom to which both R_2 and L'' are bound forms a ring;
- R_3 is hydrogen, alkyl, alkenyl, alkynyl, arylalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, cycloalkylalkyl, aryl, or heteroaryl, any of which is unsubstituted or substituted;
- each L_4 is independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, heteroarylene, or $[-R_4\text{-}K\text{-}R_4-]_n$, any of which is unsubstituted or substituted;
- each R_4 is alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene, any of which is unsubstituted or substituted;
- each K is independently O, S, SO, SO_2, CO, CO_2, $CONR_3$, OSO_2NR_3, NR_{3q}, $CONR_{3q}$, $OCONR_{3q}$, or OSO_2NR_{3q}, wherein each R_{3q} is independently a point of attachment to R_1 or R_2;
- each n is independently 1, 2, 3, 4, or 5;
- each R_7 is independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, aryl, or heteroaryl, any of which is unsubstituted or substituted, or part of a cyclic structure with a D residue;
- each R_8 is independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, aryl, or heteroaryl, any of which is unsubstituted or substituted, or part of a cyclic structure with an E residue;
- each v and w is independently an integer from 0-1000, from 1-1000, or 3-1000;
- each x, y and z is independently 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10; and
- u is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

In some embodiments, L is a macrocycle-forming linker of the formula $-L_1-L_2-$. In some embodiments, L_1 and L_2 are independently alkylene, alkenylene, alkynylene, heteroalkylene,

cycloalkylene, heterocycloalkylene, arylene, heteroarylene, or [-R_4-K-R_4-]$_n$, any of which is unsubstituted or substituted.

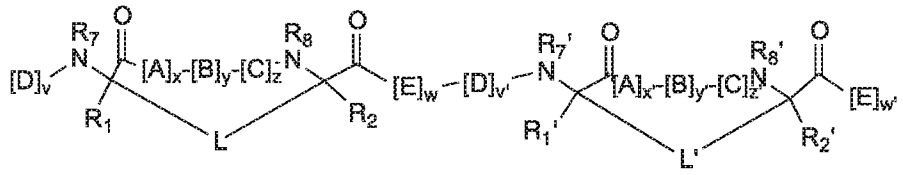
In one example, at least one of R_1 and R_2 is alkyl that is unsubstituted or substituted with halo–. In another example, both R_1 and R_2 are independently alkyl that is unsubstituted or substituted with halo–. In some embodiments, at least one of R_1 and R_2 is methyl. In other embodiments, R_1 and R_2 are methyl.

In some embodiments, x+y+z is at least 2 or at least 3. In other embodiments, x+y+z is 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10. Each occurrence of A, B, C, D, or E in a macrocycle or macrocycle precursor is independently selected. For example, a sequence represented by the formula $[A]_x$, when x is 3, encompasses embodiments where the amino acids are not identical, e.g. Gln–Asp–Ala as well as embodiments where the amino acids are identical, e.g. Gln–Gln–Gln. This applies for any value of x, y, or z in the indicated ranges. Similarly, when u is greater than 1, each compound may encompass moieties which are the same or different. For example, a compound may comprise moieties comprising different linker lengths or chemical compositions.

In some embodiments, the compound comprises a secondary structure that is a helix where R_8 is –H, allowing intrahelical hydrogen bonding. In some embodiments, at least one of A, B, C, D, or E is an α,α-disubstituted amino acid. In one example, B is an α,α-disubstituted amino acid. For instance, at least one of A, B, C, D, or E is 2-aminoisobutyric acid. In other embodiments, at least one of A, B, C, D or E is  .

In other embodiments, the length of the macrocycle-forming linker L as measured from a first Cα to a second Cα is selected to stabilize a desired secondary peptide structure, such as a helix formed by residues of the compound including, but not necessarily limited to, those between the first Cα to a second Cα.

In some embodiments, the compound of Formula (II) has the Formula (IIb):

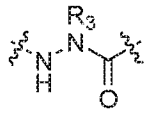


(Formula IIb)

wherein:

- each A, C, D, and E is independently an amino acid, wherein A, B, C, D, and E,

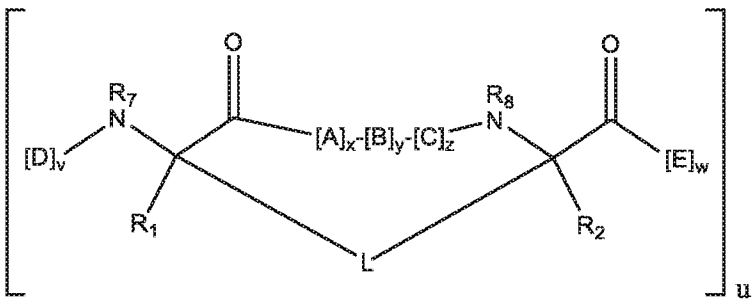
taken together with the crosslinked amino acids connected by the macrocycle-forming linkers L and L', form the amino acid sequence of a target peptide;

- each B is independently an amino acid,  , [-NH-L_4-CO-], [-NH-L_4-SO_2-], or [-NH-L_4-];
- L is a macrocycle-forming linker of the formula –L_1–L_2–;
- each R_1 is independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, any of which is unsubstituted or substituted, or together with L and the atom to which both R_1 and L are bound forms a ring;
- each R_2 is independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, any of which is unsubstituted or substituted, or together with L and the atom to which both R_2 and L are bound forms a ring;
- R_3 is hydrogen, alkyl, alkenyl, alkynyl, arylalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, cycloalkylalkyl, aryl, or heteroaryl, any of which is unsubstituted or substituted;
- L' is a macrocycle-forming linker of the formula –L_1'–L_2'–;
- each R_1' is independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, any of which is unsubstituted or substituted, or together with L' and the atom to which both R_1' and L' are bound forms a ring;
- each R_2' is independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, any of which is unsubstituted or substituted, or together with L' and the atom to which both R_2' and L' are bound forms a ring;
- L_1', L_2', and L_4 are independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, heteroarylene, or [-R_4-K-R_4-]$_n$, any of which is unsubstituted or substituted;
- each R_4 is alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene, any of which is unsubstituted or substituted;
- each K is independently O, S, SO, SO_2, CO, CO_2, $CONR_3$, OSO_2NR_3, NR_{3q}, $CONR_{3q}$, $OCONR_{3q}$, or OSO_2NR_{3q}, wherein each R_{3q} is independently a point of attachment to R_1, R_2, R_1', or R_2';

- each n is independently 1, 2, 3, 4, or 5;
- each R_7 is independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, aryl, or heteroaryl, any of which is unsubstituted or substituted, or part of a cyclic structure with a D residue;
- each R_8 is independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, aryl, or heteroaryl, any of which is unsubstituted or substituted, or part of a cyclic structure with an E residue;
- each R_7' is independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, aryl, or heteroaryl, any of which is unsubstituted or substituted, or part of a cyclic structure with a D residue;
- each R_8' is independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, aryl, or heteroaryl, any of which is unsubstituted or substituted, or part of a cyclic structure with an E residue;
- each x, y and z is independently 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10;
- each x', y' and z' is independently 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10;
- each v and w is independently an integer from 0-1000, from 1-1000, or 3-1000;
- each v' and w' is independently an integer from 0-1000, from 1-1000, or 3-1000; and
- each n is 1, 2, 3, 4, or 5.

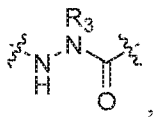
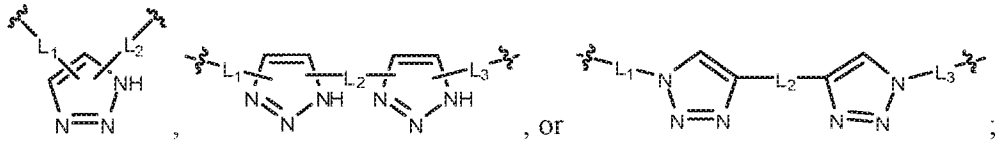
In some embodiments, the sum of x'+y'+z' is 1,2, 3, 4, 5, 6, 7, 8, 9, or 10, for example 3 or 6, at least 2, or at least 3.

In some embodiments, the compounds have the Formula (IIc):



Formula (IIc)

wherein:

- each A, C, D, and E is independently a natural or non-natural amino acid;
- each B is independently a natural or non-natural amino acid, amino acid analog,  , [-NH-L_4-CO-], [-NH-L_4-SO_2-], or [-NH-L_4-];
- each R_1 is independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, any of which is unsubstituted or substituted, or together with R_1 and the atom to which both R_1 and L are bound forms a ring;
- each R_2 is independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, any of which is unsubstituted or substituted, or together with R_2 and the atom to which both R_2 and L are bound forms a ring;
- each R_3 is independently hydrogen, alkyl, alkenyl, alkynyl, arylalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, cycloalkylalkyl, aryl, or heteroaryl, any of which is unsubstituted or substituted;
- each L is independently macrocycle-forming linker of the formula  ;
- each L_1, L_2 and L_3 is independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, heteroarylene, or [-R_4-K-R_4-]$_n$, any of which is unsubstituted or substituted;
- each R_4 is alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene, any of which is unsubstituted or substituted;
- each K is independently O, S, SO, SO_2, CO, CO_2, $CONR_3$, OSO_2NR_3, NR_{3q}, $CONR_{3q}$, $OCONR_{3q}$, or OSO_2NR_{3q}, wherein each R_{3q} is independently a point of attachment to R_1 or R_2;
- each R_7 is independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, aryl, or heteroaryl, any of which is unsubstituted or substituted, or part of a cyclic structure with a D residue;
- each R_8 is independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, aryl, or heteroaryl, any of which is unsubstituted or substituted, or part of a cyclic structure with an E residue;

- each v and w is independently an integer from 0-1000, from 1-1000, or 3-1000;
- each x, y and z is independently 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10;
- u is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10; and
- each n is 1, 2, 3, 4, or 5.

In one example, at least one of R_1 and R_2 is alkyl that is unsubstituted or substituted with halo–. In another example, both R_1 and R_2 are independently alkyl that is unsubstituted or substituted with halo–. In some embodiments, at least one of R_1 and R_2 is methyl. In other embodiments, R_1 and R_2 are methyl.

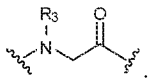
In some embodiments, x+y+z is at least 2 or at least 3. In other embodiments, x+y+z is 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10. Each occurrence of A, B, C, D, or E in a macrocycle or macrocycle precursor is independently selected. For example, a sequence represented by the formula $[A]_x$, when x is 3, encompasses embodiments where the amino acids are not identical, e.g. Gln–Asp–Ala as well as embodiments where the amino acids are identical, e.g. Gln–Gln–Gln. This applies for any value of x, y, or z in the indicated ranges.

In some embodiments, each of the first two amino acid represented by E comprises an uncharged side chain or a negatively charged side chain. In some embodiments, each of the first three amino acid represented by E comprises an uncharged side chain or a negatively charged side chain. In some embodiments, each of the first four amino acid represented by E comprises an uncharged side chain or a negatively charged side chain. In some embodiments, one or more or each of the amino acid that is *i*+1, *i*+2, *i*+3, *i*+4, *i*+5, and/or *i*+6 with respect to E comprises an uncharged side chain or a negatively charged side chain.

In some embodiments, the first C-terminal amino acid and/or the second C-terminal amino acid represented by E comprise a hydrophobic side chain. For example, the first C-terminal amino acid and/or the second C-terminal amino acid represented by E comprises a hydrophobic side chain, for example a small hydrophobic side chain. In some embodiments, the first C-terminal amino acid, the second C-terminal amino acid, and/or the third C-terminal amino acid represented by E comprise a hydrophobic side chain. For example, the first C-terminal amino acid, the second C-terminal amino acid, and/or the third C-terminal amino acid represented by E comprises a hydrophobic side chain, for example a small hydrophobic side chain. In some embodiments, one or more or each of the amino acid that is *i*+1, *i*+2, *i*+3, *i*+4, *i*+5, and/or *i*+6 with respect to E comprises an uncharged side chain or a negatively charged side

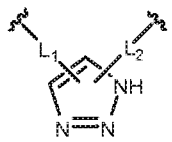
chain.

In some embodiments, each w is independently an integer from 1 to 1000. For example, the first amino acid represented by E comprises a small hydrophobic side chain. In some embodiments, w is between 2 and 1000. For example, the second amino acid represented by E comprises a small hydrophobic side chain. In some embodiments, w is between 3 and 1000. For example, the third amino acid represented by E comprises a small hydrophobic side chain. For example, the third amino acid represented by E comprises a small hydrophobic side chain. In some embodiments, w is between 4 and 1000. In some embodiments, w is between 5 and 1000. In some embodiments, w is between 6 and 1000. In some embodiments, w is between 7 and 1000. In some embodiments, w is between 8 and 1000.

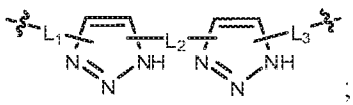
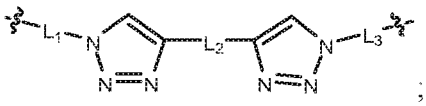
In some embodiments, the compound comprises a secondary structure that is a helix where R_8 is –H, allowing intrahelical hydrogen bonding. In some embodiments, at least one of A, B, C, D, or E is an α,α-disubstituted amino acid. In one example, B is an α,α-disubstituted amino acid. For instance, at least one of A, B, C, D, or E is 2-aminoisobutyric acid. In other embodiments, at least one of A, B, C, D, or E is  .

In other embodiments, the length of the macrocycle-forming linker L as measured from a first Cα to a second Cα is selected to stabilize a desired secondary peptide structure, such as a helix formed by residues of the compound including, but not necessarily limited to, those between the first Cα to a second Cα.

In some embodiments, L is a macrocycle-forming linker of the formula

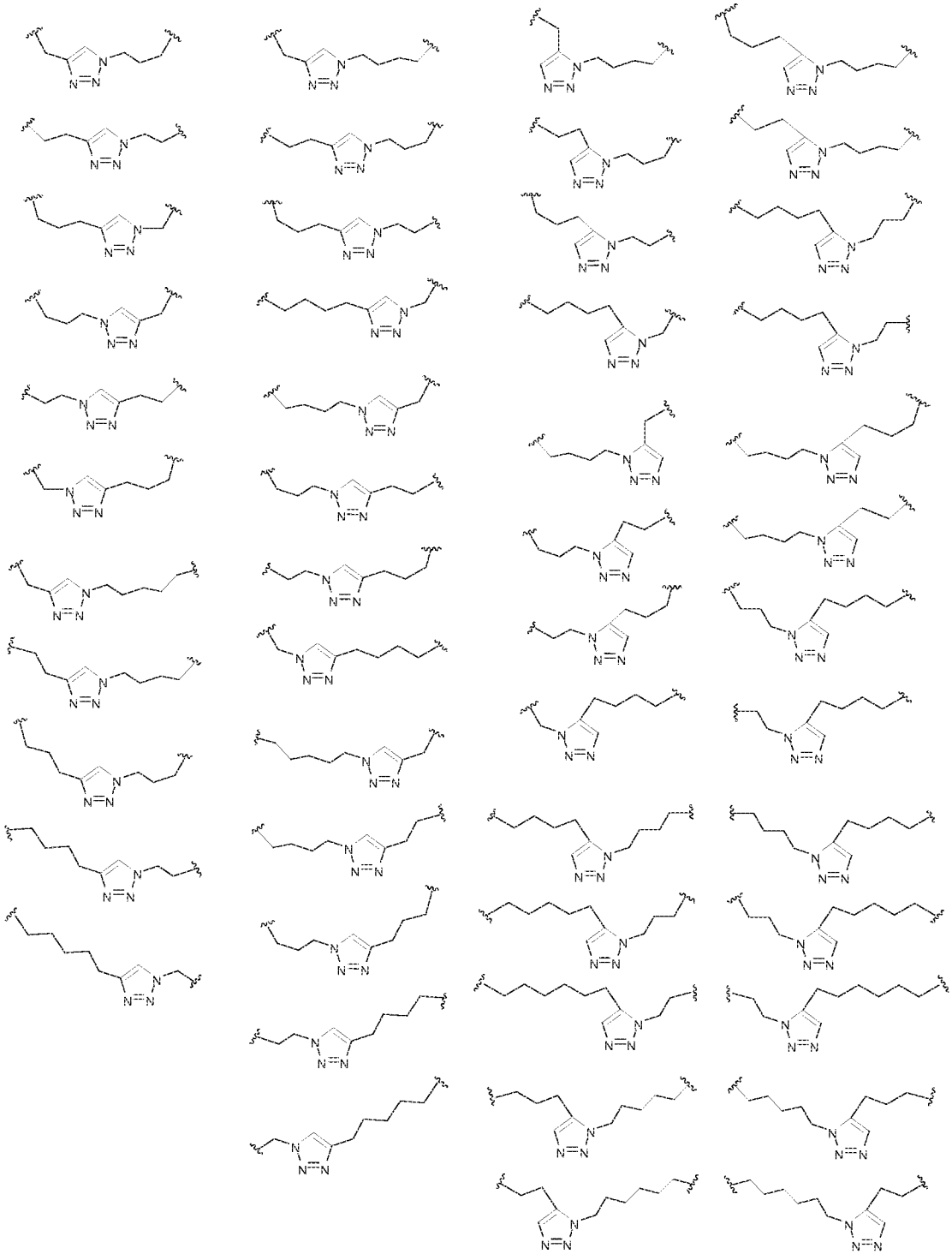


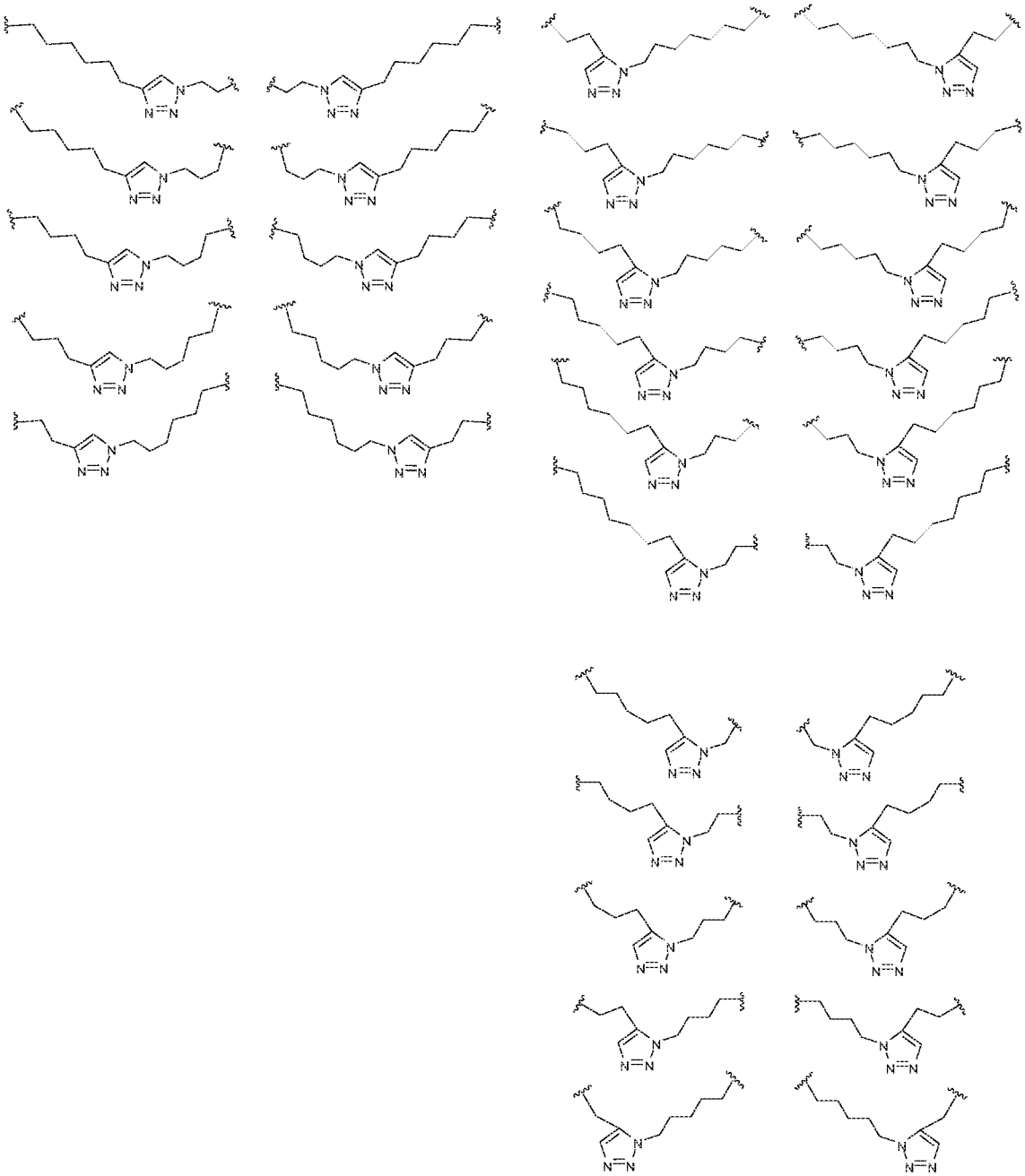
In some embodiments, L is a macrocycle-forming linker of the formula

 ; or  ;

or a tautomer thereof.

Exemplary embodiments of the macrocycle-forming linker L are shown below.





Amino acids that are used in the formation of triazole crosslinkers are represented according to the legend indicated below. Stereochemistry at the α-position of each amino acid is S unless otherwise indicated. For azide amino acids, the number of carbon atoms indicated refers to the number of methylene units between the α-carbon and the terminal azide. For alkyne amino acids, the number of carbon atoms indicated is the number of methylene units between the α-position and the triazole moiety plus the two carbon atoms within the triazole group derived from

the alkyne.

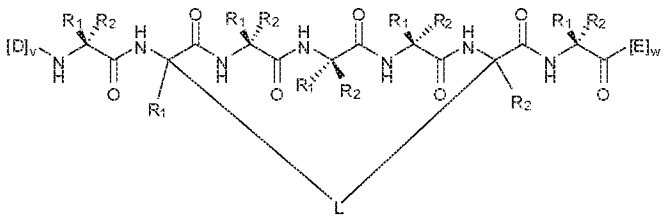
$5a5	α-Me alkyne 1,5 triazole (5 carbon)
$5n3	α-Me azide 1,5 triazole (3 carbon)
$4rn6	α-Me R-azide 1,4 triazole (6 carbon)
$4a5	α-Me alkyne 1,4 triazole (5 carbon)

Where the macrocycle-forming linker spans approximately 1 turn of an α-helix, the linkage contains approximately 4 atoms to 12 atoms, approximately 6 atoms to 10 atoms, or approximately 8 atoms. Where the macrocycle-forming linker spans approximately 2 turns of the α-helix, the linkage contains approximately 7 atoms to 15 atoms, approximately 9 atoms to 13 atoms, or approximately 11 atoms. Where the macrocycle-forming linker spans approximately 3 turns of the α-helix, the linkage contains approximately 13 atoms to 21 atoms, approximately 15 atoms to 19 atoms, or approximately 17 atoms. Where the macrocycle-forming linker spans approximately 4 turns of the α-helix, the linkage contains approximately 19 atoms to 27 atoms, approximately 21 atoms to 25 atoms, or approximately 23 atoms. Where the macrocycle-forming linker spans approximately 5 turns of the α-helix, the linkage contains approximately 25 atoms to 33 atoms, approximately 27 atoms to 31 atoms, or approximately 29 atoms. Where the macrocycle-forming linker spans approximately 1 turn of the α-helix, the resulting macrocycle forms a ring containing approximately 17 members to 25 members, approximately 19 members to 23 members, or approximately 21 members. Where the macrocycle-forming linker spans approximately 2 turns of the α-helix, the resulting macrocycle forms a ring containing approximately 29 members to 37 members, approximately 31 members to 35 members, or approximately 33 members. Where the macrocycle-forming linker spans approximately 3 turns of the α-helix, the resulting macrocycle forms a ring containing approximately 44 members to 52 members, approximately 46 members to 50 members, or approximately 48 members. Where the macrocycle-forming linker spans approximately 4 turns of the α-helix, the resulting macrocycle forms a ring containing approximately 59 members to 67 members, approximately 61 members to 65 members, or approximately 63 members. Where the macrocycle-forming linker spans approximately 5 turns of the α-helix, the resulting macrocycle forms a ring containing approximately 74 members to 82 members, approximately 76 members to 80 members, or approximately 78 members.

In any embodiment herein, each v, w, v', and w' can be, independently, 0, 1, 2, 3, 4, 5, 6,

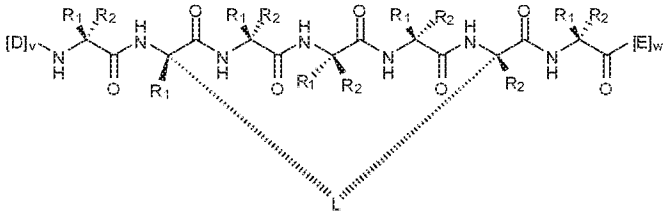
7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50. In any embodiment herein, each v, w, v', and w' can be, independently, 0-1000, 0-500, 0-400, 0-300, 0-200, 0-100, 0-50, 0-40, 0-30, 0-25, 0-20, 0-15, 0-10, 0-8, 0-6, 0-5, 1-1000, 1-500, 1-400, 1-300, 1-200, 1-100, 1-50, 1-40, 1-30, 1-25, 1-20, 1-15, 1-10, 1-8, 1-6, 1-5, 3-1000, 3-500, 3-400, 3-300, 3-200, 3-100, 3-50, 3-40, 3-30, 3-25, 3-20, 3-15, 3-10, 3-8, 3-6, or 3-5.

In one embodiment, the compound of Formula (II) is:

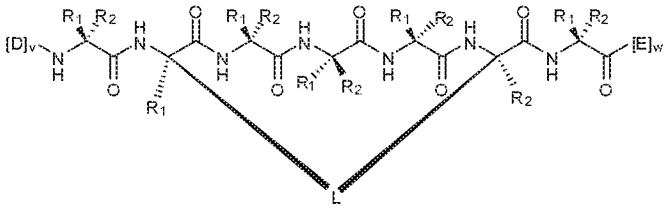


wherein each R_1 and R_2 is independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, any of which is unsubstituted or substituted.

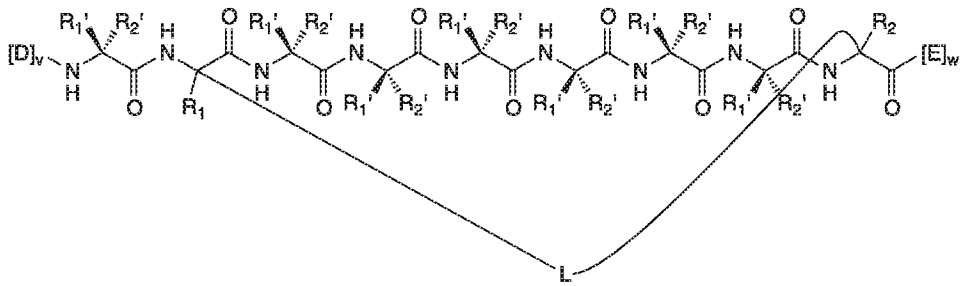
In related embodiments, the compound comprises a structure of Formula (II) which is:



or

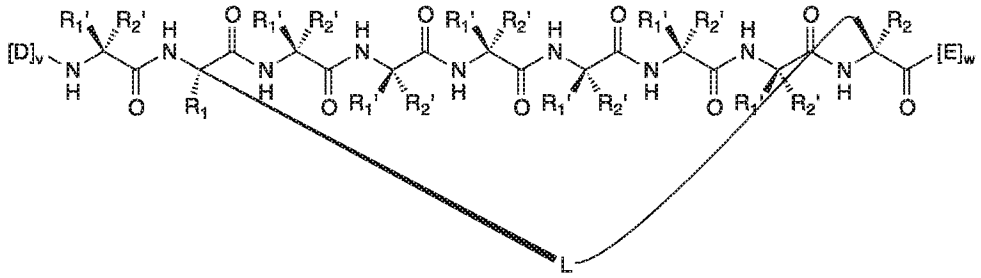


In some embodiments, the compound of Formula (II) is:



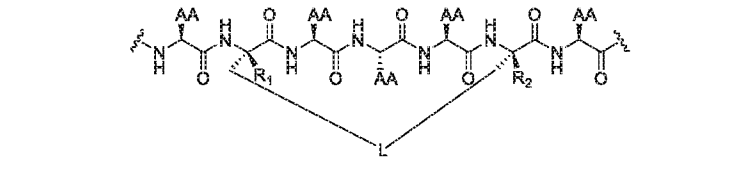
wherein each R_1 and R_2 is independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo–.

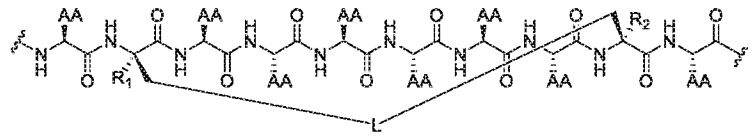
In related embodiments, the compound of Formula (II) is:

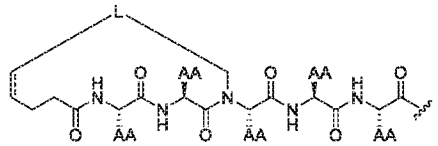


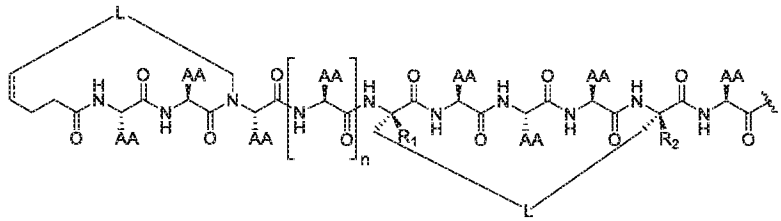
wherein each R_1' and R_2' is independently an amino acid side chain.

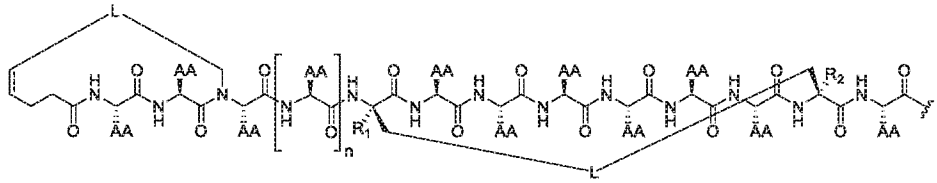
In other embodiments, the compound of Formula (II) is a compound of any of the formulas shown below:

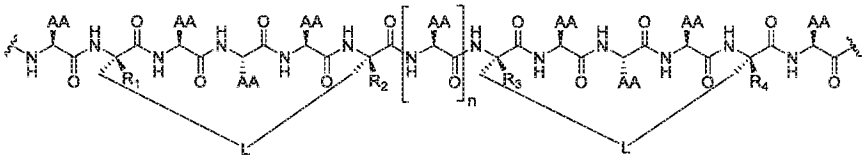


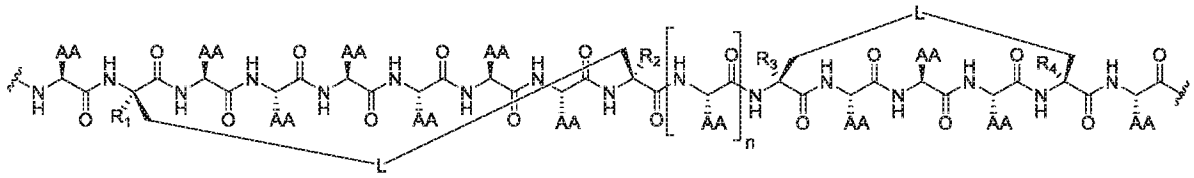


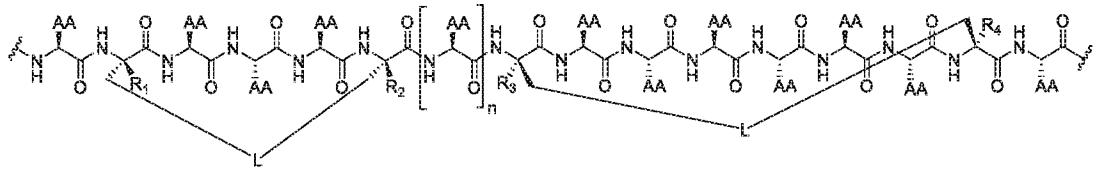


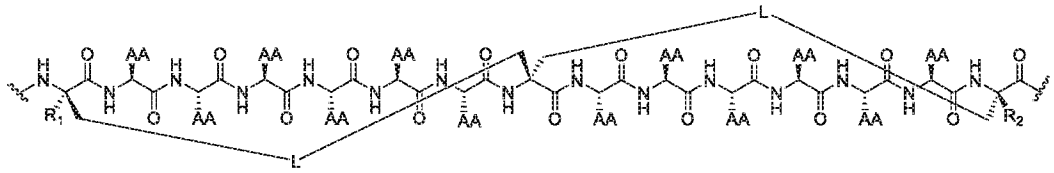






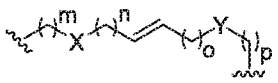




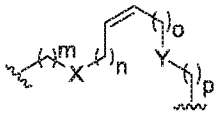


wherein "AA" represents any natural or non-natural amino acid side chain, "$\wavy$" is $[D]_v$ or $[E]_w$ as defined above, and n is an integer from 0 to 20, 50, 100, 200, 300, 400 or 500. In some embodiments, the substituent "n" shown in the preceding paragraph is 0. In other embodiments, the substituent "n" shown in the preceding paragraph is less than 50, 40, 30, 20, 10, or 5.

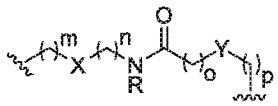
Exemplary embodiments of the macrocycle-forming linker L are shown below.



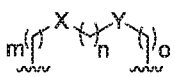
where X, Y = -CH_2-, O, S, or NH
m, n, o, p = 0-10



where X, Y = -CH_2-, O, S, or NH
m, n, o, p = 0-10



where X, Y = -CH_2-, O, S, or NH
m, n, o, p = 0-10
R = H, alkyl, other substituent



where X, Y = -CH_2-, O, S, or NH
m, n, o = 0-10

In other embodiments, [D] and/or [E] in the compound of Formula (II) are further modified in order to facilitate cellular uptake. In some embodiments, lipidating or PEGylating a compound facilitates cellular uptake, increases bioavailability, increases blood circulation, alters pharmacokinetics, decreases immunogenicity, and/or decreases the needed frequency of administration.

In other embodiments, at least one of [D] and [E] in the compound of Formula (II) represents a moiety comprising an additional macrocycle-forming linker such that the compound comprises at least two macrocycle-forming linkers. In a specific embodiment, a compound comprises two macrocycle-forming linkers. In one embodiment, u is 2.

In some embodiments, L is a macrocycle-forming linker of the formula $–L_1–L_2–$. In some embodiments, L_1 and L_2 are independently alkylene, alkenylene, alkynylene, heteroalkylene,

cycloalkylene, heterocycloalkylene, arylene, heteroarylene, or [-R_4-K-R_4-]$_n$, any of which is unsubstituted or substituted; each R_4 is alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene, any of which is unsubstituted or substituted; each K is independently O, S, SO, SO_2, CO, CO_2, $CONR_3$, OSO_2NR_3, NR_{3q}, $CONR_{3q}$, $OCONR_{3q}$, or OSO_2NR_{3q}, wherein each R_{3q} is independently a point of attachment to R_1 or R_2; and each n is independently 1, 2, 3, 4, or 5.

In an embodiment of any of the Formulas described herein, L_1 and L_2, either alone or in combination, form a triazole or a thioether.

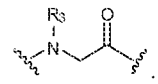
In an embodiment of any of the Formulas described herein, L_1 and L_2, either alone or in combination, do not form a triazole or a thioether.

In other embodiments, the length of the macrocycle-forming linker L as measured from a first α-carbon to a second α-carbon is selected to stabilize a desired secondary peptide structure, such as a helix formed by residues of the compound including, but not necessarily limited to, those between the first α-carbon to a second α-carbon.

In one example, at least one of R_1 and R_2 is alkyl, unsubstituted or substituted with halo–. In another example, both R_1 and R_2 are independently alkyl, unsubstituted or substituted with halo–. In some embodiments, at least one of R_1 and R_2 is methyl. In other embodiments, R_1 and R_2 are methyl.

In some embodiments, x+y+z is at least 2 or at least 3. In other embodiments, x+y+z is 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10. Each occurrence of A, B, C, D, or E in a macrocycle or macrocycle precursor is independently selected. For example, a sequence represented by the formula $[A]_x$, when x is 3, encompasses embodiments where the amino acids are not identical, e.g. Gln–Asp–Ala, as well as embodiments where the amino acids are identical, e.g. Gln–Gln–Gln. This applies for any value of x, y, or z in the indicated ranges. Similarly, when u is greater than 1, each compound may encompass compounds which are the same or different. For example, a compound may comprise compounds comprising different linker lengths or chemical compositions.

In some embodiments, the compound comprises a secondary structure that is a helix where R_8 is –H, allowing intrahelical hydrogen bonding. In some embodiments, at least one of A, B, C, D, or E is an α,α-disubstituted amino acid. In one example, B is an α,α-disubstituted amino acid. For instance, at least one of A, B, C, D, or E is 2-aminoisobutyric acid. In other

embodiments, at least one of A, B, C, D or E is  .

In some embodiments, w is from 1 to 1000. For example, the first amino acid represented by E comprises a small hydrophobic side chain. In some embodiments, w is from 2 to 1000. For example, the second amino acid represented by E comprises a small hydrophobic side chain. In some embodiments, w is from 3 to 1000. For example, the third amino acid represented by E can comprise a small hydrophobic side chain. For example, the third amino acid represented by E can comprise a small hydrophobic side chain. In some embodiments, w is from 4 and 1000. In some embodiments, w is from 5 and 1000. In some embodiments, w is from 6 and 1000. In some embodiments, w is from 7 and 1000. In some embodiments, w is from 8 and 1000. In some embodiments, w is an integer from 3-10, for example 3-6, 3-8, 6-8, or 6-10. In some embodiments, w is 3. In other embodiments, w is 6. In some embodiments, v is an integer from 1-10, for example 2-5. In some embodiments, v is 2. In some embodiments, v is 3.

In some embodiments, each of the first two amino acid represented by E comprises an uncharged side chain or a negatively charged side chain. In some embodiments, each of the first three amino acid represented by E comprises an uncharged side chain or a negatively charged side chain. In some embodiments, each of the first four amino acid represented by E comprises an uncharged side chain or a negatively charged side chain.

In some embodiments, the first C-terminal amino acid and/or the second C-terminal amino acid represented by E comprise a hydrophobic side chain. For example, the first C-terminal amino acid and/or the second C-terminal amino acid represented by E comprises a hydrophobic side chain, for example a small hydrophobic side chain. In some embodiments, the first C-terminal amino acid, the second C-terminal amino acid, and/or the third C-terminal amino acid represented by E comprise a hydrophobic side chain. For example, the first C-terminal amino acid, the second C-terminal amino acid, and/or the third C-terminal amino acid represented by E comprises a hydrophobic side chain, for example a small hydrophobic side chain.

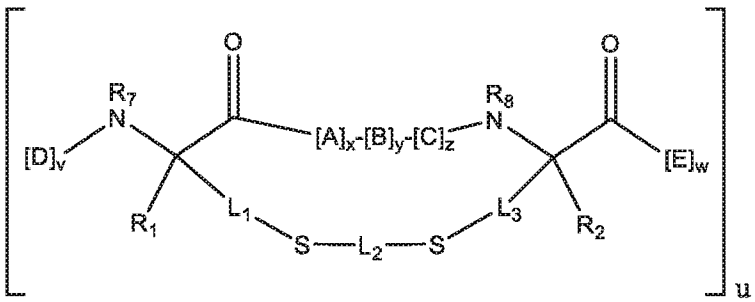
In some embodiments, one or more or each of the amino acid that is *i*+1, *i*+2, *i*+3, *i*+4, *i*+5, and/or *i*+6 with respect to a first E comprises an uncharged side chain or a negatively charged side chain. In some embodiments, each E is independently an amino acid selected from the group consisting of Ala (alanine), D–Ala (D–alanine), Aib (α–aminoisobutyric acid), Sar (N–

methyl glycine), and Ser (serine).

In other embodiments, [D] and/or [E] in the compound of Formula I, Ia, Ib, or Ic are further modified in order to facilitate cellular uptake. In some embodiments, lipidating or PEGylating a compound facilitates cellular uptake, increases bioavailability, increases blood circulation, alters pharmacokinetics, decreases immunogenicity and/or decreases the needed frequency of administration.

In other embodiments, at least one of [D] and [E] in the compound of Formula I, Ia, Ib, or Ic represents a moiety comprising an additional macrocycle-forming linker such that the compound comprises at least two macrocycle-forming linkers. In a specific embodiment, a compound comprises two macrocycle-forming linkers. In an embodiment, u is 2.

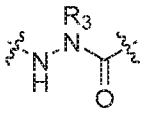
In other embodiments, the invention provides compounds of Formula (III):



Formula (III)

wherein:

- each A, C, D, and E is independently a natural or non-natural amino acid;
- each B is independently a natural or non-natural amino acid, amino acid analog,



, [-NH-L_4-CO-], [-NH-L_4-SO_2-], or [-NH-L_4-];

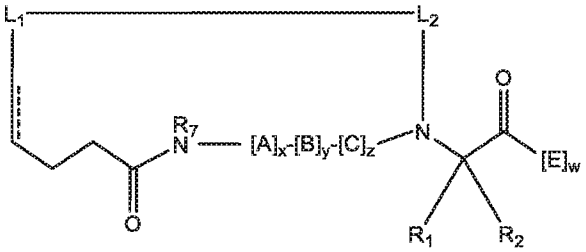
- each R_1 is independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, any of which is unsubstituted or substituted, or together with R_1 and the atom to which R_1 and L are bound forms a ring;
- each R_2 is independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, any of which is unsubstituted or substituted, or together with R_2 and the atom to which R_2 and L are bound forms a ring;
- each R_3 is independently hydrogen, alkyl, alkenyl, alkynyl, arylalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, cycloalkylalkyl, aryl, or heteroaryl, any of which is unsubstituted or

substituted;

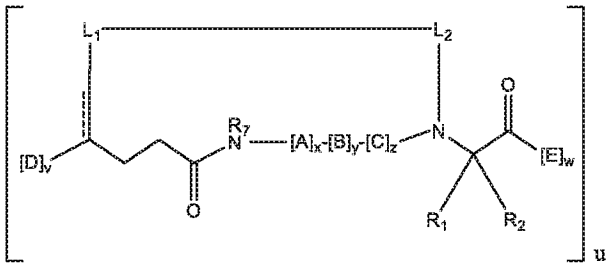
- each L_1, L_2, L_3 and L_4 is independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, heteroarylene or $[-R_4-K-R_4-]_n$, any of which is unsubstituted or substituted;
- each K is independently O, S, SO, SO_2, CO, CO_2, $CONR_3$, OSO_2NR_3, NR_{3q}, $CONR_{3q}$, $OCONR_{3q}$, or OSO_2NR_{3q}, wherein each R_{3q} is independently a point of attachment to R_1 or R_2;
- each R_4 is independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene, any of which is unsubstituted or substituted;
- each R_7 is independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, aryl, or heteroaryl, any of which is unsubstituted or substituted, or part of a cyclic structure with a D residue;
- each R_8 is independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, aryl, or heteroaryl, any of which is unsubstituted or substituted, or part of a cyclic structure with an E residue;
- each v and w is independently an integer from 0-1000, from 1-1000, or 3-1000;
- each x, y and z is independently 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10;
- u is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10; and
- each n is independently 1, 2, 3, 4, or 5.

In some embodiments, the length of the macrocycle-forming linker $[-L_1-S-L_2-S-L_3-]$ as measured from a first α-carbon to a second α-carbon is selected to stabilize a desired secondary peptide structure, such as a helix (including, but not limited to a 3_{10} helix or an α-helix) formed by residues of the compound including, but not necessarily limited to, those between the first α-carbon to a second α-carbon. In some embodiments, the thiol moieties are the side chains of the amino acid residues L-cysteine, D-cysteine, α-methyl-L cysteine, α-methyl-D-cysteine, L-homocysteine, D-homocysteine, α-methyl-L-homocysteine, or α-methyl-D-homocysteine. A bis-alkylating reagent is of the general formula $X-L_2-Y$, wherein L_2 is a linker moiety and X and Y are leaving groups that are displaced by -SH moieties to form bonds with L_2. In some embodiments, X and Y are halogens, such as I, Br, or Cl.

In other embodiments, the invention provides compounds of Formula (IV) or (IVa):



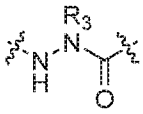
Formula (IV)



Formula (IVa)

wherein:

- each A, C, D, and E is independently a natural or non-natural amino acid;
- each B is independently a natural or non-natural amino acid, amino acid analog,



, [-NH-L_4-CO-], [-NH-L_4-SO_2-], or [-NH-L_4-];

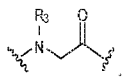
- each R_1 is independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, any of which is unsubstituted or substituted, or part of a cyclic structure with an E residue;
- each R_2 is independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, any of which is unsubstituted or substituted, or part of a cyclic structure with an E residue;
- each R_3 is independently hydrogen, alkyl, alkenyl, alkynyl, arylalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, cycloalkylalkyl, aryl, or heteroaryl, any of which is unsubstituted or substituted;
- each L is independently a macrocycle-forming linker of the formula –L_1–L_2–;
- each L_1, L_2, and L_4 is independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, heteroarylene, or [-R_4-K-R_4-]$_n$, any of which is unsubstituted or substituted;

- each R_4 is independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene, any of which is unsubstituted or substituted;
- each K is independently O, S, SO, SO_2, CO, CO_2, $CONR_3$, OSO_2NR_3, NR_{3q}, $CONR_{3q}$, $OCONR_{3q}$, or OSO_2NR_{3q}, wherein each R_{3q} is independently a point of attachment to R_1 or R_2;
- each R_7 is independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, aryl, or heteroaryl, any of which is unsubstituted or substituted;
- each v and w is independently integers from 0-1000, from 1-1000, or 3-1000;
- each x, y and z is independently 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10;
- u is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10; and
- each n is independently 1, 2, 3, 4, 5.

In one example, L_1 and L_2, either alone or in combination, do not form a triazole or a thioether.

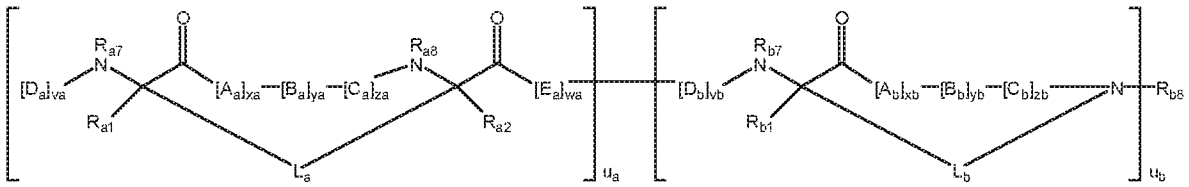
In one example, at least one of R_1 and R_2 is alkyl that is unsubstituted or substituted with halo–. In another example, both R_1 and R_2 are independently alkyl that is unsubstituted or substituted with halo–. In some embodiments, at least one of R_1 and R_2 is methyl. In other embodiments, R_1 and R_2 are methyl.

In some embodiments, x+y+z is at least 1. In other embodiments, x+y+z is at least 2. In other embodiments, x+y+z is 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10. Each occurrence of A, B, C, D, or E in a macrocycle or macrocycle precursor is independently selected. For example, a sequence represented by the formula $[A]_x$, when x is 3, encompasses embodiments where the amino acids are not identical, e.g. Gln–Asp–Ala, as well as embodiments where the amino acids are identical, e.g. Gln–Gln–Gln. This applies for any value of x, y, or z in the indicated ranges.

In some embodiments, the compound comprises a secondary structure which is an α-helix and R_8 is –H, allowing intrahelical hydrogen bonding. In some embodiments, at least one of A, B, C, D or E is an α,α-disubstituted amino acid. In one example, B is an α,α-disubstituted amino acid. For example, at least one of A, B, C, D or E is 2-aminoisobutyric acid. In other embodiments, at least one of A, B, C, D or E is  .

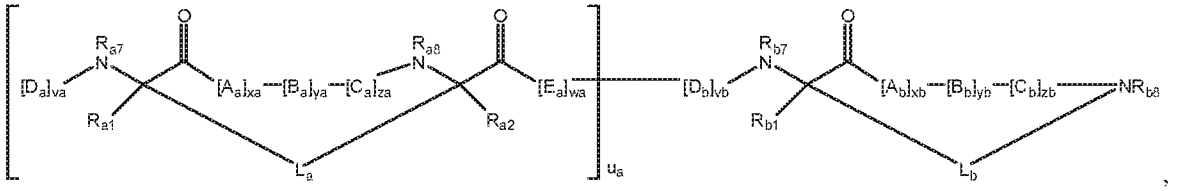
In other embodiments, the length of the macrocycle-forming linker L as measured from a first Cα to a second Cα is selected to stabilize a desired secondary peptide structure, such as an α-helix formed by residues of the compound including, but not necessarily limited to, those between the first Cα to a second Cα.

In some embodiments, the compound has the Formula (V) or Formula (Va):



Formula (V)

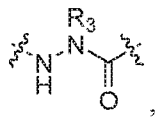
or



Formula (Va)

wherein:

- each A_a, C_a, D_a, E_a, A_b, C_b, and D_b is independently a natural or non-natural amino acid;

- each B_a and B_b is independently a natural or non-natural amino acid,  , [-NH-L_4-CO-], [-NH-L_4-SO_2-], or [-NH-L_4-];

- each R_{a1} is independently, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, any of which is unsubstituted or substituted; or H; or R_{a1} forms a macrocycle-forming linker L' connected to the alpha position of one of the D_a or E_a amino acids; or together with L_a forms a ring that is unsubstituted or substituted;

- each R_{a2} is independently, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, any of which is unsubstituted or substituted; or H; or R_{a2} forms a macrocycle-forming linker L' connected to the alpha position of one of the D_a or E_a amino acids; or together with L_a forms a ring that is unsubstituted or substituted;

- each R_{b1} is independently, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl,

cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, any of which is unsubstituted or substituted; or H; or R_{b1} forms a macrocycle-forming linker L' connected to the alpha position of one of the D_b amino acids; or together with L_b forms a ring that is unsubstituted or substituted;

- each R_3 is independently, alkyl, alkenyl, alkynyl, arylalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, cycloalkylalkyl, cycloaryl, or heterocycloaryl, any of which is unsubstituted or substituted, or H;
- each L_a is independently a macrocycle-forming linker, and optionally forms a ring with R_{a1} or R_{a2} that is unsubstituted or substituted;
- each L_b is independently a macrocycle-forming linker, and optionally forms a ring with R_{b1} that is unsubstituted or substituted;
- each L' is independently a macrocycle-forming linker;
- each L_4 is independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, cycloarylene, heterocycloarylene, or $[-R_4-K-R_4-]_n$, any of which is unsubstituted or substituted;
- each R_4 is independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene, any of which is unsubstituted or substituted;
- each K is independently O, S, SO, SO_2, CO, CO_2, OCO_2, NR_3, $CONR_3$, $OCONR_3$, OSO_2NR_3, NR_{3q}, $CONR_{3q}$, $OCONR_{3q}$, or OSO_2NR_{3q}, wherein each R_{3q} is independently a point of attachment to R_{a1}, R_{a2}, or R_{b1};
- each R_{a7} is independently alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, any of which is unsubstituted or substituted; or H; or part of a cyclic structure with a D_a amino acid;
- each R_{b7} is independently alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, any of which is unsubstituted or substituted; or H; or part of a cyclic structure with a D_b amino acid;
- each R_{a8} is independently alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, any of which is unsubstituted or substituted; or H; or part of a cyclic structure with an E_a amino acid;
- each R_{b8} is independently alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, any of which is

unsubstituted or substituted; or H; or an amino acid sequence of 1-1000 amino acid residues;

- each va and vb is independently an integer from 0-1000;
- each wa and wb is independently an integer from 0-1000;
- each ua and ub is independently 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10, wherein ua+ub is at least 1;
- each xa and xb is independently 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10;
- each ya and yb is independently 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10;
- each za and zb is independently 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10; and
- each n is independently 1, 2, 3, 4, or 5,

or a pharmaceutically-acceptable salt thereof.

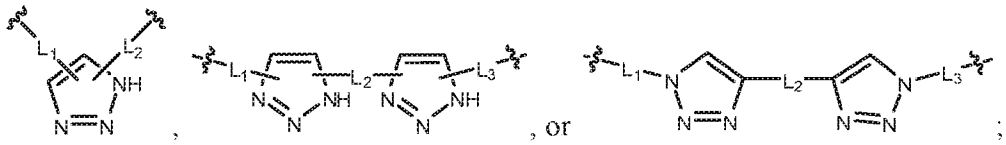
In some embodiments, the compound of the invention has the formula defined above, wherein:

- each L_a is independently a macrocycle-forming linker of the formula $–L_1–L_2–$, and optionally forms a ring with R_{a1} or R_{a2} that is unsubstituted or substituted;
- each L_b is independently a macrocycle-forming linker of the formula $–L_1–L_2–$, and optionally forms a ring with R_{b1} that is unsubstituted or substituted;
- each L' is independently a macrocycle-forming linker of the formula $–L_1–L_2–$;
- each L_1 and L_2 is independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, cycloarylene, heterocycloarylene, or $[-R_4-K-R_4-]_n$, any of which is unsubstituted or substituted;
- each R_4 is independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene, any of which is unsubstituted or substituted;
- each K is independently O, S, SO, SO_2, CO, CO_2, OCO_2, NR_3, $CONR_3$, $OCONR_3$, OSO_2NR_3, NR_{3q}, $CONR_{3q}$, $OCONR_{3q}$, or OSO_2NR_{3q}, wherein each R_{3q} is independently a point of attachment to R_{a1}, R_{a2}, or R_{b1};

or a pharmaceutically-acceptable salt thereof.

In some embodiments, the compound has the formula defined above wherein each L_a and L_b is independently a triazole-containing macrocycle-forming linker. In some embodiments, the compound has the formula defined above, wherein:

- each L_a and L_b is independently a macrocycle-forming linker of the formula:



- each L_1, L_2, and L_3 is independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, cycloarylene, heterocycloarylene, or [-R_4-K-R_4-]$_n$, any of which is unsubstituted or substituted;
- each R_4 is independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene, any of which is unsubstituted or substituted;
- each K is independently O, S, SO, SO_2, CO, CO_2, OCO_2, NR_3, $CONR_3$, $OCONR_3$, OSO_2NR_3, NR_{3q}, $CONR_{3q}$, $OCONR_{3q}$, or OSO_2NR_{3q}, wherein each R_{3q} is independently a point of attachment to R_{a1}, R_{a2}, or R_{b1}; and
- each n is independently 1, 2, 3, 4, or 5,

or a pharmaceutically-acceptable salt thereof.

In some embodiments, the compound has the formula defined above, wherein:

- each L_a and L_b is independently a macrocycle-forming linker of the formula –L_1–SR_9R_{10}–L_2–$SR_{11}R_{12}$–L_3–, wherein each L_1, L_2, and L_3 is independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, cycloarylene, heterocycloarylene, or [-R_4-K-R_4-]$_n$, any of which is unsubstituted or substituted; and each R_9, R_{10}, R_{11}, and R_{12} is independently absent or O;
- each R_4 is independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene, any of which is unsubstituted or substituted;
- each K is independently O, S, SO, SO_2, CO, CO_2, OCO_2, NR_3, $CONR_3$, $OCONR_3$, OSO_2NR_3, NR_{3q}, $CONR_{3q}$, $OCONR_{3q}$, or OSO_2NR_{3q}, wherein each R_{3q} is independently a point of attachment to R_{a1}, R_{a2}, or R_{b1}; and
- each n is independently 1, 2, 3, 4, or 5,

or a pharmaceutically-acceptable salt thereof.

In some embodiments, the compound has the formula defined above wherein one or both L_a and L_b is independently a bis-thioether-containing macrocycle-forming linker. In some embodiments, each L_a and L_b is independently a macrocycle-forming linker of the formula –L_1–

S–L_2–S–L_3–.

In some embodiments, the compound has the formula defined above wherein one or both L_a and L_b is independently a bis-sulfone-containing macrocycle-forming linker. In some embodiments, each L_a and L_b is independently a macrocycle-forming linker of the formula –L_1–SO_2–L_2–SO_2–L_3–.

In some embodiments, the compound has the formula defined above wherein one or both L_a and L_b is independently a bis-sulfoxide-containing macrocycle-forming linker. In some embodiments, each L_a and L_b is independently a macrocycle-forming linker of the formula –L_1–S(O)–L_2–S(O)–L_3–.

In some embodiments, a compound of the invention comprises one or more secondary structures. In some embodiments, the compound comprises a secondary structure that is an α-helix. In some embodiments, the compound comprises a secondary structure that is a β-hairpin turn.

In some embodiments, u_a is 0. In some embodiments, u_a is 0, and L_b is a triazole-containing macrocycle-forming linker that crosslinks an α-helical secondary structure. In some embodiments, u_a is 0, and L_b is a hydrocarbon-containing macrocycle-forming linker that crosslinks an α-helical secondary structure.

In some embodiments, u_b is 0. In some embodiments, u_b is 0, and L_a is a triazole-containing macrocycle-forming linker that crosslinks an α-helical secondary structure. In some embodiments, u_b is 0, and L_a is a hydrocarbon-containing macrocycle-forming linker that crosslinks an α-helical secondary structure.

In some embodiments, the compound comprises only α-helical secondary structures.

In other embodiments, the compound comprises a combination of secondary structures, wherein the secondary structures are α-helical and β-hairpin structures. In some embodiments, L_a and L_b are a combination of hydrocarbon-, triazole, or sulfur-containing macrocycle-forming linkers. In some embodiments, the compound comprises L_a and L_b, wherein L_a is a hydrocarbon-containing macrocycle-forming linker that crosslinks a β-hairpin structure, and L_b is a triazole-containing macrocycle-forming linker that crosslinks an α-helical structure. In some embodiments, the compound comprises L_a and L_b, wherein L_a is a hydrocarbon-containing macrocycle-forming linker that crosslinks an α-helical structure, and L_b is a triazole-containing macrocycle-forming linker that crosslinks a β-hairpin structure. In some embodiments, the

compound comprises L_a and L_b, wherein L_a is a triazole-containing macrocycle-forming linker that crosslinks an α-helical structure, and L_b is a hydrocarbon-containing macrocycle-forming linker that crosslinks a β-hairpin structure. In some embodiments, the compound comprises L_a and L_b, wherein L_a is a triazole-containing macrocycle-forming linker that crosslinks a β-hairpin structure, and L_b is a hydrocarbon-containing macrocycle-forming linker that crosslinks an α-helical structure.

In some embodiments, u_a+u_b is at least 1. In some embodiments, $u_a+u_b = 2$.

In some embodiments, u_a is 1, and u_b is 1. In some embodiments, u_a is 1, u_b is 1, L_a is a triazole-containing macrocycle-forming linker that crosslinks an α-helical secondary structure, and L_b is a triazole-containing macrocycle-forming linker that crosslinks an α-helical secondary structure. In some embodiments, u_a is 1, u_b is 1, L_a is a triazole-containing macrocycle-forming linker that crosslinks an α-helical secondary structure, and L_b is a triazole-containing macrocycle-forming linker that crosslinks a β-hairpin secondary structure. In some embodiments, u_a is 1, u_b is 1, L_a is a triazole-containing macrocycle-forming linker that crosslinks a β-hairpin secondary structure, and L_b is a triazole-containing macrocycle-forming linker that crosslinks an α-helical secondary structure.

In some embodiments, u_a is 1, u_b is 1, L_a is a triazole-containing macrocycle-forming linker that crosslinks an α-helical secondary structure, and L_b is a hydrocarbon-containing macrocycle-forming linker that crosslinks an α-helical structure. In some embodiments, u_a is 1, u_b is 1, L_a is a triazole-containing macrocycle-forming linker that crosslinks an α-helical secondary structure, and L_b is a hydrocarbon-containing macrocycle-forming linker that crosslinks a β-hairpin structure. In some embodiments, u_a is 1, u_b is 1, L_a is a triazole-containing macrocycle-forming linker that crosslinks a β-hairpin secondary structure, and L_b is a hydrocarbon-containing macrocycle-forming linker that crosslinks an α-helical structure.

In some embodiments, u_a is 1, u_b is 1, L_a is a hydrocarbon-containing macrocycle-forming linker that crosslinks an α-helical secondary structure, and L_b is a triazole-containing macrocycle-forming linker that crosslinks an α-helical secondary structure. In some embodiments, u_a is 1, u_b is 1, L_a is a hydrocarbon-containing macrocycle-forming linker that crosslinks an α-helical secondary structure, and L_b is a triazole-containing macrocycle-forming linker that crosslinks a β-hairpin secondary structure. In some embodiments, u_a is 1, u_b is 1, L_a is a hydrocarbon-containing macrocycle-forming linker that crosslinks a β-hairpin secondary

structure, and L_b is a triazole-containing macrocycle-forming linker that crosslinks an α-helical secondary structure.

In some embodiments, u_a is 1, u_b is 1, L_a is a triazole-containing macrocycle-forming linker that crosslinks an α-helical secondary structure, and L_b is a sulfur-containing macrocycle-forming linker.

In some embodiments, u_a is 1, u_b is 1, L_a is a sulfur-containing macrocycle-forming linker, and L_b is a triazole-containing macrocycle-forming linker with an α-helical secondary structure.

In some embodiments, u_a is 1, u_b is 1, L_a is a hydrocarbon-containing macrocycle-forming linker with an α-helical secondary structure, and L_b is a sulfur-containing macrocycle-forming linker.

In some embodiments, u_a is 1, u_b is 1, L_a is a sulfur-containing macrocycle-forming linker, and L_b is a hydrocarbon-containing macrocycle-forming linker with an α-helical secondary structure.

In some embodiments, u_a is 1, u_b is 1, L_a is a sulfur-containing macrocycle-forming linker, and L_b is a sulfur-containing macrocycle-forming linker.

In some embodiments, u_a is 1, u_b is 1, L_a is a hydrocarbon-containing macrocycle-forming linker that crosslinks an α-helical structure, and L_b is a hydrocarbon-containing macrocycle-forming linker that crosslinks an α-helical structure. In some embodiments, u_a is 1, u_b is 1, L_a is a hydrocarbon-containing macrocycle-forming linker that crosslinks an α-helical structure, and L_b is a hydrocarbon-containing macrocycle-forming linker that crosslinks a β-hairpin structure. In some embodiments, u_a is 1, u_b is 1, L_a is a hydrocarbon-containing macrocycle-forming linker that crosslinks a β-hairpin structure, and L_b is a hydrocarbon-containing macrocycle-forming linker that crosslinks an α-helical structure.

In some embodiments, R_{b1} is H.

In some embodiments, each v and w is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20. In some embodiments, w is an integer from 3-1000, for example 3-500, 3-200, 3-100, 3-50, 3-30, 3-20, or 3-10. In some embodiments, the sum of x+y+z is 3 or 6. In some embodiments, the sum of x+y+z is 3. In other embodiments, the sum of x+y+z is 6.

Unless otherwise stated, any compounds (including compounds, macrocycle precursors, and other compositions) are also meant to encompass compounds which differ only in the

presence of one or more isotopically enriched atoms. For example, compounds having the described structures except for the replacement of a hydrogen by a deuterium or tritium, or the replacement of a carbon by ^{13}C- or ^{14}C-enriched carbon are within the scope of this disclosure.

In some embodiments, the compounds disclosed herein can contain unnatural proportions of atomic isotopes at one or more of atoms that constitute such compounds. For example, the compounds can be radiolabeled with radioactive isotopes, such as for example tritium (^{3}H), iodine-125 (^{125}I) or carbon-14 (^{14}C). In other embodiments, one or more carbon atoms are replaced with a silicon atom. All isotopic variations of the compounds disclosed herein, whether radioactive or not, are contemplated herein.

A compound described herein can be at least 1% pure, at least 2% pure, at least 3% pure, at least 4% pure, at least 5% pure, at least 6% pure, at least 7% pure, at least 8% pure, at least 9% pure, at least 10% pure, at least 11% pure, at least 12% pure, at least 13% pure, at least 14% pure, at least 15% pure, at least 16% pure, at least 17% pure, at least 18% pure, at least 19% pure, at least 20% pure, at least 21% pure, at least 22% pure, at least 23% pure, at least 24% pure, at least 25% pure, at least 26% pure, at least 27% pure, at least 28% pure, at least 29% pure, at least 30% pure, at least 31% pure, at least 32% pure, at least 33% pure, at least 34% pure, at least 35% pure, at least 36% pure, at least 37% pure, at least 38% pure, at least 39% pure, at least 40% pure, at least 41% pure, at least 42% pure, at least 43% pure, at least 44% pure, at least 45% pure, at least 46% pure, at least 47% pure, at least 48% pure, at least 49% pure, at least 50% pure, at least 51% pure, at least 52% pure, at least 53% pure, at least 54% pure, at least 55% pure, at least 56% pure, at least 57% pure, at least 58% pure, at least 59% pure, at least 60% pure, at least 61% pure, at least 62% pure, at least 63% pure, at least 64% pure, at least 65% pure, at least 66% pure, at least 67% pure, at least 68% pure, at least 69% pure, at least 70% pure, at least 71% pure, at least 72% pure, at least 73% pure, at least 74% pure, at least 75% pure, at least 76% pure, at least 77% pure, at least 78% pure, at least 79% pure, at least 80% pure, at least 81% pure, at least 82% pure, at least 83% pure, at least 84% pure, at least 85% pure, at least 86% pure, at least 87% pure, at least 88% pure, at least 89% pure, at least 90% pure, at least 91% pure, at least 92% pure, at least 93% pure, at least 94% pure, at least 95% pure, at least 96% pure, at least 97% pure, at least 98% pure, at least 99% pure, at least 99.1% pure, at least 99.2% pure, at least 99.3% pure, at least 99.4% pure, at least 99.5% pure, at least 99.6% pure, at least 99.7% pure, at least 99.8% pure, or at least 99.9% pure

on a chemical, optical, isomeric, enantiomeric, or diastereomeric basis. Purity can be assessed, for example, by HPLC, MS, LC/MS, melting point, or NMR.

Two or more peptides can share a degree of homology. A pair of peptides can have, for example, up to about 20% pairwise homology, up to about 25% pairwise homology, up to about 30% pairwise homology, up to about 35% pairwise homology, up to about 40% pairwise homology, up to about 45% pairwise homology, up to about 50% pairwise homology, up to about 55% pairwise homology, up to about 60% pairwise homology, up to about 65% pairwise homology, up to about 70% pairwise homology, up to about 75% pairwise homology, up to about 80% pairwise homology, up to about 85% pairwise homology, up to about 90% pairwise homology, up to about 95% pairwise homology, up to about 96% pairwise homology, up to about 97% pairwise homology, up to about 98% pairwise homology, up to about 99% pairwise homology, up to about 99.5% pairwise homology, or up to about 99.9% pairwise homology. A pair of peptides can have, for example, at least about 20% pairwise homology, at least about 25% pairwise homology, at least about 30% pairwise homology, at least about 35% pairwise homology, at least about 40% pairwise homology, at least about 45% pairwise homology, at least about 50% pairwise homology, at least about 55% pairwise homology, at least about 60% pairwise homology, at least about 65% pairwise homology, at least about 70% pairwise homology, at least about 75% pairwise homology, at least about 80% pairwise homology, at least about 85% pairwise homology, at least about 90% pairwise homology, at least about 95% pairwise homology, at least about 96% pairwise homology, at least about 97% pairwise homology, at least about 98% pairwise homology, at least about 99% pairwise homology, at least about 99.5% pairwise homology, at least about 99.9% pairwise homology.

Various methods and software programs can be used to determine the homology between two or more peptides, such as NCBI BLAST, Clustal W, MAFFT, Clustal Omega, AlignMe, Praline, or another suitable method or algorithm.

In some embodiments, the compound comprises at least one helical motif, such as a 3_{10} or an α-helix motif. For example, A, B and/or C in the compound of Formula I, II, or III include one or more helices. As a general matter, helices include from 3 to 4 amino acid residues per turn. In some embodiments, the helix of the compound includes 1 to 5 turns and, therefore, 3 to 20 amino acid residues. In specific embodiments, the helix includes 1 turn, 2 turns, 3 turns, 4 turns, or 5 turns. In some embodiments, the macrocycle-forming linker stabilizes a helix motif included

within the compound. Thus, in some embodiments, the length of the macrocycle-forming linker L from a first α-carbon to a second α-carbon is selected to increase the stability of a helix. In some embodiments, the macrocycle-forming linker spans from 1 turn to 5 turns of the helix. In some embodiments, the macrocycle-forming linker spans approximately 1 turn, 2 turns, 3 turns, 4 turns, or 5 turns of the helix. In some embodiments, the length of the macrocycle-forming linker is approximately 5 Å to 9 Å per turn of the helix, or approximately 6 Å to 8 Å per turn of the helix. Where the macrocycle-forming linker spans approximately 1 turn of a helix, the length is equal to approximately 5 carbon-carbon bonds to 13 carbon-carbon bonds, approximately 7 carbon-carbon bonds to 11 carbon-carbon bonds, or approximately 9 carbon-carbon bonds. Where the macrocycle-forming linker spans approximately 2 turns of a helix, the length is equal to approximately 8 carbon-carbon bonds to 16 carbon-carbon bonds, approximately 10 carbon-carbon bonds to 14 carbon-carbon bonds, or approximately 12 carbon-carbon bonds. Where the macrocycle-forming linker spans approximately 3 turns of a helix, the length is equal to approximately 14 carbon-carbon bonds to 22 carbon-carbon bonds, approximately 16 carbon-carbon bonds to 20 carbon-carbon bonds, or approximately 18 carbon-carbon bonds. Where the macrocycle-forming linker spans approximately 4 turns of a helix, the length is equal to approximately 20 carbon-carbon bonds to 28 carbon-carbon bonds, approximately 22 carbon-carbon bonds to 26 carbon-carbon bonds, or approximately 24 carbon-carbon bonds. Where the macrocycle-forming linker spans approximately 5 turns of a helix, the length is equal to approximately 26 carbon-carbon bonds to 34 carbon-carbon bonds, approximately 28 carbon-carbon bonds to 32 carbon-carbon bonds, or approximately 30 carbon-carbon bonds. Where the macrocycle-forming linker spans approximately 1 turn of a helix, the linkage contains approximately 4 atoms to 12 atoms, approximately 6 atoms to 10 atoms, or approximately 8 atoms. Where the macrocycle-forming linker spans approximately 2 turns of the helix, the linkage contains approximately 7 atoms to 15 atoms, approximately 9 atoms to 13 atoms, or approximately 11 atoms. Where the macrocycle-forming linker spans approximately 3 turns of the helix, the linkage contains approximately 13 atoms to 21 atoms, approximately 15 atoms to 19 atoms, or approximately 17 atoms. Where the macrocycle-forming linker spans approximately 4 turns of the helix, the linkage contains approximately 19 atoms to 27 atoms, approximately 21 atoms to 25 atoms, or approximately 23 atoms. Where the macrocycle-forming linker spans approximately 5 turns of the helix, the linkage contains approximately 25 atoms to

33 atoms, approximately 27 atoms to 31 atoms, or approximately 29 atoms. Where the macrocycle-forming linker spans approximately 1 turn of the helix, the resulting macrocycle forms a ring containing approximately 17 members to 25 members, approximately 19 members to 23 members, or approximately 21 members. Where the macrocycle-forming linker spans approximately 2 turns of the helix, the resulting macrocycle forms a ring containing approximately 29 members to 37 members, approximately 31 members to 35 members, or approximately 33 members. Where the macrocycle-forming linker spans approximately 3 turns of the helix, the resulting macrocycle forms a ring containing approximately 44 members to 52 members, approximately 46 members to 50 members, or approximately 48 members. Where the macrocycle-forming linker spans approximately 4 turns of the helix, the resulting macrocycle forms a ring containing approximately 59 members to 67 members, approximately 61 members to 65 members, or approximately 63 members. Where the macrocycle-forming linker spans approximately 5 turns of the helix, the resulting macrocycle forms a ring containing approximately 74 members to 82 members, approximately 76 members to 80 members, or approximately 78 members.

In some embodiments, the stabilized peptides can have 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 amino acids. Peptides can contain one or more asymmetric centers and thus occur as racemates and racemic mixtures, single enantiomers, individual diastereomers and diastereomeric mixtures and geometric isomers (e.g. *Z* or *cis* and *E* or *trans*) of any olefins present. For example, peptides disclosed herein can exist in particular geometric or stereoisomeric forms, including, for example, cis- and trans-isomers, R- and S-enantiomers, diastereomers, (D)-isomers, (L)-isomers, the racemic mixtures thereof, and other mixtures thereof. Enantiomers can be free (e.g., substantially free) of their corresponding enantiomer, and/or may also be optically enriched. "Optically enriched," as used herein, means that the compound is made up of a significantly greater proportion of one enantiomer. In certain embodiments substantially free means that a composition contains at least about 90% by weight of a preferred enantiomer. In other embodiments the compound is made up of at least about 95%, 98%, or 99% by weight of a preferred enantiomer. Preferred enantiomers may be isolated from racemic mixtures using techniques known in the art, including, but not limited to, for example, chiral high pressure liquid chromatography (HPLC) and the formation and crystallization of chiral salts or prepared by asymmetric syntheses (see, e.g., Jacques, et al, Enantiomers,

Racemates and Resolutions (Wiley Interscience, New York, 1981); Wilen, S. H., et al., Tetrahedron 33:2725 (1977); Eliel, EX. Stereochemistry of Carbon Compounds (McGraw- Hill, NY, 1962); Wilen, S.H. Tables of Resolving Agents and Optical Resolutions p. 268 (EX. Eliel, Ed., Univ. of Notre Dame Press, Notre Dame, IN 1972). All such isomeric forms of these compounds are expressly included in the present invention.

Peptides can also be represented in multiple tautomeric forms, in such instances, the invention expressly includes all tautomeric forms of the compounds described herein (e.g., isomers in equilibrium (e.g., keto-enol), wherein alkylation at multiple sites can yield regioisomers), regioisomers, and oxidation products of the compounds disclosed herein (the invention expressly includes all such reaction products). All such isomeric forms of such compounds are included as are all crystal forms.

The term "halo" refers to any radical of fluorine, chlorine, bromine or iodine. The term "alkyl" refers to a hydrocarbon chain that may be a straight chain or branched chain, containing the indicated number of carbon atoms. For example, C_1-C_{10} indicates that the group may have from 1 to 10 (inclusive) carbon atoms in it. In the absence of any numerical designation, "alkyl" is a chain (straight or branched) having 1 to 20 (inclusive) carbon atoms in it. The term "alkylene" refers to a divalent alkyl (i.e., -R-).

The term "alkenyl" refers to a hydrocarbon chain that may be a straight chain or branched chain having one or more carbon-carbon double bonds in either Z or E geometric configurations. The alkenyl moiety contains the indicated number of carbon atoms. For example, C_2-C_{10} indicates that the group may have from 2 to 10 (inclusive) carbon atoms in it. The term "lower alkenyl" refers to a C_2-C_8 alkenyl chain. In the absence of any numerical designation, "alkenyl" is a chain (straight or branched) having 2 to 20 (inclusive) carbon atoms in it.

The term "alkynyl" refers to a hydrocarbon chain that may be a straight chain or branched chain having one or more carbon-carbon triple bonds. The alkynyl moiety contains the indicated number of carbon atoms. For example, C_2-C_{10} indicates that the group may have from 2 to 10 (inclusive) carbon atoms in it. The term "lower alkynyl" refers to a C_2-C_8 alkynyl chain. In the absence of any numerical designation, "alkynyl" is a chain (straight or branched) having 2 to 20 (inclusive) carbon atoms in it.

The term "aryl" refers to a 6-carbon monocyclic or 10-carbon bicyclic aromatic ring system wherein 0, 1, 2, 3, 4, or 5 atoms of each ring may be substituted by a substituent.

Examples of aryl groups include phenyl, naphthyl and the like. The term "arylalkyl" or the term "aralkyl" refers to alkyl substituted with an aryl. The term "arylalkoxy" refers to an alkoxy substituted with aryl.

The term "cycloalkyl" as employed herein includes saturated and partially unsaturated cyclic hydrocarbon groups having 3 to 12 carbons, preferably 3 to 8 carbons, and more preferably 3 to 6 carbons, wherein the cycloalkyl group additionally may be optionally substituted. Preferred cycloalkyl groups include, without limitation, cyclopropyl, cyclobutyl, cyclopentyl, cyclopentenyl, cyclohexyl, cyclohexenyl, cyclohexadienyl, cycloheptyl, cycloheptadienyl, cycloheptatrienyl, cyclooctyl, cyclooctenyl, cyclooctadienyl, cyclooctatrienyl, and cyclooctynyl.

The term "heteroaryl" refers to an aromatic 5-8 membered monocyclic, 8-12 membered bicyclic, or 11-14 membered tricyclic ring system having 1-3 heteroatoms if monocyclic, 1-6 heteroatoms if bicyclic, or 1-9 heteroatoms if tricyclic, said heteroatoms selected from O, N, or S (e.g., carbon atoms and 1-3, 1-6, or 1-9 heteroatoms of N, O, or S if monocyclic, bicyclic, or tricyclic, respectively), wherein 0, 1, 2, 3, or 4 atoms of each ring may be substituted by a substituent. Examples of heteroaryl groups include pyrrolyl, pyridyl, furyl or furanyl, imidazolyl, 1,2,3-triazolyl, 1,2,4-triazolyl, benzimidazolyl, pyridazyl, pyrimidyl, thiophenyl, quinolinyl, indolyl, thiazolyl, oxazolyl, isoxazolyl and the like. The term "heteroarylalkyl" or the term "heteroaralkyl" refers to an alkyl substituted with a heteroaryl. The term "heteroarylalkoxy" refers to an alkoxy substituted with heteroaryl.

The term "heterocyclyl" refers to a nonaromatic 5-8 membered monocyclic, 8-12 membered bicyclic, or 11-14 membered tricyclic ring system having 1-3 heteroatoms if monocyclic, 1-6 heteroatoms if bicyclic, or 1-9 heteroatoms if tricyclic, said heteroatoms selected from O, N, or S (e.g., carbon atoms and 1-3, 1-6, or 1-9 heteroatoms of N, O, or S if monocyclic, bicyclic, or tricyclic, respectively), wherein 0, 1, 2 or 3 atoms of each ring may be substituted by a substituent. Examples of heterocyclyl groups include piperazinyl, pyrrolidinyl, dioxanyl, aziridinyl, oxiryl, thiiryl, morpholinyl, tetrahydrofuranyl, and the like.

The term "substituents" refers to a group "substituted" on an alkyl, cycloalkyl, aryl, heterocyclyl, or heteroaryl group at any atom of that group. Suitable substituents include, without limitation, halo, hydroxy, mercapto, oxo, nitro, haloalkyl, alkyl, alkaryl, aryl, aralkyl, alkoxy,

thioalkoxy, aryloxy, amino, alkoxycarbonyl, amido, carboxy, alkanesulfonyl, alkylcarbonyl, azido, and cyano groups.

While hydrocarbon tethers have been described, other tethers are also envisioned. For example, the tether can include one or more of an ether, thioether, ester, amine, or amide moiety. In some cases, a naturally occurring amino acid side chain can be incorporated into the tether. For example, a tether can be coupled with a functional group such as the hydroxyl in serine, the thiol in cysteine, the primary amine in lysine, the acid in aspartate or glutamate, or the amide in asparagine or glutamine. Accordingly, it is possible to create a tether using naturally occurring amino acids rather than using a tether that is made by coupling two non-naturally occurring amino acids. It is also possible to use a single non-naturally occurring amino acid together with a naturally occurring amino acid.

It is further envisioned that the length of the tether can be varied. For instance, a shorter length of tether can be used where it is desirable to provide a relatively high degree of constraint on the secondary alpha-helical structure, whereas, in some instances, it is desirable to provide less constraint on the secondary alpha-helical structure, and thus a longer tether may be desired.

Additionally, while examples of tethers spanning from amino acids *i* to *i+3*, *i* to *i+4*, and *i* to *i+7* have been described in order to provide a tether that is primarily on a single face of the alpha helix, the tethers can be synthesized to span any combinations of numbers of amino acids.

In some instances, alpha disubstituted amino acids are used in the polypeptide to improve the stability of the alpha helical secondary structure. However, alpha disubstituted amino acids are not required, and instances using mono-alpha substituents (e.g., in the tethered amino acids) are also envisioned.

The stapled polypeptides can include a drug (e.g., an antibiotic; see below), a toxin, a derivative of polyethylene glycol; a second polypeptide; a carbohydrate, etc. Where a polymer or other agent is linked to the stapled polypeptide is can be desirable for the composition to be substantially homogeneous.

The addition of polyethelene glycol (PEG) moieties can improve the pharmacokinetic and pharmacodynamic properties of the polypeptide. For example, PEGylation can reduce renal clearance and can result in a more stable plasma concentration. PEG is a water soluble polymer and can be represented as linked to the polypeptide as formula:

XO--$(CH_2CH_2O)_n$--CH_2CH_2--Y where n is 2 to 10,000 and X is H or a terminal modification, e.g., a C_{1-4} alkyl; and Y is an amide, carbamate or urea linkage to an amine group (including but not limited to, the epsilon amine of lysine or the N-terminus) of the polypeptide. Y may also be a maleimide linkage to a thiol group (including but not limited to, the thiol group of cysteine). Other methods for linking PEG to a polypeptide, directly or indirectly, are known to those of ordinary skill in the art. The PEG can be linear or branched. Various forms of PEG including various functionalized derivatives are commercially available.

PEG having degradable linkages in the backbone can be used. For example, PEG can be prepared with ester linkages that are subject to hydrolysis. Conjugates having degradable PEG linkages are described, e.g., in WO 99/34833; WO 99/14259, and U.S. 6,348,558.

In certain embodiments, macromolecular polymer (e.g., PEG) is attached to an agent described herein through an intermediate linker. In certain embodiments, the linker is made up of from 1 to 20 amino acids linked by peptide bonds, wherein the amino acids are selected from the 20 naturally occurring amino acids. Some of these amino acids may be glycosylated, as is well understood by those in the art. In other embodiments, the 1 to 20 amino acids are selected from glycine, alanine, proline, asparagine, glutamine, and lysine. In other embodiments, a linker is made up of a majority of amino acids that are sterically unhindered, such as glycine and alanine. Non-peptide linkers are also possible. For example, alkyl linkers such as $-NH(CH_2)_nC(O)-$, wherein n = 2-20 can be used. These alkyl linkers may further be substituted by any non-sterically hindering group such as lower alkyl (e.g., C_1-C_6) lower acyl, halogen (e.g., Cl, Br), CN, NH_2, phenyl, etc. U.S. Pat. No. 5,446,090 describes a bifunctional PEG linker and its use in forming conjugates having a peptide at each of the PEG linker termini.

Methods of synthesizing the compounds of the described herein are known in the art. Nevertheless, the following exemplary method may be used. It will be appreciated that the various steps may be performed in an alternate sequence or order to give the desired compounds. Synthetic chemistry transformations and protecting group methodologies (protection and deprotection) useful in synthesizing the compounds described herein are known in the art and include, e.g., those such as described in R. Larock, Comprehensive Organic Transformations, VCH Publishers (1989); T.W. Greene and P.G.M. Wuts, Protective Groups in Organic Synthesis, 3d. Ed., John Wiley and Sons (1999); L. Fieser and M. Fieser, Fieser and Fieser's Reagents for

Organic Synthesis, John Wiley and Sons (1994); and L. Paquette, ed., Encyclopedia of Reagents for Organic Synthesis, John Wiley and Sons (1995), and subsequent editions thereof.

The peptides of this invention can be made by chemical synthesis methods, which are well known to the ordinarily skilled artisan. See, e.g., Fields et al., Chapter 3 in Synthetic Peptides: A User's Guide, ed. Grant, W. H. Freeman & Co., New York, N.Y., 1992, p. 77. Hence, peptides can be synthesized using the automated Merrifield techniques of solid phase synthesis with the α-NH_2 protected by either t-Boc or Fmoc chemistry using side chain protected amino acids on, e.g., an Applied Biosystems Peptide Synthesizer™ Model 430A or 431.

One manner of making of the peptides described herein is using solid phase peptide synthesis (SPPS). The C-terminal amino acid is attached to a cross-linked polystyrene resin via an acid labile bond with a linker molecule. This resin is insoluble in the solvents used for synthesis, making it relatively simple and fast to wash away excess reagents and by-products. The N-terminus is protected with the Fmoc group, which is stable in acid, but removable by base. Any side chain functional groups are protected with base stable, acid labile groups.

Longer peptides could be made by conjoining individual synthetic peptides using native chemical ligation. Alternatively, the longer synthetic peptides can be synthesized by well-known recombinant DNA techniques. Such techniques are provided in well-known standard manuals with detailed protocols. To construct a gene encoding a peptide of this invention, the amino acid sequence is reverse translated to obtain a nucleic acid sequence encoding the amino acid sequence, preferably with codons that are optimum for the organism in which the gene is to be expressed. Next, a synthetic gene is made, typically by synthesizing oligonucleotides which encode the peptide and any regulatory elements, if necessary. The synthetic gene is inserted in a suitable cloning vector and transfected into a host cell. The peptide is then expressed under suitable conditions appropriate for the selected expression system and host. The peptide is purified and characterized by standard methods.

The peptides can be made in a high-throughput, combinatorial fashion, e.g., using a high-throughput multiple channel combinatorial synthesizer available from Advanced Chemtech.

Peptide bonds can be replaced, e.g., to increase physiological stability of the peptide, by: a retro-inverso bonds (C(O)-NH); a reduced amide bond (NH-CH_2); a thiomethylene bond (S-CH_2 or CH_2-S); an oxomethylene bond (O-CH_2 or CH_2-O); an ethylene bond (CH_2-CH_2); a thioamide bond (C(S)-NH); a trans-olefin bond (CH=CH); a fluoro substituted trans-olefin bond

(CF=CH); a ketomethylene bond (C(O)-CHR) or CHR-C(O) wherein R is H or CH_3; and a fluoro-ketomethylene bond (C(O)-CFR or CFR-C(O) wherein R is H or F or CH_3.

The polypeptides can be further modified by: acetylation, amidation, biotinylation, cinnamoylation, farnesylation, fluoresceination, formylation, myristoylation, palmitoylation, phosphorylation (Ser, Tyr or Thr), stearoylation, succinylation and sulfurylation. As indicated above, peptides can be conjugated to, e.g., polyethylene glycol (PEG); alkyl groups (e.g., C_1-C_{20} straight or branched alkyl groups); fatty acid radicals; and combinations thereof.

α, α-Disubstituted non-natural amino acids containing olefinic side chains of varying length can be synthesized by known methods (Williams et al. J. Am. Chem. Soc., 113:9276, 1991; Schafmeister et al., J. Am. Chem Soc., 122:5891, 2000; and Bird et al., Methods Enzymol., 446:369, 2008; Bird et al, Current Protocols in Chemical Biology, 2011). For peptides where an *i* linked to *i*+7 staple is used (two turns of the helix stabilized), either: a) one S_5 amino acid and one R_8 is used or b) one S_8 amino acid and one R_5 amino acid is used. R_8 is synthesized using the same route, except that the starting chiral auxillary confers the R-alkyl-stereoisomer. Also, 8-iodooctene is used in place of 5-iodopentene. Inhibitors are synthesized on a solid support using solid-phase peptide synthesis (SPPS) on MBHA resin (see, e.g., WO 2010/148335).

Fmoc-protected α-amino acids (other than the olefinic amino acids Fmoc-S_5-OH, Fmoc-R_8-OH, Fmoc-R_8-OH, Fmoc-S_8-OH and Fmoc-R_5-OH), 2-(6-chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU), and Rink Amide MBHA are commercially available from, e.g., Novabiochem (San Diego, CA). Dimethylformamide (DMF), N-methyl-2-pyrrolidinone (NMP), N,N-diisopropylethylamine (DIEA), trifluoroacetic acid (TFA), 1,2-dichloroethane (DCE), fluorescein isothiocyanate (FITC), and piperidine are commercially available from, e.g., Sigma-Aldrich. Olefinic amino acid synthesis is reported in the art (see, e.g., Williams et al., Org. Synth., 80:31, 2003).

The compounds of this invention may be modified by appending appropriate functionalities to enhance selective biological properties (including, e.g., hydrophobicity and/or the position/occurrence of hydrophobic patches). Such modifications are known in the art and include those which increase biological penetration into a given biological compartment (e.g., blood, lymphatic system, central nervous system), increase oral availability, increase solubility to allow administration by injection, alter metabolism and alter rate of excretion.

An antimicrobial peptide selective for microbial versus mammalian membranes (i.e., a peptide able to kill or inhibit the growth of a microbe while also having a relatively low ability to lyse or inhibit the growth of a mammalian cell) may, e.g., possess a MIC for one or more microbes more than about 1.5-fold lower, more than about 2-fold lower, more than about 2.5-fold lower, more than about 3-fold lower, more than about 4-fold lower, more than about 5-fold lower, more than about 6-fold lower, more than about 7-fold lower, more than about 8-fold lower, more than about 9-fold lower, more than about 10-fold lower, more than about 15-fold lower, or more than about 20-fold lower than the MIC of the corresponding parent (i.e., unmodified) non-internally cross-linked peptide for the same one or more microbes. An antimicrobial peptide selective for microbial versus mammalian membranes can have a MIC of, for example, about 1 μg/ml, about 2 μg/ml, about 3 μg/ml, about 4 μg/ml, about 5 μg/ml, about 6 μg/ml, about 7 μg/ml, about 8 μg/ml, about 9 μg/ml, about 10 μg/ml, about 12 μg/ml, about 14 μg/ml, about 16 μg/ml, about 18 μg/ml, about 20 μg/ml, about 22 μg/ml, about 24 μg/ml, about 26 μg/ml, about 28 μg/ml, or about 30 μg/ml.

In addition, an antimicrobial peptide selective for microbial versus mammalian membranes may lyse, e.g., less than about 50%, less than about 40%, less than about 30%, less than about 25%, less than about 20%, less than about 15%, less than about 10%, less than about 5%, less than about 2.5%, less than about 2%, or less than about 1% of red blood cells (RBCs) in a RBC hemolytic activity assay when administered at its MIC for one or more microbes. An antimicrobial peptide selective for microbial versus mammalian membranes may lyse, e.g., less than about 50%, less than about 40%, less than about 30%, less than about 25%, less than about 20%, less than about 15%, less than about 10%, less than about 5%, less than about 2.5%, less than about 2%, or less than about 1% of red blood cells (RBCs) in a RBC hemolytic activity assay when administered at a concentration, e.g., greater than or approximately equal to 1.5-fold, 2-fold, 2.5-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, or 10-fold its MIC for one or more microbes. The RBC hemolytic activity of an antimicrobial peptide selective for microbial versus mammalian membranes may be less than, approximately equal to, less than 1.5-fold greater, less than 2-fold greater, less than 2.5-fold greater, less than 3-fold greater, less than 4-fold greater, less than 5-fold greater, less than 6-fold greater, less than 7-fold greater, less than 8-fold greater, less than 9-fold greater, or less than 10-fold greater than the RBC hemolytic activity of the corresponding parent (i.e., unmodified) non-internally cross-linked peptide.

Hydrophobic patches within a peptide or protein may be identified using techniques generally known in the art, including, e.g., computational prediction/ simulation (e.g., using ExPASy ProtScale, Scooby-domain prediction, PSIPRED, hydrophobic cluster analysis, Kyte Doolittle plotting, and/or SPLIT) and/or experimental determination (e.g., using techniques involving NMR spectroscopy, electron microscopy, homology modeling, small-angle X-ray and/or neutron scattering (SAXS/SANS), and/or X-ray crystallography) of the structure of the peptide or protein.

Pharmaceutically acceptable salts of the compounds of this invention include those derived from pharmaceutically acceptable inorganic and organic acids and bases. Examples of suitable acid salts include acetate, adipate, benzoate, benzenesulfonate, butyrate, citrate, digluconate, dodecylsulfate, formate, fumarate, glycolate, hemisulfate, heptanoate, hexanoate, hydrochloride, hydrobromide, hydroiodide, lactate, maleate, malonate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, nitrate, palmoate, phosphate, picrate, pivalate, propionate, salicylate, succinate, sulfate, tartrate, tosylate, trifluoromethylsulfonate, and undecanoate. Salts derived from appropriate bases include alkali metal (e.g., sodium), alkaline earth metal (e.g., magnesium), ammonium and N-(alkyl)4+ salts. This invention also envisions the quaternization of any basic nitrogen-containing groups of the compounds disclosed herein. Water or oil-soluble or dispersible products may be obtained by such quaternization.

Methods suitable for obtaining (e.g., synthesizing), stapling, and purifying the peptides disclosed herein are also known in the art (see, e.g., Bird et. al., Methods in Enzymol., 446:369-386 (2008); Bird et al, Current Protocols in Chemical Biology, 2011; Walensky et al., Science, 305:1466-1470 (2004); Schafmeister et al., J. Am. Chem. Soc., 122:5891-5892 (2000); U.S. Patent Application Serial No. 12/525,123, filed March 18, 2010; and U.S. Patent No. 7,723,468, issued May 25, 2010, each of which are hereby incorporated by reference in their entirety) and are described herein.

In some embodiments, the peptides are substantially free of non-stapled peptide contaminants or are isolated. Methods for purifying peptides include, for example, synthesizing the peptide on a solid-phase support. Following cyclization, the solid-phase support may be isolated and suspended in a solution of a solvent such as DMSO, DMSO/dichloromethane mixture, or DMSO/NMP mixture. The DMSO/dichloromethane or DMSO/NMP mixture may comprise about 30%, 40%, 50%, or 60% DMSO. In a specific embodiment, a 50%/50%

DMSO/NMP solution is used. The solution may be incubated for a period of 1, 6, 12 or 24 hours, following which the resin may be washed, for example with dichloromethane or NMP. In one embodiment, the resin is washed with NMP. Shaking and bubbling an inert gas into the solution may be performed.

Properties of the cross-linked polypeptides of the invention can be assayed, for example, using the methods described below.

Assays to Determine α-Helicity: Compounds are dissolved in an aqueous solution (e.g. 5 mM potassium phosphate solution at pH 7, or distilled H_2O, to concentrations of 25-50 μM). Circular dichroism (CD) spectra are obtained on a spectropolarimeter (e.g., Jasco J-710, Aviv) using standard measurement parameters (e.g. temperature, 20°C; wavelength, 190-260 nm; step resolution, 0.5 nm; speed, 20 nm/sec; accumulations, 10; response, 1 sec; bandwidth, 1 nm; path length, 0.1 cm). The α-helical content of each peptide is calculated by dividing the mean residue ellipticity by the reported value for a model helical decapeptide (Yang et al., Methods Enzymol. 130:208 (1986)).

Assays to Determine Melting Temperature (Tm): Cross-linked or the unmodified template peptides are dissolved in distilled H_2O or other buffer or solvent (e.g. at a final concentration of 50 μM) and Tm is determined by measuring the change in ellipticity over a temperature range (e.g. 4 to 95 °C) on a spectropolarimeter (e.g., Jasco J-710, Aviv) using standard parameters (e.g. wavelength 222 nm; step resolution, 0.5 nm; speed, 20 nm/sec; accumulations, 10; response, 1 sec; bandwidth, 1 nm; temperature increase rate: 1°C/min; path length, 0.1 cm).

In Vitro Protease Resistance Assays: The amide bond of the peptide backbone is susceptible to hydrolysis by proteases, thereby rendering peptidic compounds vulnerable to rapid degradation in vivo. Peptide helix formation, however, typically buries and/or twists and/or shields the amide backbone and therefore may prevent or substantially retard proteolytic cleavage. The peptidomimetic macrocycles of the present invention may be subjected to in vitro enzymatic proteolysis (e.g. trypsin, chymotrypsin, pepsin) to assess for any change in degradation rate compared to a corresponding uncrosslinked or alternatively stapled polypeptide. For example, the peptidomimetic macrocycle and a corresponding uncrosslinked polypeptide are incubated with trypsin agarose and the reactions quenched at various time points by centrifugation and subsequent HPLC injection to quantitate the residual substrate by ultraviolet

absorption at 280 nm. Briefly, the peptidomimetic macrocycle and peptidomimetic precursor (5 mcg) are incubated with trypsin agarose (Pierce) (S/E ~125) for 0, 10, 20, 90, and 180 minutes. Reactions are quenched by tabletop centrifugation at high speed; remaining substrate in the isolated supernatant is quantified by HPLC-based peak detection at 280 nm. The proteolytic reaction displays first order kinetics and the rate constant, *k*, is determined from a plot of ln[S] versus time.

Peptidomimetic macrocycles and/or a corresponding uncrosslinked polypeptide can be each incubated with fresh mouse, rat and/or human serum (e.g. 1-2 mL) at 37°C for, e.g., 0, 1, 2, 4, 8, and 24 hours. Samples of differing macrocycle concentration may be prepared by serial dilution with serum. To determine the level of intact compound, the following procedure may be used: The samples are extracted, for example, by transferring 100 µL of sera to 2 ml centrifuge tubes followed by the addition of 10 µL of 50% formic acid and 500 µL acetonitrile and centrifugation at 14,000 RPM for 10 min at 4+/-2°C. The supernatants are then transferred to fresh 2 ml tubes and evaporated on Turbovap under N_2<10 psi, 37°C. The samples are reconstituted in 100 µL of 50:50 acetonitrile:water and submitted to LC-MS/MS analysis. Equivalent or similar procedures for testing *ex vivo* stability are known and may be used to determine stability of macrocycles in serum.

In Vivo *Protease Resistance Assays:* A key benefit of peptide stapling is the translation of in vitro protease resistance into markedly improved pharmacokinetics *in vivo*. Structurally-stabilized I-TAMPs with potent and selective antimicrobial activity are screened for protease stability *in vivo*, e.g., using previously published methods (see, e.g., Bird et al, PNAS, 2010).

Pharmaceutical Compositions

One or more of the stabilized peptides disclosed herein (e.g., those derived from I-TAMPs having one or more of SEQ ID NOs: 1–13, including SEQ ID NOs: 14–36) can be formulated for use as or in pharmaceutical compositions. Such compositions can be formulated or adapted for administration to a subject via any route, e.g., any route approved by the Food and Drug Administration (FDA). Exemplary methods are described in the FDA Data Standards Manual (DSM).

The pharmaceutical compositions of this invention may be administered, e.g., orally, parenterally, by inhalation spray or nebulizer, topically, rectally, nasally, buccally, vaginally, via

an implanted reservoir, by injection (e.g., intravenously, intra-arterially, subdermally, intraperitoneally, intramuscularly, and/or subcutaneously), in an ophthalmic preparation, or via transmucosal administration. Suitable dosages may range from about 0.001 to about 100 mg/kg of body weight, or according to the requirements of the particular drug. The pharmaceutical compositions of this invention may contain any conventional non-toxic pharmaceutically-acceptable carriers, adjuvants or vehicles. In some cases, the pH of the formulation may be adjusted with pharmaceutically acceptable acids, bases or buffers to enhance the stability of the formulated compound or its delivery form. The term parenteral as used herein includes subcutaneous, intracutaneous, intravenous, intramuscular, intraarticular, intra-arterial, intrasynovial, intrasternal, intrathecal, intralesional and intracranial injection or infusion techniques. Alternatively or in addition, the present invention may be administered according to any of the methods as described in the FDA DSM.

As used herein, the compounds of this invention, including the compounds of formulae described herein, are defined to include pharmaceutically acceptable derivatives or prodrugs thereof. A "pharmaceutically acceptable derivative or prodrug" means any pharmaceutically acceptable salt, ester, salt of an ester, or other derivative of a compound or agent disclosed herein which, upon administration to a recipient, is capable of providing (directly or indirectly) a compound of this invention. Particularly favored derivatives and prodrugs are those that increase the bioavailability of the compounds of this invention when such compounds are administered to a mammal (e.g., by allowing an orally administered compound to be more readily absorbed into the blood) or which enhance delivery of the parent compound to a biological compartment (e.g., the brain or lymphatic system) relative to the parent species. Preferred prodrugs include derivatives where a group which enhances aqueous solubility or active transport through the gut membrane is appended to the structure of formulae described herein.

In some instances, pharmaceutical compositions can include an effective amount of one or more stabilized peptides. The terms "effective amount" and "effective to treat," as used herein, refer to an amount or a concentration of one or more compounds or a pharmaceutical composition described herein utilized for a period of time (including acute or chronic administration and periodic or continuous administration) that is effective within the context of its administration for causing an intended effect or physiological outcome (e.g., treatment of infection).

The methods herein contemplate administration of an effective amount of compound or compound composition to achieve the desired or stated effect. Typically, the pharmaceutical compositions of this invention will be administered from about 1 to about 6 times per day or alternatively, as a continuous infusion. Such administration can be used as a chronic or acute therapy. The amount of active ingredient that may be combined with the carrier materials to produce a single dosage form will vary depending upon the host treated and the particular mode of administration. A typical preparation will contain from about 5% to about 95% active compound (w/w). Alternatively, such preparations contain from about 20% to about 80% active compound.

Dosing can be determined using various techniques. The selected dosage level can depend upon a variety of factors including the activity of the particular compound employed, the route of administration, the time of administration, the rate of excretion or metabolism of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compound employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts. The dosage values can also vary with the severity of the condition to be alleviated. For any particular subject, specific dosage regimens can be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions.

In some embodiments, a suitable daily dose of a compound of the disclosure can be that amount of the compound which is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above. The precise time of administration and amount of any particular compound that will yield the most effective treatment in a given patient will depend upon the activity, pharmacokinetics, and bioavailability of a particular compound, physiological condition of the patient (including age, sex, disease type and stage, general physical condition, responsiveness to a given dosage and type of medication), route of administration, and the like.

A physician or veterinarian can prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the compounds of the disclosure employed in the pharmaceutical composition at levels lower than

that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved.

Pharmaceutical compositions described herein can be in unit dosage forms suitable for single administration of precise dosages. In unit dosage form, the formulation is divided into unit doses containing appropriate quantities of one or more compounds. The unit dosage can be in the form of a package containing discrete quantities of the formulation. Non-limiting examples are liquids in vials or ampoules. Aqueous suspension compositions can be packaged in single-dose non-reclosable containers. Multiple-dose reclosable containers can be used, for example, in combination with a preservative. Formulations for parenteral injection can be presented in unit dosage form, for example, in ampoules, or in multi dose containers with a preservative.

A compound described herein can be present in a composition in a range of from about 1 mg to about 2000 mg; from about 100 mg to about 2000 mg; from about 10 mg to about 2000 mg; from about 5 mg to about 1000 mg, from about 10 mg to about 500 mg, from about 50 mg to about 250 mg, from about 100 mg to about 200 mg, from about 1 mg to about 50 mg, from about 50 mg to about 100 mg, from about 100 mg to about 150 mg, from about 150 mg to about 200 mg, from about 200 mg to about 250 mg, from about 250 mg to about 300 mg, from about 300 mg to about 350 mg, from about 350 mg to about 400 mg, from about 400 mg to about 450 mg, from about 450 mg to about 500 mg, from about 500 mg to about 550 mg, from about 550 mg to about 600 mg, from about 600 mg to about 650 mg, from about 650 mg to about 700 mg, from about 700 mg to about 750 mg, from about 750 mg to about 800 mg, from about 800 mg to about 850 mg, from about 850 mg to about 900 mg, from about 900 mg to about 950 mg, or from about 950 mg to about 1000 mg.

A compound described herein can be present in a composition in an amount of about 1 mg, about 2 mg, about 3 mg, about 4 mg, about 5 mg, about 10 mg, about 15 mg, about 20 mg, about 25 mg, about 30 mg, about 35 mg, about 40 mg, about 45 mg, about 50 mg, about 55 mg, about 60 mg, about 65 mg, about 70 mg, about 75 mg, about 80 mg, about 85 mg, about 90 mg, about 95 mg, about 100 mg, about 125 mg, about 150 mg, about 175 mg, about 200 mg, about 250 mg, about 300 mg, about 350 mg, about 400 mg, about 450 mg, about 500 mg, about 550 mg, about 600 mg, about 650 mg, about 700 mg, about 750 mg, about 800 mg, about 850 mg, about 900 mg, about 950 mg, about 1000 mg, about 1050 mg, about 1100 mg, about 1150 mg, about 1200 mg, about 1250 mg, about 1300 mg, about 1350 mg, about 1400 mg, about 1450 mg,

about 1500 mg, about 1550 mg, about 1600 mg, about 1650 mg, about 1700 mg, about 1750 mg, about 1800 mg, about 1850 mg, about 1900 mg, about 1950 mg, or about 2000 mg.

In some embodiments, a dose can be expressed in terms of an amount of the drug divided by the mass of the subject, for example, milligrams of drug per kilograms of subject body mass. In some embodiments, a compound is administered in an amount ranging from about 5 mg/kg to about 50 mg/kg, 250 mg/kg to about 2000 mg/kg, about 10 mg/kg to about 800 mg/kg, about 50 mg/kg to about 400 mg/kg, about 100 mg/kg to about 300 mg/kg, or about 150 mg/kg to about 200 mg/kg.

Dosage can be based on the amount of the compound per kg body weight of the patient. Alternatively, the dosage of the subject disclosure can be determined by reference to the plasma concentrations of the compound. For example, the maximum plasma concentration (C_{max}) and the area under the plasma concentration-time curve from time 0 to infinity (AUC) can be used.

In some embodiments, the subject is a human subject and the amount of the compound administered is 0.01-100 mg per kilogram body weight of the human subject. For example, in various examples, the amount of the compound administered is about .01-50 mg/kg, about 0.01-20 mg/kg, about 0.01-10 mg/kg, about 0.1-100 mg/kg, about 0.1-50 mg/kg, about 0.1-20 mg/kg, about 0.1-10 mg/kg, about 0.5-100 mg/kg, about 0.5-50 mg/kg, about 0.5-20 mg/kg, about 0.5-10 mg/kg, about 1-100 mg/kg, about 1-50 mg/kg, about 1-20 mg/kg, about 1-10 mg/kg body weight of the human subject. In one embodiment, about 0.5 mg-10 mg of the compound per kilogram body weight of the human subject is administered. In some examples the amount of the compound administered is about 0.16 mg, about 0.32 mg, about 0.64 mg, about 1.28 mg, about 3.56 mg, about 7.12 mg, about 14.24 mg, or about 20 mg per kilogram body weight of the human subject. In some examples the amount of the compound administered is about 0.16 mg, about 0.32 mg, about 0.64 mg, about 1.28 mg, about 3.56 mg, about 7.12 mg, or about 14.24 mg per kilogram body weight of the human subject. In some examples the amount of the compound administered is about 0.16 mg per kilogram body weight of the human subject. In some examples the amount of the compound administered is about 0.32 mg per kilogram body weight of the human subject. In some examples the amount of the compound administered is about 0.64 mg per kilogram body weight of the human subject. In some examples the amount of the compound administered is about 1.28 mg per kilogram body weight of the human subject. In some examples the amount of the compound administered is about 3.56 mg per kilogram body

weight of the human subject. In some examples the amount of the compound administered is about 7.12 mg per kilogram body weight of the human subject. In some examples the amount of the compound administered is about 14.24 mg per kilogram body weight of the human subject.

In some embodiments about 0.5- about 20 mg or about 0.5- about 10 mg of the compound per kilogram body weight of the human subject is administered two times a week. For example about 0.5- about 1 mg, about 0.5- about 5 mg, about 0.5- about 10 mg, about 0.5- about 15 mg, about 1- about 5 mg, about 1- about 10 mg, about 1- about 15 mg, about 1- about 20 mg, about 5- about 10 mg, about 1- about 15 mg, about 5- about 20 mg, about 10- about 15 mg, about 10- about 20 mg, or about 15- about 20 mg of the compound per kilogram body weight of the human subject is administered about twice a week. In some examples, about 1 mg, about 1.5 mg, about 2 mg, about 2.5 mg, about 3 mg, about 3.5 mg, about 4 mg, about 4.5 mg, about 5 mg, about 5.5 mg, about 6 mg, about 6.5 mg, about 7 mg, about 7.5 mg, about 8 mg, about 8.5 mg, about 9 mg, about 9.5 mg, about 10 mg, about 10.5 mg, about 11 mg, about 11.5 mg, about 12 mg, about 12.5 mg, about 13 mg, about 13.5 mg, about 14 mg, about 14.5 mg, about 15 mg, about 15.5 mg, about 16 mg, about 16.5 mg, about 17 mg, about 17.5 mg, about 18 mg, about 18.5 mg, about 19 mg, about 19.5 mg, or about 20 mg of the compound per kilogram body weight of the human subject is administered two times a week. In some examples, the amount of the compound administered is about 1.25 mg, about 2.5 mg, about 5 mg, about 10 mg, or about 20 mg per kilogram body weight of the human subject and the compound is administered two times a week. In some examples, the amount of the compound administered is about 1.25 mg, about 2.5 mg, about 5 mg or about 10 mg per kilogram body weight of the human subject. The compound can be administered once a week, two times a week, three, four, five, six, or seven times a week. The compound can be administered once every 3 weeks.

In some embodiments, the compound is administered gradually over a period of time. A desired amount of compound can, for example can be administered gradually over a period of from about 0.1 h -24 h. In some cases, a desired amount of compound is administered gradually over a period of 0.1 h, 0.5 h, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 3.5 h, 4 h, 4.5 h, 5 h, 6 h, 7 h, 8 h, 9 h, 10 h, 11 h, 12 h, 13 h, 14 h, 15 h, 16 h, 17 h, 18 h, 19 h, 20 h, 21 h, 22 h, 23 h, or 24 h. In some examples, a desired amount of compound is administered gradually over a period of 0.25 -12 h, for example over a period of 0.25-1 h, 0.25-2 h, 0.25-3 h, 0.25-4 h, 0.25-6 h, 0.25-8 h, or 0.25-10 h. In some examples, a desired amount of compound is administered gradually over a period of

0.25-2 h. In some examples, a desired amount of compound is administered gradually over a period of 0.25-1 h. In some examples, a desired amount of compound is administered gradually over a period of 0.25 h, 0.3 h, 0.4 h, 0.5 h, 0.6 h, 0.7 h, 0.8 h, 0.9 h, 1.0 h, 1.1 h, 1.2 h, 1.3 h, 1.4 h, 1.5 h, 1.6 h, 1.7 h, 1.8 h, 1.9 h, or 2.0 h. In some examples, a desired amount of compound is administered gradually over a period of 1 h. In some examples, a desired amount of compound is administered gradually over a period of 2 h.

Administration of the compounds can continue as long as necessary. In some embodiments, one or more compound of the disclosure is administered for more than 1 day, more than 1 week, more than 1 month, more than 2 months, more than 3 months, more than 4 months, more than 5 months, more than 6 months, more than 7 months, more than 8 months, more than 9 months, more than 10 months, more than 11 months, more than 12 months, more than 13 months, more than 14 months, more than 15 months, more than 16 months, more than 17 months, more than 18 months, more than 19 months, more than 20 months, more than 21 months, more than 22 months, more than 23 months, or more than 24 months. In some embodiments, one or more compound of the disclosure is administered for less than 1 week, less than 1 month, less than 2 months, less than 3 months, less than 4 months, less than 5 months, less than 6 months, less than 7 months, less than 8 months, less than 9 months, less than 10 months, less than 11 months, less than 12 months, less than 13 months, less than 14 months, less than 15 months, less than 16 months, less than 17 months, less than 18 months, less than 19 months, less than 20 months, less than 21 months, less than 22 months, less than 23 months, or less than 24 months.

In some embodiments, the compound is administered on day 1, 8, 15, and 28 of a 28 day cycle. In some embodiments, the compound is administered on day 1, 8, 15, and 28 of a 28 day cycle and administration is continued for two cycles. In some embodiments, the compound is administered on day 1, 8, 15, and 28 of a 28 day cycle and administration is continued for three cycles. In some embodiments, the compound is administered on day 1, 8, 15, and 28 of a 28 day cycle and administration is continued for 4, 5, 6, 7, 8, 9, 10, or more cycles.

In some embodiments, the compound is administered on day 1, 8, 11, and 21 of a 21-day cycle. In some embodiments, the compound is administered on day 1, 8, 11, and 21 of a 21-day cycle and administration is continued for two cycles. In some embodiments, the compound is administered on day 1, 8, 11, and 21 of a 21-day cycle and administration is continued for three

cycles. In some embodiments, the compound is administered on day 1, 8, 11, and 21 of a 21-day cycle and administration is continued for 4, 5, 6, 7, 8, 9, 10, or more cycles.

In some embodiments, one or more compound of the disclosure is administered chronically on an ongoing basis. In some embodiments administration of one or more compound of the disclosure is continued until documentation of disease progression, unacceptable toxicity, or patient or physician decision to discontinue administration. In some embodiments, an effective dose of a stapled AMP can include, but is not limited to, e.g., about, 0.00001, 0.0001, 0.001, 0.01, 0.1, 1 or 10-10000; 0.00001, 0.0001, 0.001, 0.01, 0.1, 1 or 10-5000; 0.00001, 0.0001, 0.001, 0.01, 0.1, 1 or 10-2500; 0.00001, 0.0001, 0.001, 0.01, 0.1, 1 or 10-1000; 0.00001, 0.0001, 0.001, 0.01, 0.1, 1 or 10-900; 0.00001, 0.0001, 0.001, 0.01, 0.1, 1 or 10-800; 0.00001, 0.0001, 0.001, 0.01, 0.1, 1 or 10-700; 0.00001, 0.0001, 0.001, 0.01, 0.1, 1 or 10-600; 0.00001, 0.0001, 0.001, 0.01, 0.1, 1 or 10-500; 0.00001, 0.0001, 0.001, 0.01, 0.1, 1 or 10-400; 0.00001, 0.0001, 0.001, 0.01, 0.1, 1 or 10-300; 0.00001, 0.0001, 0.001, 0.01, 0.1, 1 or 10-200; 0.00001, 0.0001, 0.001, 0.01, 0.1, 1 or 10-100; 0.00001, 0.0001, 0.001, 0.01, 0.1, 1 or 10-90; 0.00001, 0.0001, 0.001, 0.01, 0.1, 1 or 10-80; 0.00001, 0.0001, 0.001, 0.01, 0.1, 1 or 10-70; 0.00001, 0.0001, 0.001, 0.01, 0.1, 1 or 10-60; 0.00001, 0.0001, 0.001, 0.01, 0.1, 1 or 10-50; 0.00001, 0.0001, 0.001, 0.01, 0.1, 1 or 10-40; 0.00001, 0.0001, 0.001, 0.01, 0.1, 1 or 10-30; 0.00001, 0.0001, 0.001, 0.01, 0.1, 1 or 10-20; 0.00001, 0.0001, 0.001, 0.01, 0.1, 1 or 10-30; 0.00001, 0.0001, 0.001, 0.01, 0.1, 1-15, 0.00001, 0.0001, 0.001, 0.01, 0.1, 1 or 10-30; 0.00001, 0.0001, 0.001, 0.01, 0.1, 1 -10, 0.00001, 0.0001, 0.001, 0.01, 0.1, 1 or 10-30; or 0.00001, 0.0001, 0.001, 0.01, 0.1, 1-5 mg/kg/day.

Pharmaceutical compositions of this invention can include one or more peptides and any pharmaceutically acceptable carrier and/or vehicle. In some instances, pharmaceuticals can further include one or more additional therapeutic agents in amounts effective for achieving a modulation of disease or disease symptoms. Appropriate therapeutic agents can also be administered chemically (covalently or non-covalently) bound to the stabilized I-TAMPs of this document Such additional therapeutic agents may include antimicrobial agents (e.g., antibiotics) known in the art. When co-administered, stapled AMPs of the invention operate in conjunction with antimicrobial agents to produce mechanistically additive or synergistic antimicrobial effects.

Examples of antibiotics suitable for co-administration with (as separate entities or chemically bound (covalently or non-covalently) to) the stapled peptides disclosed herein (as separate agents or chemically bound to the stapled peptides) include, but are not limited to, **quinolones** (e.g., levofloxacin, norfloxacin, ofloxacin, ciprofloxacin, perfloxacin, lomefloxacin, fleroxacin, sparfloxacin, grepafloxacin, trovafloxacin, clinafloxacin, gemifloxacin, enoxacin, sitafloxacin, nadifloxacin, tosulfloxacin, cinnoxacin, rosoxacin, miloxacin, moxifloxacin, gatifloxacin, cinnoxacin, enoxacin, fleroxacin, lomafloxacin, lomefloxacin, miloxacin, nalidixic acid, nadifloxacin, oxolinic acid, pefloxacin, pirimidic acid, pipemidic acid, rosoxacin, rufloxacin, temafloxacin, tosufloxacin, trovafloxacin, besifloxacin); **β-lactams** including cephalosporins (e.g., cefacetrile, cefixime, cefadroxil, cefaloglycin, cefalonium, cefaloridine, cefalotin, cefapirin, cefcapene, cefdaloxime, cefdinir, cefditoren, cefatrizine, cefetamet, cefazaflur, cefazedone, cefazolin, cefaradine, cefroxadine, ceftezole, cefteram, ceftibuten, ceftiofur, ceftiolene, ceftizoxime, cefaclor, cefprozil, cefuroxime, cefuzonam, cefmenoxime, cefodizime, cefotaxime, cefovecin, cefpimizole, cefpirome, cefquinome, ceftobiprole, cefpodoxime, ceftazidime, ceftaroline, cefclidine, cefepime, cefluprenam, cefoselis, cefozopran, cephalexin, cephaloridine, cefamandole, cefsulodin, cefonicid, cefoperazine, cefoperazone, cefoprozil, ceftriaxone), penicillins and penicillin derivatives (e.g., penicillin G, penicillin V, procaine penicillin, benzathine penicillin, benzathine benzylpenicillin, ampicillin, epicillin, amoxicillin, benzylpenicillin, clometocillin, phenoxymethylpenicillin, oxacillin, methicillin, dicloxacillin, flucloxacillin, temocillin, azlocillin, carbenicillin, ricarcillin, mezlocillin, piperacillin, apalcillin, hetacillin, bacampicillin, sulbenicillin,, mecicilam, pevmecillinam, ciclacillin, talapicillin, aspoxicillin, azidocillin, cloxacillin, nafcillin, pivampicillin, penamecillin, mecillinam, propicillin, pheneticillin, ticarcillin temocillin), carbapenems (e.g., thienamycin, tomopenem, lenapenem, tebipenem, razupenem, imipenem, meropenem, ertapenem, doripenem, panipenem (betamipron), biapenem), carbacephems (e.g., loracarbef), penems (e.g., faropenem), cephamycins (e.g., cefbuperazone, cefmetazole, cefminox, cefotetan, cefoxitin), monobactams (e.g., aztreonam, nocardicin A, tabtoxin, tigemonam), and oxacephems (e.g., flomoxef, latamoxef); **lipopeptide** antibiotics (e.g., amphomycin, aspartocin, brevistin, cerexin A, cerexin B, glumamycin, laspartomycin, tsushimycin, zaomycin, daptomycin); **polymyxin** antibiotics (e.g., polymyxin B, colistin (polymyxin E), polymyxin M); **aminoglycosides** (e.g., gentamicin, amikacin, tobramycin, debekacin, kanamycin, neomycin, netilmicin, paromomycin, sisomycin,

spectinomycin, streptomycin); **glycopeptides** (e.g., vancomycin, teicoplanin, telavancin, ramoplanin, daptomycin, decaplanin, bleomycin); **macrolides** (e.g., azithromycin, clarithromycin, erythromycin, fidaxomicin, telithromycin, carbomycin A, josamycin, kitasamycin, midecamycin/midecamycinacetate, oleandomycin, solithromycin, spiramycin, troleandomycin, tylosin/tylocine, roxithromycin, dirithromycin, troleandomycin, spectinomycin, methymycin, neomethymycin, erythronolid, megalomycin, picromycin, narbomycin, oleandomycin, triacetyl-oleandomycin, laukamycin, kujimycin A, albocyclin, cineromycin B); **ansamycins** (e.g., streptovaricin, geldanamycin, herbimycin, rifamycin, rifampin, rifabutin, rifapentine, rifamixin); **linezolid**; **pristinamycin**; and **sulfonamides** (e.g., sulfanilamide, sulfacetarnide, sulfapyridine, sulfathiazole, sulfadiazine, sulfamerazine, sulfadimidine, sulfasomidine, sulfasalazine, mafenide, sulfamethoxazole, sulfamethoxypyridazine, sulfadimethoxine, sulfasymazine, sulfadoxine, sulfametopyrazine, sulfaguanidine, succinylsulfathiazole, phthalylsulfathiazole).

When the compositions of this invention comprise a combination of a compound of the formulae described herein and one or more additional therapeutic or prophylactic agents, both the compound and the additional agent should be present at dosage levels of between about 1% to about 100%, or between about 5% to about 95% of the dosage normally administered in a monotherapy regimen. The additional agents may be administered separately, as part of a multiple dose regimen, from the compounds of this invention. Alternatively, those agents may be part of a single dosage form, mixed together with the compounds of this invention in a single composition.

The term "pharmaceutically acceptable carrier or adjuvant" refers to a carrier or adjuvant that may be administered to a patient, together with a compound of this invention, and which does not destroy the pharmacological activity thereof and is nontoxic when administered in doses sufficient to deliver a therapeutic amount of the compound.

Pharmaceutically acceptable carriers, adjuvants and vehicles that may be used in the pharmaceutical compositions of this invention include, but are not limited to, ion exchangers, alumina, aluminum stearate, lecithin, self-emulsifying drug delivery systems (SEDDS) such as d-α-tocopherol polyethyleneglycol 1000 succinate, surfactants used in pharmaceutical dosage forms such as Tweens or other similar polymeric delivery matrices, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium

sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, polyethylene glycol, sodium carboxymethylcellulose, polyacrylates, waxes, polyethylene-polyoxypropylene-block polymers, polyethylene glycol and wool fat. Cyclodextrins such as α-, β-, and γ-cyclodextrin, may also be advantageously used to enhance delivery of compounds of the formulae described herein.

The pharmaceutical compositions of this invention may contain any conventional non-toxic pharmaceutically-acceptable carriers, adjuvants or vehicles. In some cases, the pH of the formulation may be adjusted with pharmaceutically acceptable acids, bases or buffers to enhance the stability of the formulated compound or its delivery form. The term parenteral as used herein includes parenteral, epidural, subcutaneous, intra-cutaneous, intra-venous, intra-muscular, intra-articular, intra-arterial, intra-synovial, intra-sternal, intra-thecal, intra-lesional and intra-cranial injection or infusion techniques.

An effective amount of a compound of the disclosure can be administered in either single or multiple doses by any of the accepted modes of administration. Regardless of the route of administration selected, the compounds of the present disclosure, and/or the pharmaceutical compositions of the present disclosure, are formulated into pharmaceutically-acceptable dosage forms. The compounds according to the disclosure can be formulated for administration in any convenient way for use in human or veterinary medicine, by analogy with other pharmaceuticals.

In one aspect, the disclosure provides pharmaceutical formulation comprising a therapeutically-effective amount of one or more of the compounds described above, formulated together with one or more pharmaceutically acceptable carriers (additives) and/or diluents. In one embodiment, one or more of the compounds described herein are formulated for parenteral administration for parenteral administration, one or more compounds disclosed herein can be formulated as aqueous or nonaqueous solutions, dispersions, suspensions or emulsions or sterile powders which can be reconstituted into sterile injectable solutions or dispersions just prior to use. Such formulations can comprise sugars, alcohols, antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents. These compositions can also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms

upon the subject compounds can be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It can also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form can be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin. If desired the formulation can be diluted prior to use with, for example, an isotonic saline solution or a dextrose solution. In some examples, the compound is formulated as an aqueous solution and is administered intravenously.

Pharmaceutical compositions can be in the form of a solution or powder for injection. Such compositions may be formulated according to techniques known in the art using suitable dispersing or wetting agents (such as, for example, Tween 80) and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are mannitol, water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed including synthetic mono- or diglycerides. Fatty acids, such as oleic acid and its glyceride derivatives are useful in the preparation of injectables, as are natural pharmaceutically-acceptable oils, such as olive oil or castor oil, especially in their polyoxyethylated versions. These oil solutions or suspensions may also contain a long-chain alcohol diluent or dispersant, or carboxymethyl cellulose or similar dispersing agents which are commonly used in the formulation of pharmaceutically acceptable dosage forms such as emulsions and or suspensions. Other commonly used surfactants such as Tweens, Spans, and/or other similar emulsifying agents or bioavailability enhancers which are commonly used in the manufacture of pharmaceutically acceptable solid, liquid, or other dosage forms may also be used for the purposes of formulation.

Pharmaceutical compositions can be orally administered in any orally acceptable dosage form including, but not limited to, capsules, tablets, emulsions and aqueous suspensions, dispersions and solutions. In the case of tablets for oral use, carriers which are commonly used include lactose and corn starch. Lubricating agents, such as magnesium stearate, are also typically added. For oral administration in a capsule form, useful diluents include lactose and

dried corn starch. When aqueous suspensions and/or emulsions are administered orally, the active ingredient may be suspended or dissolved in an oily phase is combined with emulsifying and/or suspending agents. If desired, certain sweetening and/or flavoring and/or coloring agents may be added.

The pharmaceutical compositions of this invention may also be administered in the form of suppositories for rectal administration. These compositions can be prepared by mixing a compound of this invention with a suitable non-irritating excipient which is solid at room temperature but liquid at the rectal temperature and therefore will melt in the rectum to release the active components. Such materials include, but are not limited to, cocoa butter, beeswax and polyethylene glycols.

Alternatively or in addition, pharmaceutical compositions can be administered by nasal aerosol or inhalation. Such compositions are prepared according to techniques well-known in the art of pharmaceutical formulation and may be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons, and/or other solubilizing or dispersing agents known in the art.

In some instances, one or more peptides disclosed herein can be conjugated, for example, to a carrier protein. Such conjugated compositions can be monovalent or multivalent. For example, conjugated compositions can include one peptide disclosed herein conjugated to a carrier protein. Alternatively, conjugated compositions can include two or more peptides disclosed herein conjugated to a carrier.

As used herein, when two entities are "conjugated" to one another they are linked by a direct or indirect covalent or non-covalent interaction. In certain embodiments, the association is covalent. In other embodiments, the association is non-covalent. Non- covalent interactions include hydrogen bonding, van der Waals interactions, hydrophobic interactions, magnetic interactions, electrostatic interactions, etc. An indirect covalent interaction is when two entities are covalently connected, optionally through a linker group.

Carrier proteins can include any protein that increases or enhances immunogenicity in a subject. Exemplary carrier proteins are described in the art (see, e.g., Fattom et al., Infect. Immun., 58:2309-2312, 1990; Devi et al., Proc. Natl. Acad. Sci. USA 88:7175-7179, 1991; Li et al., Infect. Immun. 57:3823-3827, 1989; Szu et al., Infect. Immun. 59:4555-4561,1991; Szu et al., J. Exp. Med. 166:1510-1524, 1987; and Szu et al., Infect. Immun. 62:4440-4444, 1994).

Polymeric carriers can be a natural or a synthetic material containing one or more primary and/or secondary amino groups, azido groups, or carboxyl groups. Carriers can be water soluble.

Methods of Treatment

The disclosure includes methods of using the peptides herein for the prophylaxis and/or treatment of infection. The terms "treat", "treating" or "treatment" as used herein, refers to partially or completely alleviating, inhibiting, ameliorating, and/or relieving the disease or condition from which the subject is suffering. This means any manner in which one or more of the symptoms of a disease or disorder (e.g., cancer) are ameliorated or otherwise beneficially altered. As used herein, amelioration of the symptoms of a particular disorder (e.g., infection) refers to any lessening, whether permanent or temporary, lasting or transient that can be attributed to or associated with treatment by the compositions and methods of the present invention. In some embodiments, treatment can promote or result in, for example, a decrease in the number of microbial cells or organisms (e.g., in a subject) relative to the number of microbial cells or organisms prior to treatment; a decrease in the viability (e.g., the average/mean viability) of microbial cells or organisms (e.g., in a subject) relative to the viability (e.g., the average/mean viability) of microbial cells or organisms (e.g., in the subject) prior to treatment; and/or reductions in one or more symptoms associated with one or more infections in a subject relative to the subject's symptoms prior to treatment.

Examples of bacteria internally cross-linked AMPs are active against include, without limitation, *Staphylococci* (e.g., *S. aureus*, *S. intermedius*, *S. epidermidis*, and other coagulase negative *Staphylococci*), *Neisseriae* (e.g., *N. gonorrheae* and *N. meningitidis*), *Streptococci* (e.g., Group A *Streptococcus* (e.g., *S. pyogenes*), Group B *Streptococcus* (e.g., *S. agalactiae*), Group C *Streptococcus*, Group G *Streptococcus*, *S. pneumoniae*, and viridans *Streptococci*), *Chlamydia trachomatis*, *Treponemae* (e.g., *T. pallidum*, *T. pertenue*, and *T. cerateum*), *Haemophilus* bacteria (e.g., *H. ducreyi*, *H. influenzae*, and *H. aegyptius*), *Bordetellae* (e.g., *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*), *Gardnerella vaginalis*, *Bacillus* (e.g., *B. anthracis and B. cereus*), *Mycobacteria (*e.g., *M. tuberculosis* and *M. leprae), Listeria monocytogenes*, *Borrelia burgdorferi, Actinobacillus pleuropneumoniae, Helicobacter pylori, Clostridium* (e.g. *C. perfringens*, *C. septicum*, *C. novyi*, and *C. tetani*), *Escherichia coli*, *Porphyromonas gingivalis*, *Vibrio cholerae*, Salmonella bacteria (e.g., *S. enteriditis*, *S. typhimurium*, and *S. typhi*), Shigella

bacteria, Francisella bacteria, Yersinia bacteria (e.g. *Y. pestis* and *Y. enterocolitica*), Burkholderia bacteria, Pseudomonas bacteria, and Brucella bacteria. Mycoplasmal organisms AMPs are active against include, e.g., *M. pneumoniae*, *M. fermentans*, *M. hominis*, and *M. penetrans*.

Examples of fungal (including yeast) organisms internally cross-linked AMPs are active against include, but are not limited to, *Candida albicans*, other *Candida* species, *Cryptococcus neoformans*, *Histoplasma capsulatum*, and *Pneumocystis carinii*.

Examples of protozoan parasites internally cross-linked AMPs are active against include, without limitation, *Trichomonas vaginalis*, *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, *Entamoeba histolytica*, *Toxoplasma brucei*, *Toxoplasma gondii*, and *Leishmania major*.

Examples of viruses internally cross-linked AMPs may be employed against include, but are not limited to cells infected with, human immunodeficiency virus (HIV) 1 and 2, human lymphotropic virus (HTLV), measles virus, rabies virus, hepatitis virus A, B, and C, rotaviruses, rhinoviruses, influenza virus, parainfluenza virus, respiratory syncytial virus, adenoviruses, parvoviruses (e.g., parvovirus B19), roseola virus, enteroviruses, papilloma viruses, retroviruses, herpesviruses (e.g., herpes simplex virus, varicella zoster virus, Epstein Barr virus (EBV), human cytomegalovirus (CMV), human herpesvirus 6, 7 and 8), poxviruses (e.g., variola major and variola minor, vaccinia, and monkeypox virus), feline leukemia virus, feline immunodeficiency virus, and simian immunodeficiency virus. While the structurally stabilized I-TAMPs translocate into microbial cell and exert their biological activities within the microbial cells, it is envisioned that structurally stabilized AMPs having appropriate intra-mammalian cell activities (e.g., anti-intracellular virus or other intra-mammalian cell anti-microbial activity) with the ability to translocate into relevant mammalian cells, without causing significant lysis of the mammalian cells, can be developed.

Disorders that can be treated by the compositions, formulations, and/or methods described herein include, but are not limited to, infectious diseases. Infectious diseases can be caused by pathogens, such as bacteria, viruses, fungi or parasites. In some embodiments, an infectious disease can be passed from person to person. In some embodiments, an infectious disease can be transmitted by bites from insects or animals. In some embodiments, an infectious

disease can be acquired by ingesting contaminated food or water or being exposed to organisms in the environment. Some infectious diseases can be prevented by vaccines.

In specific embodiments, infectious diseases that can be treated by the compositions, formulations, and/or methods described herein include, but are not limited to, Acinetobacter infections, Actinomycosis, African sleeping sickness (African trypanosomiasis), AIDS (Acquired immunodeficiency syndrome), Amebiasis, Anaplasmosis, Angiostrongyliasis, Anisakiasis, Anthrax, Arcanobacterium haemolyticum infection, Argentine hemorrhagic fever, Ascariasis, Aspergillosis, Astrovirus infection, Babesiosis, Bacillus cereus infection, Bacterial pneumonia, Bacterial vaginosis, Bacteroides infection, Balantidiasis, Bartonellosis, Baylisascaris infection, BK virus infection, Black piedra, Blastocystosis, Blastomycosis, Bolivian hemorrhagic fever, Botulism (and Infant botulism), Brazilian hemorrhagic fever, Brucellosis, Bubonic plague, Burkholderia infection, Buruli ulcer, Calicivirus infection (Norovirus and Sapovirus), Campylobacteriosis, Candidiasis (Moniliasis; Thrush), Capillariasis, Carrion's disease, Cat-scratch disease, Cellulitis, Chagas Disease (American trypanosomiasis), Chancroid, Chickenpox, Chikungunya, Chlamydia, Chlamydophila pneumoniae infection (Taiwan acute respiratory agent or TWAR), Cholera, Chromoblastomycosis, Chytridiomycosis, Clonorchiasis, Clostridium difficile colitis, Coccidioidomycosis, Colorado tick fever (CTF), Common cold (Acute viral rhinopharyngitis; Acute coryza), Creutzfeldt-Jakob disease (CJD), Crimean-Congo hemorrhagic fever (CCHF), Cryptococcosis, Cryptosporidiosis, Cutaneous larva migrans (CLM), Cyclosporiasis, Cysticercosis, Cytomegalovirus infection, Dengue fever, Desmodesmus infection, Dientamoebiasis, Diphtheria, Diphyllobothriasis, Dracunculiasis, Ebola hemorrhagic fever, Echinococcosis, Ehrlichiosis, Enterobiasis (Pinworm infection), Enterococcus infection, Enterovirus infection, Epidemic typhus, Erythema infectiosum (Fifth disease), Exanthem subitum (Sixth disease), Fasciolasis, Fasciolopsiasis, Fatal familial insomnia (FFI), Filariasis, Food poisoning by Clostridium perfringens, Free-living amebic infection, Fusobacterium infection, Gas gangrene (Clostridial myonecrosis), Geotrichosis, Gerstmann-Sträussler-Scheinker syndrome (GSS), Giardiasis, Glanders, Gnathostomiasis, Gonorrhea, Granuloma inguinale (Donovanosis), Group A streptococcal infection, Group B streptococcal infection, Haemophilus influenzae infection, Hand, foot and mouth disease (HFMD), Hantavirus Pulmonary Syndrome (HPS), Heartland virus disease, Helicobacter pylori infection, Hemolytic-uremic syndrome (HUS), Hemorrhagic fever with renal syndrome (HFRS), Hepatitis A,

Hepatitis B, Hepatitis C, Hepatitis D, Hepatitis E, Herpes simplex, Histoplasmosis, Hookworm infection, Human bocavirus infection, Human ewingii ehrlichiosis, Human granulocytic anaplasmosis (HGA), Human metapneumovirus infection, Human monocytic ehrlichiosis, Human papillomavirus (HPV) infection, Human parainfluenza virus infection, Hymenolepiasis, Epstein-Barr Virus Infectious Mononucleosis (Mono), Influenza (flu), Isosporiasis, Kawasaki disease, Keratitis, Kingella kingae infection, Kuru, Lassa fever, Legionellosis (Legionnaires' disease), Legionellosis (Pontiac fever), Leishmaniasis, Leprosy, Leptospirosis, Listeriosis, Lyme disease (Lyme borreliosis), Lymphatic filariasis (Elephantiasis), Lymphocytic choriomeningitis, Malaria, Marburg hemorrhagic fever (MHF), Measles, Middle East respiratory syndrome (MERS), Melioidosis (Whitmore's disease), Meningitis, Meningococcal disease, Metagonimiasis, Microsporidiosis, Molluscum contagiosum (MC), Monkeypox, Mumps, Murine typhus (Endemic typhus), Mycoplasma pneumonia, Mycetoma (disambiguation), Myiasis, Neonatal conjunctivitis (Ophthalmia neonatorum), Variant Creutzfeldt-Jakob disease (vCJD, nvCJD), Nocardiosis, Onchocerciasis (River blindness), Opisthorchiasis, Paracoccidioidomycosis (South American blastomycosis), Paragonimiasis, Pasteurellosis, Pediculosis capitis (Head lice), Pediculosis corporis (Body lice), Pediculosis pubis (Pubic lice, Crab lice), Pelvic inflammatory disease (PID), Pertussis (Whooping cough), Plague, Pneumococcal infection, Pneumocystis pneumonia (PCP), Pneumonia, Poliomyelitis, Prevotella infection, Primary amoebic meningoencephalitis (PAM), Progressive multifocal leukoencephalopathy, Psittacosis, Q fever, Rabies, Relapsing fever, Respiratory syncytial virus infection, Rhinosporidiosis, Rhinovirus infection, Rickettsial infection, Rickettsialpox, Rift Valley fever (RVF), Rocky Mountain spotted fever (RMSF), Rotavirus infection, Rubella, Salmonellosis, SARS (Severe Acute Respiratory Syndrome), Scabies, Schistosomiasis, Sepsis, Shigellosis (Bacillary dysentery), Shingles (Herpes zoster), Smallpox (Variola), Sporotrichosis, Staphylococcal food poisoning, Staphylococcal infection, Strongyloidiasis, Subacute sclerosing panencephalitis, Syphilis, Taeniasis, Tetanus (Lockjaw), Tinea barbae (Barber's itch), Tinea capitis (Ringworm of the Scalp), Tinea corporis (Ringworm of the Body), Tinea cruris (Jock itch), Tinea manum (Ringworm of the Hand), Tinea nigra, Tinea pedis (Athlete's foot), Tinea unguium (Onychomycosis), Tinea versicolor (Pityriasis versicolor), Toxocariasis (Ocular Larva Migrans (OLM)), Toxocariasis (Visceral Larva Migrans (VLM)), Trachoma, Toxoplasmosis, Trichinosis, Trichomoniasis, Trichuriasis (Whipworm infection), Tuberculosis, Tularemia,

Typhoid fever, Typhus fever, Ureaplasma urealyticum infection, Valley fever, Venezuelan equine encephalitis, Venezuelan hemorrhagic fever, Vibrio vulnificus infection, Vibrio parahaemolyticus enteritis, Viral pneumonia, West Nile Fever, White piedra (Tinea blanca), Yersinia pseudotuberculosis infection, Yersiniosis, Yellow fever, and Zygomycosis.

The compositions, formulations, and/or methods described herein can be used to treat a pathogen. In some embodiments, the pathogen can be a virus, bacterium, prion, a fungus, or a parasite. In specific embodiments, the pathogen described herein include, but are not limited to, Acinetobacter baumannii, Actinomyces israelii, Actinomyces gerencseriae and Propionibacterium propionicus, Trypanosoma brucei, HIV (Human immunodeficiency virus), Entamoeba histolytica, Anaplasma species, Angiostrongylus, Anisakis, Bacillus anthracis, Arcanobacterium haemolyticum, Junin virus, Ascaris lumbricoides, Aspergillus species, Astroviridae family, Babesia species, Bacillus cereus, bacterial vaginosis microbiota, Bacteroides species, Balantidium coli, Bartonella, Baylisascaris species, BK virus, Piedraia hortae, Blastocystis species, Blastomyces dermatitidis, Machupo virus, Clostridium botulinum, Sabia, Brucella species, Enterobacteriaceae, Burkholderia cepacia and other Burkholderia species, Mycobacterium ulcerans, Caliciviridae family, Campylobacter species, Candida albicans and other Candida species, Capillaria philippinensis, Capillaria hepatica, Capillaria aerophila, Bartonella bacilliformis, Bartonella henselae, Group A Streptococcus and Staphylococcus, Trypanosoma cruzi, Haemophilus ducreyi, Varicella zoster virus (VZV), Alphavirus, Chlamydia trachomatis, Chlamydophila pneumoniae, Vibrio cholera, Fonsecaea pedrosoi, Batrachochytrium dendrabatidis, Clonorchis sinensis, Clostridium difficile, Coccidioides immitis and Coccidioides posadasii, Colorado tick fever virus (CTFV), rhinoviruses and coronaviruses, PRNP, Crimean-Congo hemorrhagic fever virus, Cryptococcus neoformans, Cryptosporidium species, Ancylostoma braziliense; multiple other parasites, Cyclospora cayetanensis, Taenia solium, Cytomegalovirus, Dengue viruses (DEN-1, DEN-2, DEN-3 and DEN-4) – Flaviviruses, Green algae Desmodesmus armatus, Dientamoeba fragilis, Corynebacterium diphtheria, Diphyllobothrium, Dracunculus medinensis, Ebolavirus (EBOV), Echinococcus species, Ehrlichia species, Enterobius vermicularis, Enterococcus species ,Enterovirus species, Rickettsia prowazekii, Parvovirus B19, Human herpesvirus 6 (HHV-6) and Human herpesvirus 7 (HHV-7), Fasciola hepatica and Fasciola gigantica, Fasciolopsis buski, PRNP, Filarioidea superfamily, Clostridium perfringens, Fusobacterium species, Clostridium perfringens, other Clostridium

species, Geotrichum candidum, Giardia lamblia, Burkholderia mallei, Gnathostoma spinigerum and Gnathostoma hispidum, Neisseria gonorrhoeae, Klebsiella granulomatis, Streptococcus pyogenes, Streptococcus agalactiae, Haemophilus influenza, Enteroviruses, mainly Coxsackie A virus and Enterovirus 71 (EV71), Sin Nombre virus, Heartland virus, Helicobacter pylori, Escherichia coli O157:H7, O111 and O104:H4, Bunyaviridae family, Hepatitis A virus ,Hepatitis B virus, Hepatitis C virus, Hepatitis D Virus, Hepatitis E virus, Herpes simplex virus 1 and 2 (HSV-1 and HSV-2), Histoplasma capsulatum, Ancylostoma duodenale and Necator americanus, Human bocavirus (HBoV), Ehrlichia ewingii, Anaplasma phagocytophilum, Human metapneumovirus (hMPV), Ehrlichia chaffeensis, Human papillomavirus (HPV), Human parainfluenza viruses (HPIV), Hymenolepis nana and Hymenolepis diminuta, Epstein-Barr Virus (EBV), Orthomyxoviridae family, Isospora belli, Kingella kingae, Lassa virus, Legionella pneumophila, Leishmania species, Mycobacterium leprae, Mycobacterium lepromatosis, Leptospira species, Listeria monocytogenes, Borrelia burgdorferi, Borrelia garinii, Borrelia afzelii, Wuchereria bancrofti, Brugia malayi, Lymphocytic choriomeningitis virus (LCMV), Plasmodium species, Marburg virus, Measles virus, Middle East respiratory syndrome coronavirus, Burkholderia pseudomallei, Neisseria meningitides, Metagonimus yokagawai, Microsporidia phylum, Molluscum contagiosum virus (MCV), Monkeypox virus, Mumps virus, Rickettsia typhi, Mycoplasma pneumoniae, Actinomycetoma, Eumycetoma, parasitic dipterous fly larvae, Chlamydia trachomatis, Neisseria gonorrhoeae, Nocardia asteroids, Nocardia species, Onchocerca volvulus, Opisthorchis viverrini and Opisthorchis felineus, Paracoccidioides brasiliensis, Pediculus humanus capitis, Phthirus pubis, Bordetella pertussis, Yersinia pestis, Streptococcus pneumoniae, Pneumocystis jirovecii, Poliovirus, Prevotella species, Naegleria fowleri, JC virus, Chlamydophila psittaci, Coxiella burnetii, Rabies virus, Borrelia hermsii, Borrelia recurrentis, Borrelia species, Respiratory syncytial virus (RSV), Rhinosporidium seeberi, Rhinovirus, Rickettsia species, Rickettsia akari, Rift Valley fever virus, Rickettsia rickettsia, Rotavirus, Rubella virus, Salmonella species, SARS coronavirus, Sarcoptes scabiei, Schistosoma species, Shigella species, Varicella zoster virus (VZV), Variola major, Variola minor, Sporothrix schenckii, Staphylococcus species, Strongyloides stercoralis, Measles virus, Treponema pallidum, Taenia species, Clostridium tetani, Trichophyton species, Trichophyton tonsurans, Epidermophyton floccosum, Trichophyton rubrum, Trichophyton mentagrophytes, Trichophyton rubrum, Hortaea werneckii, Trichophyton species, Trichophyton species,

Malassezia species, Toxocara canis, Toxocara cati, Chlamydia trachomatis, Toxoplasma gondii, Trichinella spiralis, Trichomonas vaginalis, Trichuris trichiura, Mycobacterium tuberculosis, Francisella tularensis, Salmonella enterica subsp. enterica, serovar typhi, Rickettsia, Ureaplasma urealyticum, Coccidioides immitis, Coccidioides posadasii, Venezuelan equine encephalitis virus, Guanarito virus, Vibrio vulnificus, Vibrio parahaemolyticus, multiple viruses, West Nile virus, Trichosporon beigelii, Yersinia pseudotuberculosis, Yersinia enterocolitica, Yellow fever virus, Mucorales order (Mucormycosis), and Entomophthorales order (Entomophthoramycosis).

All the methods of treatment and prophylaxis described herein may be applied to at least any or all the above-listed microbial organisms.

In some embodiments, the compounds of the invention can be toxic to one microbe. In some embodiments, the compounds of the invention can be toxic to two microbes. In some embodiments, the compounds of the invention can be toxic to three microbes. In some embodiments, the compounds of the invention can be toxic to four microbes. In some embodiments, the compounds of the invention can be toxic to five microbes.

In some embodiments, the compounds of the invention can be used to treat a microbe without damaging the host subject. In some embodiments, the compounds of the invention can be used to treat two microbes without damaging the host subject. In some embodiments, the compounds of the invention can be used to treat three microbes without damaging the host subject. In some embodiments, the compounds of the invention can be used to treat four microbes without damaging the host subject. In some embodiments, the compounds of the invention can be used to treat five microbes without damaging the host subject.

In general, methods include selecting a subject and administering to the subject an effective amount of one or more of the peptides herein, e.g., in or as a pharmaceutical composition, and optionally repeating administration as required for the prophylaxis or treatment of a microbial infection and can be administered, e.g., orally, intravenously or topically. Specific dosage and treatment regimens for any particular patient will depend upon a variety of factors, including the activity of the specific compound employed, the age, body weight, general health status, sex, diet, time of administration, rate of excretion, drug combination, the severity and course of the disease, condition or symptoms, the patient's disposition to the disease, condition or symptoms, and the judgment of the treating physician.

An effective amount can be administered in one or more administrations, applications or dosages. A therapeutically effective amount of a therapeutic compound (i.e., an effective dosage) depends on the therapeutic compounds selected. The compositions can be administered one from one or more times per day to one or more times per week; including once every other day. The skilled artisan will appreciate that certain factors may influence the dosage and timing required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present.

Moreover, treatment of a subject with a therapeutically effective amount of the therapeutic compounds described herein can include a single treatment or a series of treatments. For example, effective amounts can be administered at least once. Upon improvement of a patient's condition, a maintenance dose of a compound, composition or combination of this invention may be administered, if necessary. Subsequently, the dosage or frequency of administration, or both, may be reduced, as a function of the symptoms, to a level at which the improved condition is retained. Patients may, however, require intermittent treatment on a long-term basis upon any recurrence of disease symptoms.

In some instances, the peptides herein can further be co-administered with one or more additional therapeutic agents in amounts effective for achieving a modulation of disease or disease symptoms. Such additional therapeutic agents may include conventional antimicrobial agents (e.g., antibiotics) known in the art. When co-administered, stapled AMPs of the invention operate in conjunction with conventional antimicrobial agents to produce mechanistically additive or synergistic antimicrobial effects. Without being limited by any particular mechanism of action, certain internally cross-linked (e.g., stapled) AMPs having the ability to produce "pores" in the membranes of certain microbial organisms (including, e.g., Gram-negative bacteria) can act to facilitate and/or enhance the passage of appropriate conventional antimicrobial agents to the interiors of relevant microbial cells. For the same purpose, the internally cross-linked AMPs can be conjugated (covalently or non-covalently) to appropriate antimicrobial agents, the resulting conjugates being administered to appropriate subjects.

The ability of select internally cross-linked and intracellular targeting AMPs to also produce transient "pores" in the membranes of microbial organisms provides the basis for another utility for them. Thus, e.g., relevant microbial organisms (e.g., any of those disclosed

herein) can be contacted either in a subject or *in vitro* to an internally cross-linked AMP with the ability to produce "pores" or even lysis of the microbial organism. As result of this activity, nucleic acids (e.g., DNA and/or RNA) are released from microbial organisms into their surroundings. This phenomenon can be used as a basis for accurate, rapid, and inexpensive identification of the microbial organism. Where the contacting occurs in a subject, any of a variety of bodily fluids (e.g., blood, lymph, urine, feces, mucus, or tears) or body lavages can be tested. Where the contacting occurs *in vitro*, culture medium can be tested.

Application to Medical or Hygienic Devices

The antimicrobial peptides of the invention can be applied to, or incorporated into, various medical and/or hygienic devices (e.g., as a coating, or impregnated within a biodegradable device for exposure or release as the device degrades or dissolves after the device is inserted into a bodily canal of a vertebrate subject, inserted into a bodily cavity of a vertebrate subject, or applied to a tissue or organ of a vertebrate animal) to prevent or inhibit microbial (e.g., bacterial or biofilm) growth. Medical or hygienic devices suitable for use with the stapled peptides disclosed herein include, but are not limited to, devices that are inserted into a bodily canal of a vertebrate subject, inserted into a bodily cavity of a vertebrate subject, or applied to a tissue or organ of a vertebrate animal for the purpose of: (a) wound protection; (b) preventing or reducing unwanted, or overcoming restricted, release from the body of the vertebrate subject of a bodily fluid, bodily secretion, or excreta (e.g., blood, menses, urine, lymphatic fluid, cerebrospinal fluid, semen, saliva, vaginal secretions, mucus, or feces); (c) delivering a drug or some other therapeutic or prophylactic agent to a subject; (d) replacing absent or supplementing defective organ functions; or (e) maintaining the patency of a bodily canal (e.g., a blood vessel). Specific examples of medical or hygienic devices include, without limitation: rectal devices such as suppositories, enemas, and catheters; nasal, tracheal, or esophageal delivery devices; vaginal devices such as vaginal tampons and contraceptive devices (e.g., diaphragms or intrauterine devices (IUDs)); venous, arterial, intracranial and other needles, catheters and stents; renal dialysis accesses; surgical bandages, sutures, or dressings; ostomy devices; natural and synthetic implantable tissue matrices (see, for example, U.S. Patent No. 5,885,829, incorporated herein by reference in its entirety); pace makers and pace maker wires and leads; synthetic and natural prostheses such as hip and knee prostheses and heart valves; osmotic pumps (e.g., mini osmotic

pumps) that are implanted in body cavity (e.g., the peritoneal cavity) and provide slow delivery of a drug or some other therapeutic or prophylactic agent.

Further Applications

Examples of suitable animals for treatment are generally known in the art and include (but are not limited to), e.g., poultry and other birds (including chickens, turkeys, ducks, ostrich, emu, quail), ruminants (including goats, sheep, and cattle), fish, pigs, rabbits, mice, rats, horses, donkeys, monkeys, apes, felines (including cats), hamsters, ferrets, guinea pigs, and canines (including dogs). Examples of suitable plants for treatment are generally known in the art and include (but are not limited to), e.g., almond, apple, amaranth, artichoke, asparagus, avocado, banana and plantain, barley, beet, berries (including blueberry, blackberry, strawberry, and raspberry), breadfruit and jackfruit, brussels sprout, cabbage, carrot, cassava, cauliflower and broccoli, celery, chayote, cherry, coconut, collard and kale, corn (maize), cucumber and zucchini, dandelion, eggplant, endive and chicory, garlic, kohlrabi, grape, legume, lettuce, melons (including honeydew, cantaloupe, and watermelon), mustard, oat, oca, olive, okra, onion, orange and grapefruit, oyster plant, pear, peach, pemmican, pepper, potato and other tubers, quinoa, radish, rice, rhubarb, rye, sago, sorghum, soybean, spinach, pumpkin and other squashes, sunchoke, taro, teff, tomato, turnip, ulluco, vanilla, watercress, wheat, yam, and yautia. Examples of suitable foods for treatment are generally known in the art and include (but are not limited to), e.g., algae, mushrooms, and products derived from animals (e.g., beef, butter, eggs, (ice) cream, gravy, milk, pork, veal, yogurt) and/or plants (e.g., beer, bread, cereal, chocolate, coffee, ketchup, mustard sauce, oatmeal, juice, monosodium glutamate, salad, soda, soft drinks, soymilk, soy sauce, tea, tofu, fries, vinegar, wine) as described above.

The peptides herein can also be applied in the personal care and/or consumer products context (e.g., to health or beauty products in sterilization processes) to reduce or eliminate the risk of microbial (e.g., bacterial) contamination. Examples of suitable products for treatment are generally known in the art and include (but are not limited to), e.g., brushes, conditioners, clips, clippers, curling irons, shampoos, soaps, lotions, topical acne ointments, oils, colorants, dyes, perfumes, pins, fragrances, razors, shaving devices, deodorants, cosmetics, kitchen and/or dining devices (e.g., cutting boards, racks, containers, pots, pans, utensils), and cleaning products (e.g., brooms, mops, dustpans, sweepers) and cleaning solutions.

EXAMPLES

Example 1: Synthesis of Stapled I-TAMP Analogues

We synthesized a complete *i, i+4*, and *i, i+7* staple scanning panel of stapled buforin II peptides to identify which staple insertion position(s) yield I-TAMP analogues with optimal biological and pharmacologic properties (**FIGURE 1**). In some compositions, replacement of the central proline residue was avoided given the potential role of prolines as breakpoints in secondary structure. Also, in some panels, the phenylalanine adjacent to the proline was replaced with tryptophan for facile determination of peptide concentration by UV spectroscopy. The production of these exemplary panels allowed us to interrogate the effects of structural stabilization of various segments of the parent I-TAMP on its antimicrobial and hemolytic activity.

Example 2: Alpha-Helical Characterization of Stapled I-TAMP Analogues

To determine the α-helical content for each peptide within our panel of stapled I-TAMP analogues, we studied the peptides dissolved in 10 mM phosphate buffer (pH 7.4) using CD spectroscopy in the presence and absence of trifluoroethanol (TFE; 50% v/v), an α-helix promoting solvent. In the absence of TFE, the *i, i+4* stapled analogues displayed modestly improved α-helical structure compared to the unstapled parent I-TAMP, buforin II (F10W), which was otherwise completely disordered (**FIGURE 2 Panel A**). Upon the addition of TFE, the α-helical content increased for some of the stapled analogues and the parent I-TAMP (F10W), but not as dramatically as we previously observed for α-helical AMPs (**FIGURE 2 Panel B**), consistent with the incorporation of a helix-breaking proline within the buforin II sequence. The *i, i+7* stapled analogues displayed higher levels of α-helicity when compared to their *i, i+4* counterparts, both in the presence and absence of TFE (**FIGURE 3**).

Example 3: Hemolytic Activity of Stapled I-TAMP Peptides

One of the key obstacles that have long impeded the use of AMPs in systemic infections such as sepsis is their tendency to lyse human cells, such as red blood cells (RBCs). Thus, it is critical to minimize hemolytic activity as much as possible to achieve a therapeutic window. When we tested stapled analogues of the I-TAMP buforin II in a 1% RBC suspension in phosphate buffer, most *i, i+4* analogues displayed low hemolytic activity, although in some cases

there was a 5-fold increase in hemolysis (**TABLE 1**). Generally, the *i*, *i*+7 analogues had higher hemolytic activity, likely due to the greater hydrophobicity of the longer hydrocarbon staple (Table 1). Of note, in those circumstances where hydrocarbon staples increase hemolytic activity due to increasing hydrophobicity, the alkene moiety can be modified, e.g., by dihydroxylation, to install hydrophilic residues and potentially mitigate hemolysis.

Balancing antimicrobial potency and suppression of hemolytic activity, we identified BFStap(i+4)7 and BFStap(i+4)11 as specific exemplary candidates for further development, as they displayed the greatest increase in antimicrobial activity while maintaining low hemolytic activity.

FIGURE 8 is a graph showing the hemolysis of stapled buforin II analogues. Stapled buforin II analogs were incubated with 1% v/v human red blood cells (RBCs) in phosphate buffer for 1 hour at 37 °C and then the supernatant was collected and hemoglobin release was measured using UV absorbance at 570 nm. As opposed to a stapled lytic control peptide, the buforin II analogs displayed hemolysis levels below 5% at all the concentrations tested up to 50 μg/mL.

Table 1. Minimum inhibitory concentrations (MICs) of buforin II and stapled derivatives against *E. coli*, and the hemolytic activity of said peptides at 50 μg/ml.

Peptide	Antimicrobial Activity MIC (μg/ml)	% Hemolysis at 50 μg/ml
	E.coli	
Buforin II	>800	2.1
Buforin II(F10W)	>800	2.7
BFStap(i+4)1	50.0	2.2
BFStap(i+4)2	5.2	12.6
BFStap(i+4)3	25.0	2.9
BFStap(i+4)4	29.2	2.9
BFStap(i+4)5	66.7	3.0
BFStap(i+4)6	8.3	10.0
BFStap(i+4)7	6.3	3.4
BFStap(i+4)8	29.2	2.8
BFStap(i+4)9	50.0	2.5
BFStap(i+4)10	100	2.4
BFStap(i+4)11	6.3	3.1
BFStap(i+4)12	>100	2.4
BFStap(i+4)13	>100	2.4
BFStap(i+7)1	10.4	16.6
BFStap(i+7)2	33.3	3.1
BFStap(i+7)3	29.2	6.1
BFStap(i+7)4	3.1	57.0
BFStap(i+7)5	52.1	6.8
BFStap(i+7)6	6.3	9.5
BFStap(i+7)7	22.9	3.0
BFStap(i+7)8	12.5	3.9
BFStap(i+7)9	41.7	4.3
BFStap(i+7)10	>100	2.3

Example 4: Antimicrobial Activity of Stapled I-TAMP Peptides

To determine the antimicrobial activity of the stapled buforin II analogues compared to the unstapled sequence, we determined the MIC against a common Gram-negative pathogen, *Escherichia coli* (*E. coli*). Most studies utilize radial diffusion assays (RDAs) to study buforin II activity, but since the microbroth dilution assay is the standard technique used in the antibiotic field, we adopted the more broadly applied technique. Compared to the activity of the unstapled buforin II (F10W) and buforin II, all stapled analogues displayed higher levels of activity, even beyond what would have been anticipated due to modest structural stabilization (**TABLES 1 AND 2**). Strikingly, in certain cases, such as for BFStap(i+4)7 and BFStap(i+7)4, the observed

increase in activity compared to unmodified buforin II was over 200-fold. Of note, it was especially unexpected that stapling the N-terminal region yielded superior compounds than installing staples in the C-terminal region, which was the previously C-terminal alpha-helical region of buforin II. These results reaffirm the importance of comprehensive staple scanning to discover optimal constructs, as the staple insertion sites were not apparent or obvious based on previous structural and biological data.

Table 2. Minimum inhibitory concentrations (MICs) of buforin II and stapled derivatives against Gram-positive and Gram-negative bacterial pathogens.

Peptide	Antimicrobial Activity MIC (μg/ml)				
	E.coli	B.cereus	P.aeruginosa	S.Aureus	MRSA
Buforin II	>800	>800	>800	>800	>800
BFStap(i+4)7	6.25	12.5	12.5	12.5	25
BFStap(i+4)11	6.25	25	12.5	50	>50
BFStap(i+7)6	6.25	12.5	12.5	25	>50

Methods used in Examples

Solid phase peptide synthesis Fmoc-based solid-phase peptide synthesis was used to synthesize the antimicrobial peptides and their stapled derivatives. To achieve the various staple lengths, α-methyl, α-alkenyl amino acids were used flanking two, three or six residues. As examples, an R_5 or R_3 residue can be incorporated at position *i* and S_5 at position *i+3*, while two S_5 residues can be used at the *i* and *i+4* locations, and an R_8 at position *i* and S_5 at *i+7* (or S_8 at position *i* and R_5 at *i+7*) [20]. For the stapling reaction, Grubbs 1st generation ruthenium catalyst dissolved in dichloroethane was added to the peptides on resin. To ensure maximal conversion, three to five rounds of stapling were performed. Once stapled, the peptides were cleaved off the resin using trifluoroacetic acid, then precipitated using a hexane:ether (1:1) mixture, and then air dried and purified using LC-MS. We used UV spectroscopy to measure the amount of peptide purified based on the tryptophan extinction coefficient ε= 5690 M^{-1} cm^{-1} at 280 nm. Amino acid analysis was also used.

Circular Dichroism Spectroscopy For characterization of helical structure, the mean molar ellipticities of each peptide was determined using circular dichroism (CD) spectroscopy on an Aviv Biomedical spectrometer. Peptide stock solutions (100 μM) in 10 mM phosphate buffer (pH 7.4) and in a mixture of buffer:2,2,2-trifluoroethanol (1:1 v/v) were loaded into 1-mm fused silica cells and their ellipticity scanned from 195 to 260 nm.

Antimicrobial Activity Assay The following microbroth dilution protocol was adapted to determine the MIC of each peptide. First, Mueller-Hinton broth (MHB) was passed through an anion exchange column to remove polyanionic species and generate refined MHB. This refined broth was then used in the standard microbroth dilution protocol devised by Hancock et al. for 96 well plates [21]. Note that no BSA was used in the adapted protocol described herein, since initial studies revealed that it could interfere with peptide activity. Briefly, bacterial cells were grown overnight in refined MHB at 37°C and then diluted and allowed to grow again for several hours. Serial dilutions of peptide stocks in water (10 μl) were prepared using clear round-bottom polypropylene 96-well plates. Then 90 μl of bacteria in refined MHB was added to give a final inoculum of $5x10^5$ CFU/ml. The plates were then covered with porous tape to reduce evaporation, and incubated for 20-24 hours at 37°C. The MIC was determined by assessing the minimum peptide concentration at which no visible growth was observed.

Hemolytic Activity Assay For the determination of hemolytic activity, the following protocol was applied: human blood samples were centrifuged to isolate red blood cells (RBCs), which were then washed and suspended in phosphate-buffered saline to yield a 1% (v/v) suspension. This suspension was then added to serial dilutions of peptide stocks in water in clear round-bottom polypropylene 96-well plates and the plates incubated for 1 hour at 37°C. After incubation, the plates were centrifuged and the supernatant isolated to determine the amount of hemoglobin released using a spectrophotometer (570 nm). The minimum hemolytic concentration (MHC) was determined by assessing the peptide concentration at which there was less than 1% hemoglobin release.

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Biophysica Acta (BBA) - Biomembranes. 1838 (2014) 2228–2233. doi:10.1016/j.bbamem.2014.04.010.

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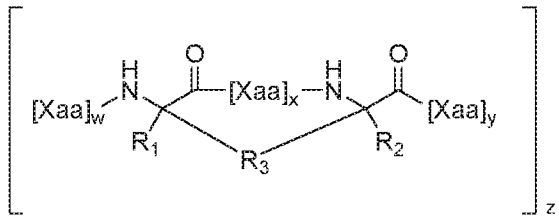
CLAIMS

What is claimed is:

1. A compound having the formula:



Formula (I)

or a pharmaceutically acceptable salt thereof,

wherein;

each R_1 and R_2 is independently H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heteroarylalkyl, or heterocyclylalkyl, any of which is substituted or unsubstituted;

each R_3 is independently alkylene, alkenylene, or alkynylene, any of which is substituted or unsubstituted;

each x is independently 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10;

each w and y is independently 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20;

z is1, 2, 3, 4, 5, 6, 7, 8, 9, or 10; and

each Xaa is independently an amino acid,

wherein the compound exhibits an intracellular antimicrobial effect against at least one microbe.

2. The compound of claim 1, wherein x is 2, 3, or 6.

3. The compound of claim 2, wherein x is 2; R_3 is C_8 alkylene, C_8 alkenylene, or C_8 alkynylene; and the sum of x, w, and y is at least 10.

4. The compound of claim 1, wherein x is 3; R_3 is C_8 alkylene, C_8 alkenylene, or C_8 alkynylene; and the sum of x, w, and y is at least 10.

5. The compound of claim 1, wherein R_3 is substituted with 1, 2, 3, 4, 5, or 6 R_4, and each R_4 is

independently –NH_3 or –OH, wherein each –NH_3 is optionally coupled with another chemical entity.

6. The compound of claim 5, wherein R_3 is substituted with 2 R_4, and both R_4 are –OH.

7. The compound of claim 5, wherein R_3 is substituted with 2 R_4, and one R_4 is an optionally substituted –NH_3 and the other R_4 is –OH.

8. The compound of claim 1, wherein at least one Xaa is an amino acid that is a non-natural amino acid.

9. The compound of claim 1, wherein the compound comprises a helix.

10. The compound of claim 9, wherein the helix is an α-helix.

11. The compound of claim 1, wherein R_3 spans from one to two turns on an α-helix.

12. The compound of claim 1, wherein the compound comprises an amino acid sequence that is at least 60% identical to an amino acid sequence selected from the group consisting of: SEQ ID NOs: 1–60.

13. The compound of claim 1, wherein the compound comprises an amino acid sequence that is at least 70% identical to an amino acid sequence selected from the group consisting of: SEQ ID NOs: 1–60.

14. The compound of claim 1, wherein the compound comprises an amino acid sequence that is at least 80% identical to an amino acid sequence selected from the group consisting of: SEQ ID NOs: 1–60.

15. The compound of claim 1, wherein the compound comprises an amino acid sequence that is at least 90% identical to an amino acid sequence selected from the group consisting of: SEQ ID

NOs: 1–60.

16. The compound of claim 1, wherein the compound comprises an amino acid sequence that is any one of: SEQ ID NOs: 1–60.

17. The compound of claim 1, wherein the compound comprises BFStap(i+4)2, BFStap(i+4)3, BFStap(i+4)4, BFStap(i+4)6, BFStap(i+4)7, BFStap(i+4)8, BFStap(i+4)11, BFStap(i+7)1, BFStap(i+7)2, BFStap(i+7)3, BFStap(i+7)4, BFStap(i+7)5, BFStap(i+7)6, BFStap(i+7)7, BFStap(i+7)8, or BFStap(i+7)9.

18. The compound of claim 1, wherein the compound comprises the sequence of BFStap(i+4)7, BFStap(i+4)11, or BFStap(i+7) 6.

19. The compound of claim 1, wherein the antimicrobial effect is a minimum inhibitory concentration between about 0.1 μM and about 50 μM.

20. The compound of claim 1, wherein the antimicrobial effect is a minimum inhibitory concentration between about 0.5 μM and about 20 μM.

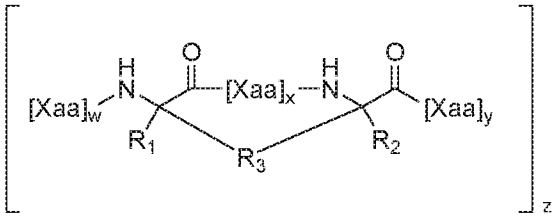
21. The compound of claim 1, wherein the microbe is a bacterial organism.

22. The compound of claim 21, wherein the bacterial organism is Gram-positive.

23. The compound of claim 21, wherein the bacterial organism is Gram-negative.

24. The compound of claim 21, wherein the bacterial organism is *Mycobacterium tuberculosis*.

25. A method of treating an infection caused by a microbe, the method comprising administering a therapeutically-effective amount of a compound having the formula:



Formula (I)

or a pharmaceutically acceptable salt thereof,

wherein;

each R_1 and R_2 is independently H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heteroarylalkyl, or heterocyclylalkyl, any of which is substituted or unsubstituted;

each R_3 is independently alkylene, alkenylene, or alkynylene, any of which is substituted or unsubstituted;

each x is independently 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10;

each w and y is independently 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20;

z is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10; and

each Xaa is independently an amino acid,

to a subject having, or at risk of having, the infection caused by the microbe, wherein the compound exhibits an intracellular antimicrobial effect against at least one microbe.

26. The method of claim 25, wherein the subject is an animal.

27. The method of claim 26, wherein the animal is a mammal.

28. The method of claim 26, wherein the animal is a human.

29. The method of claim 25, wherein the subject is a plant.

30. The method of claim 25, wherein the microbe is a bacterial organism.

31. The method of claim 30, wherein the bacterial organism is Gram-positive.

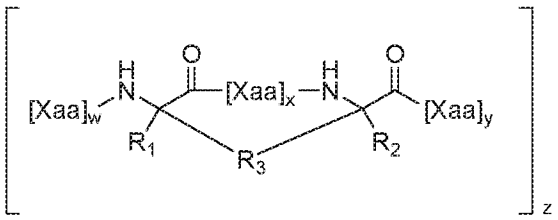
32. The method of claim 30, wherein the bacterial organism is Gram-negative.

33. The method of claim 25, further comprising administering to the subject a therapeutically-effective amount of an antibiotic.

34. The method of claim 33, wherein the compound and the antibiotic act synergistically to treat the infection.

35. The method of claim 33, wherein the compound and the antibiotic act synergistically to overcome a resistance of the infection to the antibiotic.

36. A pharmaceutical composition comprising a compound having the formula:



Formula (I)

or a pharmaceutically acceptable salt thereof,

wherein;

each R_1 and R_2 is independently H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heteroarylalkyl, or heterocyclylalkyl, any of which is substituted or unsubstituted;

each R_3 is independently alkylene, alkenylene, or alkynylene, any of which is substituted or unsubstituted;

each x is independently 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10;

each w and y is independently 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20;

z is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10; and

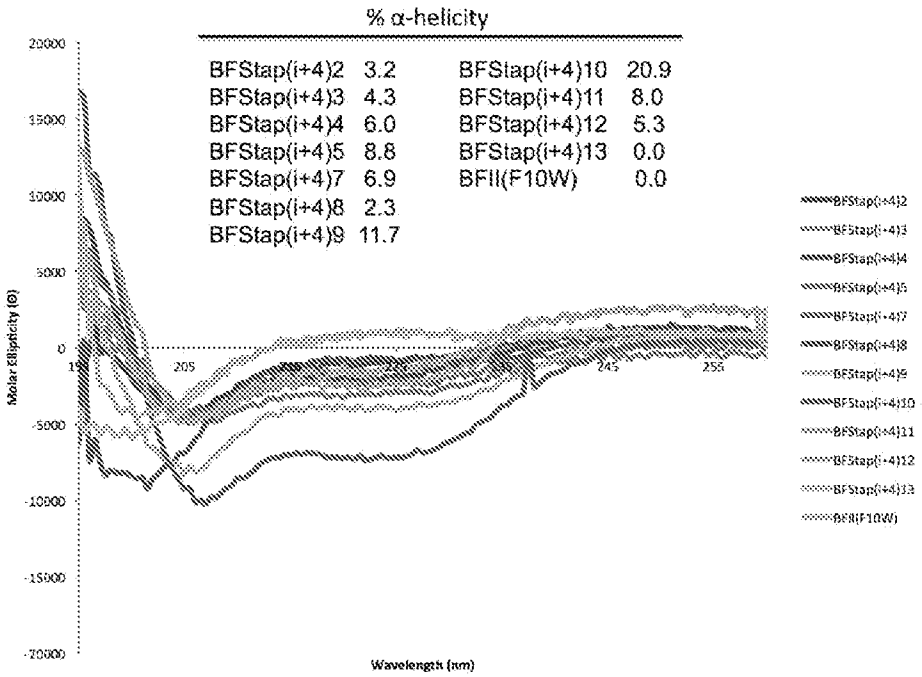
each Xaa is independently an amino acid,

wherein the compound exhibits an intracellular antimicrobial effect against at least one microbe.

BFStap(i+4)1	TRSSRAGLQ⁽ᵂ⁾PVGRVH⁽ˣ⁾LLR⁽ˣ⁾
BFStap(i+4)2	TRSSRAGLQ⁽ᵂ⁾PVGRV⁽ˣ⁾RLL⁽ˣ⁾K
BFStap(i+4)3	TRSSRAGLQ⁽ᵂ⁾PVGR⁽ˣ⁾HRL⁽ˣ⁾RK
BFStap(i+4)4	TRSSRAGLQ⁽ᵂ⁾PVG⁽ˣ⁾VHR⁽ˣ⁾LRK
BFStap(i+4)5	TRSSRAGLQ⁽ᵂ⁾PV⁽ˣ⁾RVH⁽ˣ⁾LLRK
BFStap(i+4)6	TRSSRAGLQ⁽ᵂ⁾P⁽ˣ⁾GRV⁽ˣ⁾RLLRK
BFStap(i+4)7	TRSSRAGL⁽ˣ⁾⁽ᵂ⁾PV⁽ˣ⁾RVHRLLRK
BFStap(i+4)8	TRSSRAG⁽ˣ⁾Q⁽ᵂ⁾P⁽ˣ⁾GRVHRLLRK
BFStap(i+4)9	TRSS⁽ˣ⁾AGL⁽ˣ⁾⁽ᵂ⁾PVGRVHRLLRK
BFStap(i+4)10	TRS⁽ˣ⁾RAG⁽ˣ⁾Q⁽ᵂ⁾PVGRVHRLLRK
BFStap(i+4)11	TR⁽ˣ⁾SRA⁽ˣ⁾LQ⁽ᵂ⁾PVGRVHRLLRK
BFStap(i+4)12	T⁽ˣ⁾SSR⁽ˣ⁾GLQ⁽ᵂ⁾PVGRVHRLLRK
BFStap(i+4)13	⁽ˣ⁾RSS⁽ˣ⁾AGLQ⁽ᵂ⁾PVGRVHRLLRK
BFStap(i+7)1	TRSSRAGLQ⁽ᵂ⁾PVG⁽ˢ⁾VHRLLR⁽ˣ⁾
BFStap(i+7)2	TRSSRAGLQ⁽ᵂ⁾PV⁽ˢ⁾RVHRLL⁽ˣ⁾K
BFStap(i+7)3	TRSSRAGLQ⁽ᵂ⁾P⁽ˢ⁾GRVHRL⁽ˣ⁾RK
BFStap(i+7)4	TRSSRAGL⁽ˢ⁾⁽ᵂ⁾PVGRV⁽ˣ⁾RLLRK
BFStap(i+7)5	TRSSRAG⁽ˢ⁾Q⁽ᵂ⁾PVGR⁽ˣ⁾HRLLRK
BFStap(i+7)6	TRSSRA⁽ˢ⁾LQ⁽ᵂ⁾PVG⁽ˣ⁾VHRLLRK
BFStap(i+7)7	TRSSR⁽ˢ⁾GLQ⁽ᵂ⁾PV⁽ˣ⁾RVHRLLRK
BFStap(i+7)8	TRSS⁽ˢ⁾AGLQ⁽ᵂ⁾P⁽ˣ⁾GRVHRLLRK
BFStap(i+7)9	T⁽ˢ⁾SSRAGL⁽ˣ⁾⁽ᵂ⁾PVGRVHRLLRK
BFStap(i+7)10	⁽ˢ⁾RSSRAG⁽ˣ⁾Q⁽ᵂ⁾PVGRVHRLLRK
BuforinII(F10W)	TRSSRAGLQ⁽ᵂ⁾PVGRVHRLLRK
BuforinII	TRSSRAGLQFPVGRVHRLLRK

FIG. 1

A


% α-helicity
BFStap(i+4)2 3.2
BFStap(i+4)3 4.3
BFStap(i+4)4 6.0
BFStap(i+4)5 8.8
BFStap(i+4)7 6.9
BFStap(i+4)8 2.3
BFStap(i+4)9 11.7
BFStap(i+4)10 20.9
BFStap(i+4)11 8.0
BFStap(i+4)12 5.3
BFStap(i+4)13 0.0
BFII(F10W) 0.0
Molar Ellipticity (θ)
Wavelength (nm)

B

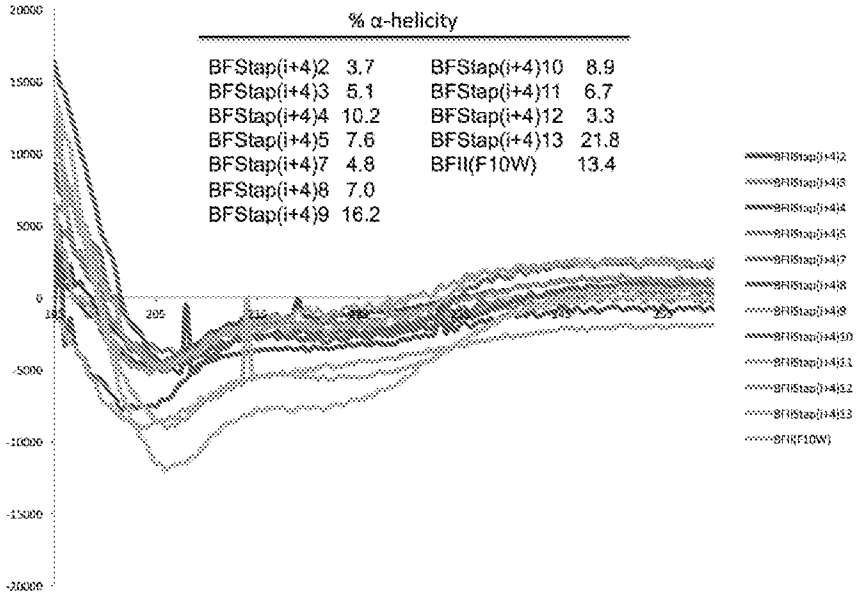
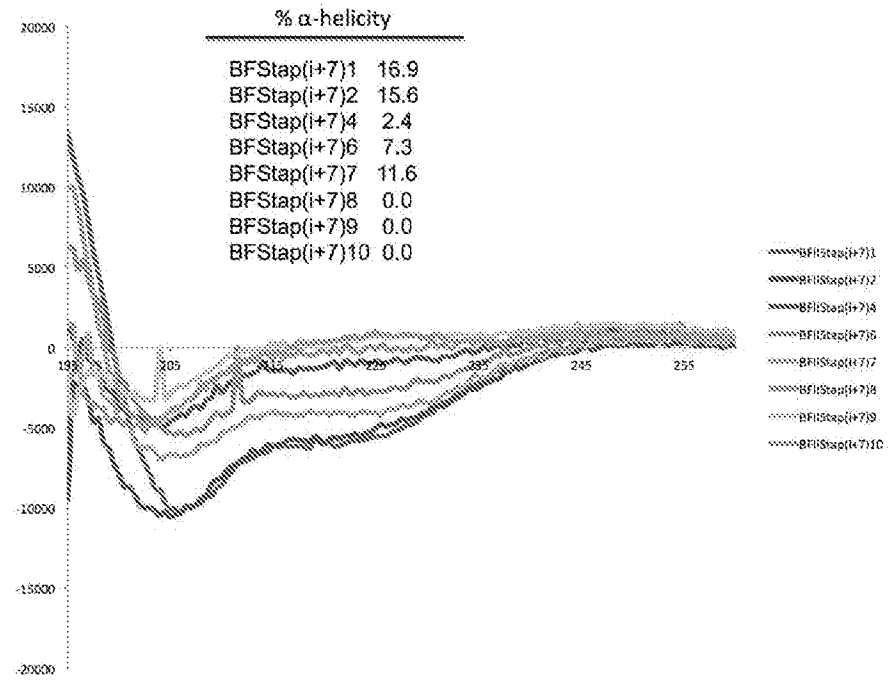

% α-helicity
BFStap(i+4)2 3.7
BFStap(i+4)3 5.1
BFStap(i+4)4 10.2
BFStap(i+4)5 7.6
BFStap(i+4)7 4.8
BFStap(i+4)8 7.0
BFStap(i+4)9 16.2
BFStap(i+4)10 8.9
BFStap(i+4)11 6.7
BFStap(i+4)12 3.3
BFStap(i+4)13 21.8
BFII(F10W) 13.4

FIG. 2

A


% α-helicity
BFStap(i+7)1 16.9
BFStap(i+7)2 15.6
BFStap(i+7)4 2.4
BFStap(i+7)6 7.3
BFStap(i+7)7 11.6
BFStap(i+7)8 0.0
BFStap(i+7)9 0.0
BFStap(i+7)10 0.0

B

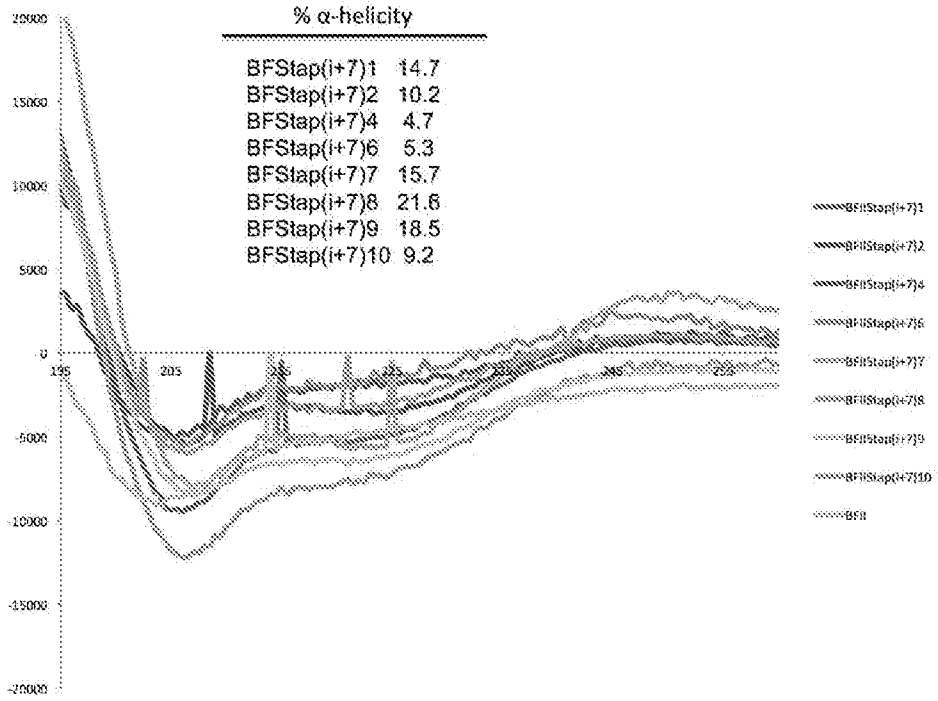

% α-helicity
BFStap(i+7)1 14.7
BFStap(i+7)2 10.2
BFStap(i+7)4 4.7
BFStap(i+7)6 5.3
BFStap(i+7)7 15.7
BFStap(i+7)8 21.6
BFStap(i+7)9 18.5
BFStap(i+7)10 9.2

FIG. 3

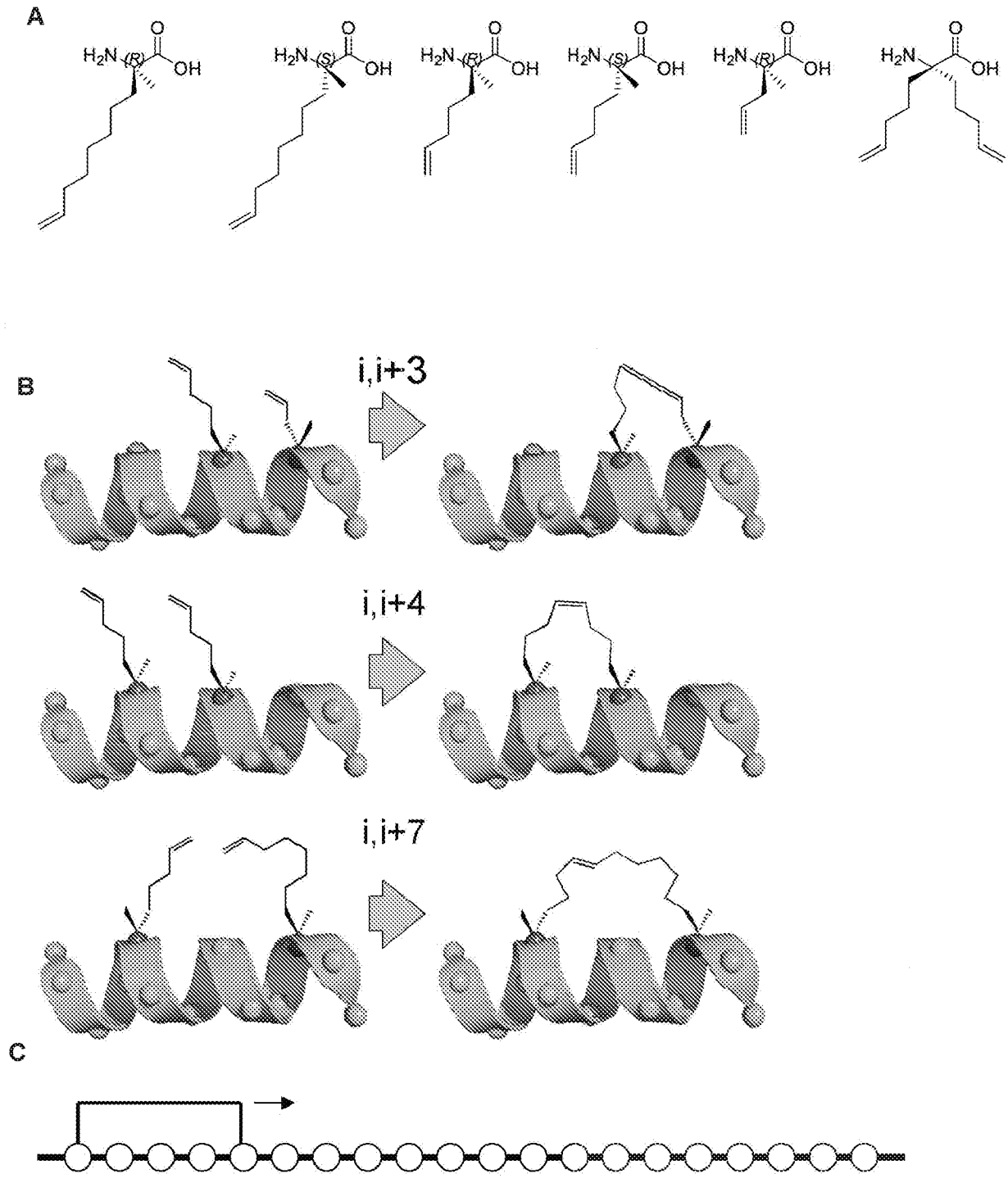


FIG. 4

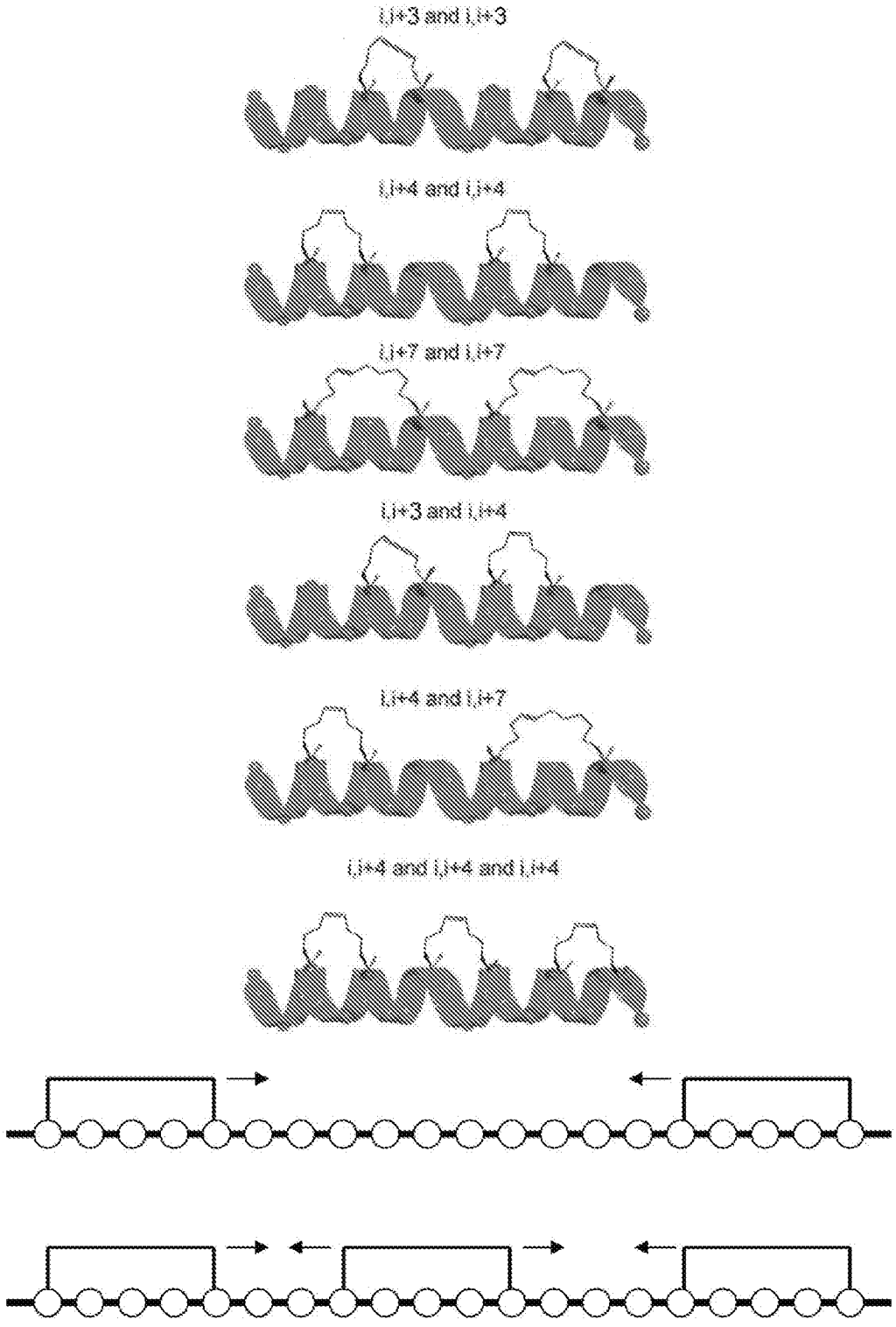

i,i+3 and i,i+3
i,i+4 and i,i+4
i,i+7 and i,i+7
i,i+3 and i,i+4
i,i+4 and i,i+7
i,i+4 and i,i+4 and i,i+4

FIG. 5

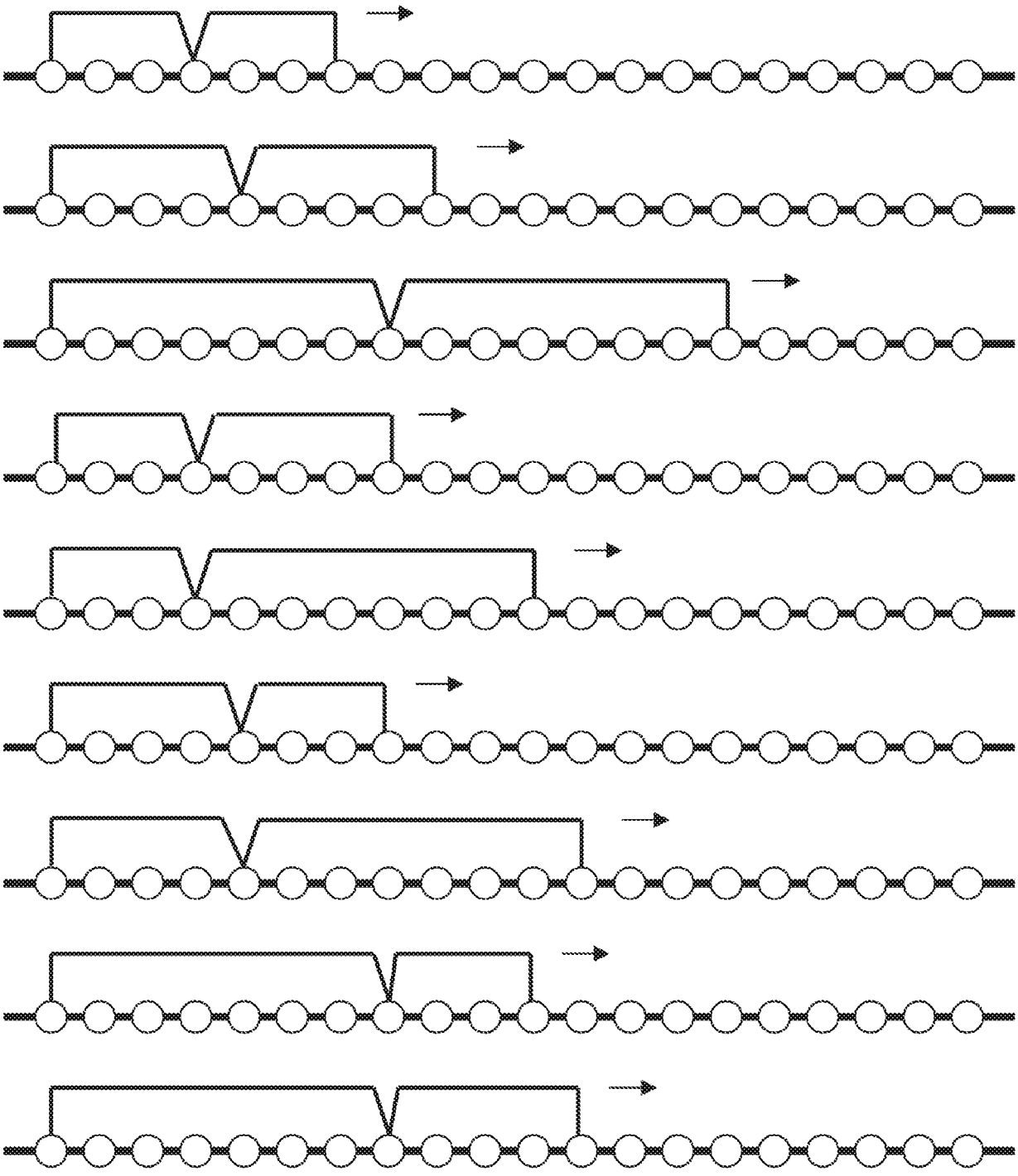


FIG. 6

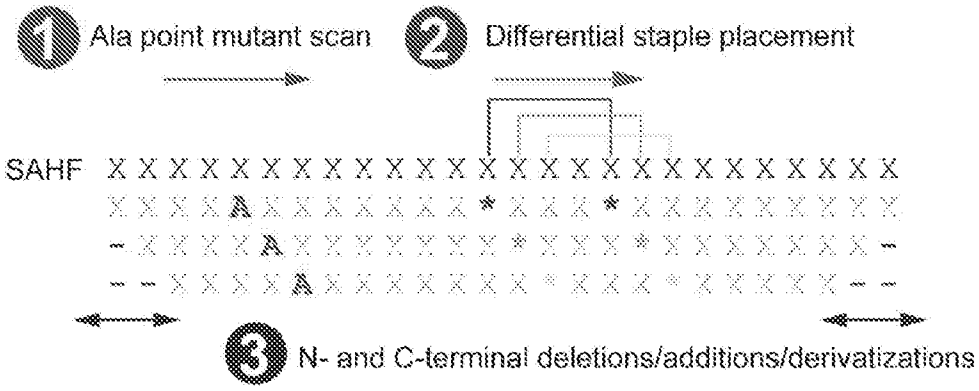

1 Ala point mutant scan
2 Differential staple placement
SAHF
3 N- and C-terminal deletions/additions/derivatizations

FIG. 7

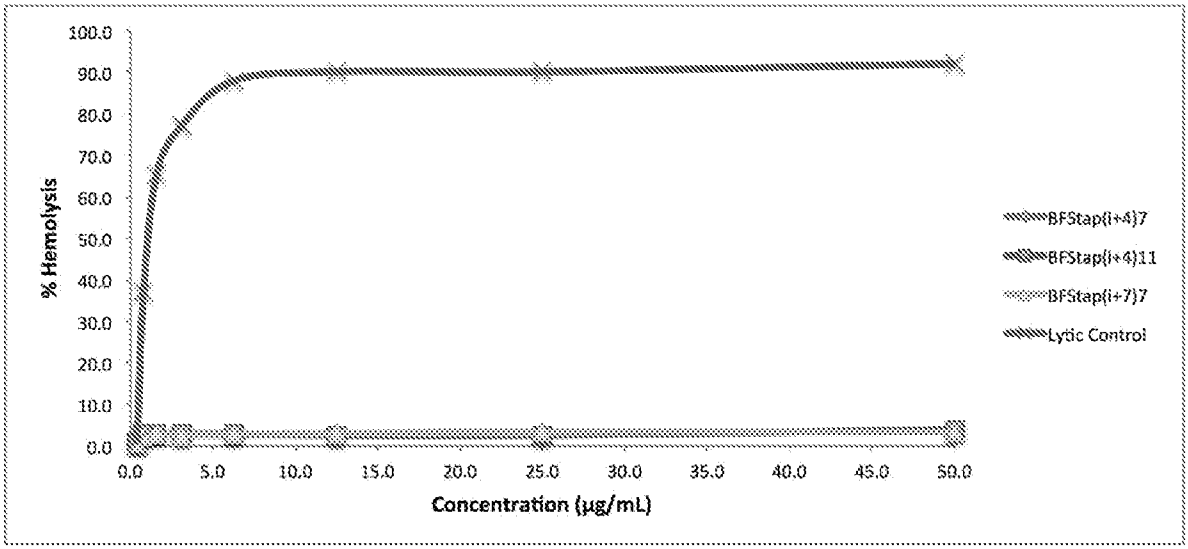

100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
% Hemolysis
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0
45.0
50.0
Concentration (µg/mL)
BFStap(i+4)7
BFStap(i+4)11
BFStap(i+7)7
Lytic Control

FIG. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US17/19953

A. CLASSIFICATION OF SUBJECT MATTER
IPC - A61K 38/08, 38/12; C07K 5/12, 14/00, 14/47 (2017.01)
CPC - A61K 38/08, 38/12; C07K 1/113, 1/1136, 7/08, 7/64, 14/001, 14/4747, 14/435

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2005/0250680 A1 (WALENSKY, LD et al.) November 10, 2005; paragraphs [0009]-[0012], [0018]-[0021], [0026], [0043], [0044], [0128], [0131], [0179], [0182], [0250], [0251]; claims 9, 17	1-4, 8-16, 19-36
Y	US 2014/0155319 A1 (MOREHOUSE SCHOOL OF MEDICINE) June 5, 2014; abstract; paragraphs [0039], [0064], [0094]; claims 1, 32, 33	1-4, 8-16, 19-36
Y	US 2003/0096949 A1 (HANCOCK, REW et al.) May 22, 2003; paragraph [0037]; claim 17	12-16
Y	WO 2015/138494 A1 (BELFORT, G et al.) September 17, 2015; abstract	24
Y	US 2015/0087512 A1 (UNIVERSITY OF FLORIDA RESEARCH FOUNDATION, INCORPORATED) March 26, 2015; abstract	29
Y	CA 2367149 C (SHAPIRO, HM et al.) September 20, 2011; page 2, lines 28-32; claim 9	33
Y	US 2006/0287232 A1 (CLAYBERGER, C et al.) December 21, 2006; abstract; claim 20	19-20
A	US 2015/0087579 A1 (ROBERT BOSCH GENSELLSCHAFT FÜR MEDIZINISCHE FORSCHUNG MBH) March 26, 2015; abstract	5-7

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 June 2017 (22.06.2017)	04 AUG 2017
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US17/19953

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
-***-Please See Supplemental Page-***-

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-16, 19-36 ; SEQ ID NO: 1

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US17/19953

-***-Continued from Box No. III Observations where unity of invention is lacking: -***-

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Groups I+, Claims 1-36; and SEQ ID NO: 1 are directed toward a pharmaceutical composition comprising a compound having Formula (I), wherein the compound exhibits an intracellular antimicrobial effect against at least one microbe.

The compound, composition and method will be searched to the extent they encompass a peptide encompassing SEQ ID NO: 1 (first exemplary peptide sequence). Applicant is invited to elect additional peptide(s), with specified SEQ ID NO: for each, to be searched. Additional peptide sequence(s) can be searched upon the payment of additional fees. It is believed that claims 1-11, 12 (in-part), 13 (in-part), 14 (in-part), 15 (in-part), 16 (in-part) and 19-36 encompass this first named invention and thus these claims will be searched without fee to the extent that they encompass SEQ ID NO: 1 (peptide sequence). Applicants must specify the claims that encompass any additionally elected peptide sequence(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be a peptide encompassing SEQ ID NO: 2 (first exemplary elected peptide sequence).

No technical features are shared between the peptide sequences of Groups I+ and, accordingly, these groups lack unity a priori.

Groups I+ share the technical features including: a pharmaceutical composition comprising a compound having Formula (I), or a pharmaceutically acceptable salt thereof, wherein; each R1 and R2 is independently H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heteroarylalkyl, or heterocyclylalkyl, any of which is substituted or unsubstituted; each R3 is independently alkylene, alkenylene, or alkynylene, any of which is substituted or unsubstituted; each x is independently 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10; each w and y is independently 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20: z is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10; and each Xaa is independently an amino acid, wherein the compound exhibits an intracellular antimicrobial effect against at least one microbe; and a method of treating an infection caused by a microbe, the method comprising administering a therapeutically-effective amount of said compound to a subject having, or at risk of having, the infection caused by the microbe.

However, these shared technical features are previously disclosed by the article 'Comparative antimicrobial activity and mechanism of action of bovine lactoferricin-derived synthetic peptides' by Liu et al. (hereinafter 'Liu') in view of US 2005/0250680 A1 to Walensky et al. (hereinafter 'Walensky').

Liu discloses a cyclic peptide compound (bovine lactoferricin (a cyclic peptide compound); page 1069, second column), wherein the compound exhibits an intracellular antimicrobial effect (wherein the compound exhibits an effect on bacterial protein synthesis (intracellular antimicrobial effect); abstract, page 1076, second column, second paragraph) against at least one microbe (against E. coli and S. aureus (at least one microbe); abstract, page 1076, second column, second paragraph); and administering said compound to a subject (performing in vivo trials with LfcinB (administering said compound to a subject); page 1077, first column, second paragraph).

Liu does not disclose a pharmaceutical composition; a compound having Formula (I), or a pharmaceutically acceptable salt thereof, wherein; each R1 and R2 is independently H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heteroarylalkyl, or heterocyclylalkyl, any of which is substituted or unsubstituted; each R3 is independently alkylene, alkenylene, or alkynylene, any of which is substituted or unsubstituted; each x is independently 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10; each w and y is independently 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20: z is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10; and each Xaa is independently an amino acid; and a method of treating an infection caused by a microbe, the method comprising administering a therapeutically-effective amount of said compound to a subject having, or at risk of having, the infection caused by the microbe.

Walensky discloses a pharmaceutical composition (a pharmaceutical composition; paragraphs [0250], [0251]) comprising a compound having Formula (I) (comprising a compound having Formula (I); paragraph [0009]): wherein; each R1 and R2 is independently H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heteroarylalkyl, or heterocyclylalkyl, (wherein; each R1 and R2 is independently H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heteroarylalkyl, or heterocyclylalky; paragraph [0011]); each R3 is independently alkylene, alkenylene, or alkynylene (R3 is independently alkylene, alkenylene, or alkynylene; paragraph [0012]); x is 2, 3, 4, 5, 6, 7, 8, 9, or 10 (x is 2, 3, 4, 5, 6, 7, 8, 9, or 10; paragraph [0018]); each w and y is independently 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 (each w and y is independently 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20; paragraph [0019]): z is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 (z is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10; paragraph [0020]); and each Xaa is independently an amino acid (each Xaa is independently an amino acid; paragraph [0021]).

It would have been obvious to a person of ordinary skill in the art at the time of the invention was made to have modified the disclosure of Liu to have provided a method of treating an infection caused by a microbe, the method comprising administering a therapeutically-effective amount of said compound to a subject having, or at risk of having, the infection caused by the microbe, such as S. aureus, on the basis of the antimicrobial activity of said compound, in order to provide an effective method of treatment for an infection by a gram positive microbe, such as multi-drug resistant S. aureus. It further would have been obvious to a person of ordinary skill in the art at the time of the invention was made to have modified the disclosure of Liu to have formed cyclic lactoferricin-derived peptides, as disclosed by Liu, using the stapling peptide formula and methods of Walensky, in order to enable the production of large amounts of the peptide in the absence of potential agglomeration produced by inter-molecular disulfide bonds, as well as enabling the production of compounds with varying loop sizes and positions in order to enable the identification of more stable and/or active analogs.

Since none of the special technical features of the Groups I+ inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by a combination of the Liu and Walensky references, unity of invention is lacking.