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(54) Title: COMPOSITIONS AND METHODS FOR ENHANCING CELLULAR TRANSPORT OF BIOMOLECULES

(57) Abstract: The present invention discloses compositions and methods for delivery of biomolecules into cells. Compositions comprise peptidomimetic macrocycles complexed or conjugated to biomolecules such as nucleic acids.

COMPOSITIONS AND METHODS FOR ENHANCING CELLULAR TRANSPORT OF BIOMOLECULES

CROSS-REFERENCE

[0001] This application claims the benefit of U.S. Provisional Application No. 61/130,934, filed June 3, 2008, which application is incorporated herein by reference.

BACKGROUND OF THE INVENTION

[0002] Interaction with intracellular components of a cell, whether pursued for research or therapeutic purposes, requires that the cellular membrane is crossed by an agent that is expected to interact with such intracellular components. However, such agents often lack the necessary balance of biological and physicochemical properties such as hydrophobicity, solubility, charge and size to cross the cell membrane. For example, highly charged molecules such as nucleic acids experience particular difficulty in passing across such membranes. In therapeutic applications, biomolecules such as polypeptides and nucleic acids show limited bioavailability due at least in part to inability to penetrate cellular membranes.

[0003] In particular, RNAi is a process whereby double-stranded RNA (dsRNA) induces the sequence-specific degradation of homologous mRNA in animals and plant cells (Hutvagner and Zamore (2002), Curr. Opin. Genet. Dev., 12, 225-232; Sharp (2001), Genes Dev., 15, 485-490). In mammalian cells, RNAi can be triggered by 21-nucleotide (nt) duplexes of small interfering RNA (siRNA) (Chiu et al. (2002), MoI. Cell., 10, 549-561; Elbashir et al. (2001), Nature, 411, 494-498), or by micro-RNAs (miRNA), functional small-hairpin RNA (shRNA), or other dsRNAs that are expressed in vivo using engineered RNA precursors such as DNA templates, e.g., with RNA polymerase III promoters (Zeng et al. (2002), MoI. Cell, 9, 1327-1333; Paddison et al. (2002), Genes Dev., 16, 948-958; Lee et al. (2002), Nature Biotechnol., 20, 500-505; Paul et al. (2002), Nature Biotechnol., 20, 505-508; Tuschl, T. (2002), Nature Biotechnol., 20, 440-448; Yu et al. (2002), Proc. Natl. Acad. Sci. USA, 99(9), 6047-6052; McManus et al. (2002), RNA, 8, 842-850; Sui et al. (2002), Proc. Natl. Acad. Sci. USA, 99(6), 5515-5520.) While RNAi has proven to be a remarkably efficient method of modulating gene expression *in vitro*, its therapeutic applications have been impeded by the difficulty of introducing dsRNA molecules into cells.

[0004] Therefore, there remains a need for methods of transporting biomolecules into cells efficiently and reliably. The present invention addresses this and other needs.

SUMMARY OF THE INVENTION

[0005] In one aspect, the present invention provides a method of modulating expression of a gene in a cell comprising contacting said cell with a peptidomimetic macrocycle and a nucleic acid. In one embodiment, the peptidomimetic macrocycle is capable of transporting the nucleic acid into the cell. The nucleic acid may be, for example, double-stranded or single-stranded, and may be RNA, DNA or a mixed RNA/DNA sequence. In one embodiment, a strand of the nucleic acid is between 19 and 23 nucleotides long. A strand of the nucleic acid may be complementary to a fragment of said gene or to a product of said gene. Alternatively, a strand of the nucleic acid is identical in sequence to a fragment of said gene or to a product of said gene.

[0006] In one embodiment, the peptidomimetic macrocycle forms a non-covalent complex with the nucleic acid. In another embodiment, the peptidomimetic macrocycle is conjugated to the nucleic acid. For example, the nucleic acid may be conjugated to an N-terminus or a C-terminus of the peptidomimetic macrocycle, or may be

conjugated to an internal amino acid of the peptidomimetic macrocycle. The peptidomimetic macrocycle may be cell-permeable.

[0007] In some embodiments, the peptidomimetic macrocycle comprises a crosslinker connecting a first amino acid to a second amino acid. The nucleic acid may be conjugated to the crosslinker. In some embodiments, the first amino acid and the second amino acid are separated by three amino acids. The crosslinker may comprise between 6 and 14 consecutive bonds, or between 8 and 12 consecutive bonds. The macrocycle may comprise a ring of about 18 atoms to 26 atoms. In other embodiments, the first amino acid and the second amino acid are separated by six amino acids. The crosslinker may comprise between 8 and 16 consecutive bonds, or between 10 and 13 consecutive bonds. The macrocycle comprises a ring of about 29 atoms to 37 atoms.

[0008] In yet other embodiments, the peptidomimetic macrocycle comprises an alpha helix. For example, the crosslinker spans 1, 2, 3, 4 or 5 turns of the α-helix. The length of the crosslinker may be about 5 A to about 9 A per turn of the α-helix.

[0009] The peptidomimetic macrocycle may carry a net neutral charge at pH 7.4, for example a net charge of 0. In other embodiments the peptidomimetic macrocycle may carry a net positive charge at pH 7.4, for example at least a net +1, +2, +3 or +4 charge. An alpha position of the first and/or second amino acid may be additionally substituted.

[0010] The present invention also provides a composition comprising a peptidomimetic macrocycle conjugated to a biomolecule. The biomolecule may be, for example, a nucleic acid, a polypeptide, an antibody, an imaging agent, a fluorescent dye or a quantum dot. The biomolecule may be conjugated to an N-terminus, C-terminus or an internal amino aid of the peptidomimetic macroycle. The biomolecule may also be conjugated to the crosslinker of the peptidomimetic macrocycle.

[0011] In another aspect, the invention relates to a method of introducing a biomolecule into a cell comprising contacting said cell with a conjugate comprising a peptidomimetic macrocycle and the biomolecule. For example, the cell is a cancer cell and/or a mammalian cell.

INCORPORATION BY REFERENCE

[0012] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0014] **FIGURE 1** shows exemplary modes of conjugating peptidomimetic macrocycles to biomolecules such as oligonucleotides.

DETAILED DESCRIPTION OF THE INVENTION

[0015] The present invention provides compositions and methods for enhancing cellular transport of biomolecules.

Definitions

[0016] The term "biological membrane" or "membrane" refers to a lipid-containing barrier which separates cells or groups of cells from extracellular space. Biological membranes include, but are not limited to, plasma membranes, cell walls, intracellular organelle membranes, such as the mitochondrial membrane, nuclear membranes, and the like.

[0017] The term "biomolecule" refers to any moiety, regardless of size, which may be conjugated to the peptidomimetic macrocycles of the invention.

[0018] The term "gene" encompasses a DNA sequence encoding a gene product or a fragment of such a DNA sequence.

[0019] A "RNAi target gene" is a gene whose expression is to be selectively inhibited or "silenced." This silencing is achieved by cleaving the mRNA of the target gene by an siRNA, e.g., an isolated siRNA or one that is created from an engineered RNA precursor. One portion or segment of a duplex stem of the siRNA RNA precursor, or one strand of the siRNA, is an anti-sense strand that is complementary, e.g., fully complementary, to a section, e.g., about 16 to about 40 or more nucleotides, of the mRNA of the target gene.

[0020] The germ "gene product" encompasses any nucleic acid sequence derived from a gene, such as a mRNA or any other regulatory sequence. Gene products include partial nucleic acid sequences, and encompass sequences that have been processed or modified by any post-transcriptional or regulatory mechanism.

[0021] The term "nucleic acid" as used herein encompasses any molecule capable of hybridizing with at least some base specificity to a DNA or RNA strand. Thus, nucleic acids include DNA, RNA, mixed DNA/RNA sequences and any analogs thereof. Nucleic acid analogs incorporating backbone and/or base modifications are specifically included in this definition. For example, peptide nucleic acids (PNA), locked nucleic acids (LNA), threose nucleic acids (TNA), expanded base DNA (xDNA or yDNA), are considered to be within the scope of the invention. Similarly, phosphorothioate or phosphonate backbone-modified nucleic acids are also encompassed.

[0022] As used herein, the term "macrocycle" refers to a molecule having a chemical structure including a ring or cycle formed by at least 9 covalently bonded atoms.

[0023] As used herein, the term "peptidomimetic macrocycle" or "crosslinked polypeptide" refers to a compound comprising a plurality of amino acid residues joined by a plurality of peptide bonds and at least one macrocycle-forming linker which forms a macrocycle between a first naturally-occurring or non-naturally-occurring amino acid residue (or analog) and a second naturally-occurring or non-naturally-occurring amino acid residue (or analog) within the same molecule. Peptidomimetic macrocycle include embodiments where the macrocycle-forming linker connects the α carbon of the first amino acid residue (or analog) to the α carbon of the second amino acid residue (or analog). The peptidomimetic macrocycles optionally include one or more non-peptide bonds between one or more amino acid residues and/or amino acid analog residues, and optionally include one or more non-naturally-occurring amino acid residues or amino acid analog residues in addition to any which form the macrocycle.

[0024] As used herein, the term "stability" refers to the maintenance of a defined secondary structure in solution by a peptidomimetic macrocycle of the invention as measured by circular dichroism, NMR or another biophysical measure, or resistance to proteolytic degradation *in vitro* or *in vivo*. Non-limiting examples of secondary structures contemplated in this invention are α-helices, β-turns, and β-pleated sheets.

[0025] As used herein, the term "helical stability" refers to the maintenance of α-helical structure by a peptidomimetic macrocycle of the invention as measured by circular dichroism or NMR. For example, in some embodiments, the peptidomimetic macrocycles of the invention exhibit 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 macrocycle lacking the R-substituent.

[0026] The term "α-amino acid" or simply "amino acid" refers to a molecule containing both an amino group and a carboxyl group bound to a carbon which is designated the α-carbon. Suitable amino acids include, without limitation, both the D-and L-isomers of the naturally-occurring amino acids, as well as non-naturally occurring amino acids prepared by organic synthesis or other metabolic routes. Unless the context specifically indicates otherwise, the term amino acid, as used herein, is intended to include amino acid analogs.

[0027] The term "naturally occurring amino acid" refers to any one of the twenty amino acids commonly found in peptides synthesized in nature, and known by the one letter abbreviations A, R, N, C, D, Q, E, G, H, I, L, K, M, F, P, S, T, W, Y and V.

[0028] The term "amino acid analog" or "non-natural amino acid" refers to a molecule which is structurally similar to an amino acid and which can be substituted for an amino acid in the formation of a peptidomimetic macrocycle. Amino acid analogs include, without limitation, compounds which are structurally identical to an amino acid, as defined herein, except for the inclusion of one or more additional methylene groups between the amino and carboxyl group (e.g., α-amino β-carboxy acids), or for the substitution of the amino or carboxy group by a similarly reactive group (e.g., substitution of the primary amine with a secondary or tertiary amine, or substitution or the carboxy group with an ester).

[0029] A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequence of a polypeptide (e.g., a BH3 domain or the p53 MDM2 binding domain) without abolishing or substantially altering its essential biological or biochemical activity (e.g., receptor binding or activation). An "essential" amino acid residue is a residue that, when altered from the wild-type sequence of the polypeptide, results in abolishing or substantially abolishing the polypeptide's essential biological or biochemical activity.

[0030] A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an 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., K, R, H), acidic side chains (e.g., D, E), uncharged polar side chains (e.g., G, N, Q, S, T, Y, C), nonpolar side chains (e.g., A, V, L, I, P, F, M, W), beta-branched side chains (e.g., T, V, I) and aromatic side chains (e.g., Y, F, W, H). Thus, a predicted nonessential amino acid residue in a BH3 polypeptide, for example, is preferably replaced with another amino acid residue from the same side chain family. Other examples of acceptable substitutions are substitutions based on isosteric considerations (e.g. norleucine for methionine) or other properties (e.g. 2-thienylalanine for phenylalanine).

[0031] The term "member" as used herein in conjunction with macrocycles or macrocycle-forming linkers refers to the atoms that form or can form the macrocycle, and excludes substituent or side chain atoms. By analogy,

cyclodecane, 1,2-difluorodecane and 1,3-dimethyl cyclodecane are all considered ten-membered macrocycles as the hydrogen or fluoro substituents or methyl side chains do not participate in forming the macrocycle.

[0032] The symbol "  " when used as part of a molecular structure refers to a single bond or a *trans* or *cis* double bond.

[0033] The term "amino acid side chain" refers to a moiety attached to the α-carbon in an amino acid. For example, the amino acid side chain for alanine is methyl, the amino acid side chain for phenylalanine is phenylmethyl, the amino acid side chain for cysteine is thiomethyl, the amino acid side chain for aspartate is carboxymethyl, the amino acid side chain for tyrosine is 4-hydroxyphenylmethyl, etc. Other non-naturally occurring amino acid side chains are also included, for example, those that occur in nature (e.g., an amino acid metabolite) or those that are made synthetically (e.g., an α,α di-substituted amino acid).

[0034] The term "α,α di-substituted amino" acid refers to a molecule or moiety containing both an amino group and a carboxyl group bound to a carbon (the α-carbon) that is attached to two natural or non-natural amino acid side chains.

[0035] The term "polypeptide" encompasses two or more naturally or non-naturally-occurring amino acids joined by a covalent bond (e.g., an amide bond). Polypeptides as described herein include full length proteins (e.g., fully processed proteins) as well as shorter amino acid sequences (e.g., fragments of naturally-occurring proteins or synthetic polypeptide fragments).

[0036] The term "macrocyclization reagent" or "macrocycle-forming reagent" as used herein refers to any reagent which may be used to prepare a peptidomimetic macrocycle of the invention by mediating the reaction between two reactive groups. Reactive groups may be, for example, an azide and alkyne, in which case macrocyclization reagents include, without limitation, Cu reagents such as reagents which provide a reactive Cu(I) species, such as CuBr, CuI or CuOTf, as well as Cu(II) salts such as $Cu(CO_2CH_3)_2$, $CuSO_4$, and $CuCl_2$ that can be converted in situ to an active Cu(I) reagent by the addition of a reducing agent such as ascorbic acid or sodium ascorbate. Macrocyclization reagents may additionally include, for example, Ru reagents known in the art such as $Cp^*RuCl(PPh_3)_2$, $[Cp^*RuCl]_4$ or other Ru reagents which may provide a reactive Ru(II) species. In other cases, the reactive groups are terminal olefins. In such embodiments, the macrocyclization reagents or macrocycle-forming reagents are metathesis catalysts including, but not limited to, stabilized, late transition metal carbene complex catalysts such as Group VIII transition metal carbene catalysts. For example, such catalysts are Ru and Os metal centers having a +2 oxidation state, an electron count of 16 and pentacoordinated. Additional catalysts are disclosed in Grubbs et al., "Ring Closing Metathesis and Related Processes in Organic Synthesis" Ace. Chem. Res. 1995, 28, 446-452, and U.S. Pat. No. 5,81 1,515. In yet other cases, the reactive groups are thiol groups. In such embodiments, the macrocyclization reagent is, for example, a linker functionalized with two thiol-reactive groups such as halogen groups.

[0037] The term "halo" or "halogen" refers to fluorine, chlorine, bromine or iodine or a radical thereof.

[0038] The term "alkyl" refers to a hydrocarbon chain that is a straight chain or branched chain, containing the indicated number of carbon atoms. For example, Ci-Cio indicates that the group has 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.

[0039] The term "alkylene" refers to a divalent alkyl *{i.e.,* -R-).

[0040] The term "alkenyl" refers to a hydrocarbon chain that is a straight chain or branched chain having one or more carbon-carbon double bonds. The alkenyl moiety contains the indicated number of carbon atoms. For example, C_2-C_{10} indicates that the group has from 2 to 10 (inclusive) carbon atoms in it. The term "lower alkenyl" refers to a C_2-C_6 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.

[0041] The term "alkynyl" refers to a hydrocarbon chain that is 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 has from 2 to 10 (inclusive) carbon atoms in it. The term "lower alkynyl" refers to a C_2-C_6 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.

[0042] The term "aryl" refers to a 6-carbon monocyclic or 10-carbon bicyclic aromatic ring system wherein 0, 1, 2, 3, or 4 atoms of each ring are 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.

[0043] "Arylalkyl" refers to an aryl group, as defined above, wherein one of the aryl group's hydrogen atoms has been replaced with a C_1-C_5 alkyl group, as defined above. Representative examples of an arylalkyl group include, but are not limited to, 2-methylphenyl, 3-methylphenyl, 4-methylphenyl, 2-ethylphenyl, 3-ethylphenyl, *A*-ethylphenyl, 2-propylphenyl, 3-propylphenyl, 4-propylphenyl, 2-butylphenyl, 3-butylphenyl, 4-butylphenyl, 2-pentylphenyl, 3-pentylphenyl, 4-pentylphenyl, 2-isopropylphenyl, 3-isopropylphenyl, 4-isopropylphenyl, 2-isobutylphenyl, 3-isobutylphenyl, 4-isobutylphenyl, 2-sec-butylphenyl, 3-sec-butylphenyl, 4-sec-butylphenyl, 2-t-butylphenyl, 3-t-butylphenyl and 4-t-butylphenyl.

[0044] "Arylamido" refers to an aryl group, as defined above, wherein one of the aryl group's hydrogen atoms has been replaced with one or more -$C(O)NH_2$ groups. Representative examples of an arylamido group include 2-C(O)NH2-phenyl, 3-$C(O)NH_2$-phenyl, 4-$C(O)NH_2$-phenyl, 2-$C(O)NH_2$-pyridyl, 3-$C(O)NH_2$-pyridyl, and 4-$C(O)NH_2$-pyridyl,

[0045] "Alkylheterocycle" refers to a C_1-C_5 alkyl group, as defined above, wherein one of the C_1-C_5 alkyl group's hydrogen atoms has been replaced with a heterocycle. Representative examples of an alkylheterocycle group include, but are not limited to, -CH_2CH_2-morpholine, -CH_2CH_2-piperidine, -$CH_2CH_2CH_2$-morpholine, and -$CH_2CH_2CH_2$-imidazole.

[0046] "Alkylamido" refers to a C_1-C_5 alkyl group, as defined above, wherein one of the C_1-C_5 alkyl group's hydrogen atoms has been replaced with a -$C(O)NH_2$ group. Representative examples of an alkylamido group include, but are not limited to, -CH_2-$C(O)NH_2$, -CH_2CH_2-$C(O)NH_2$, -$CH_2CH_2CH_2C(O)NH_2$, -$CH_2CH_2CH_2CH_2C(O)NH_2$, -$CH_2CH_2CH_2CH_2CH_2C(O)NH_2$, -$CH_2CH(C(O)NH_2)CH_3$, -$CH_2CH(C(O)NH_2)CH_2CH_3$, -$CH(C(O)NH_2)CH_2CH_3$, -$C(CH_3)_2CH_2C(O)NH_2$, -$CH_2$-$CH_2$-NH-C(O)-$CH_3$, -$CH_2$-$CH_2$-NH-C(O)-$CH_3$-OB, and -$CH_2$-$CH_2$-NH-C(O)-CH=$CH_2$.

[0047] "Alkanol" refers to a C_1-C_5 alkyl group, as defined above, wherein one of the C_1-C_5 alkyl group's hydrogen atoms has been replaced with a hydroxyl group. Representative examples of an alkanol group include, but are not limited to, -CH_2OH, -CH_2CH_2OH, -$CH_2CH_2CH_2OH$, -$CH_2CH_2CH_2CH_2OH$, -$CH_2CH_2CH_2CH_2CH_2OH$, -$CH_2CH(OH)CH_3$, -$CH_2CH(OH)CH_2CH_3$, -$CH(OH)CH_3$ and -$C(CH_3)_2CH_2OH$.

[0048] "Alkylcarboxy" refers to a C_1-C_5 alkyl group, as defined above, wherein one of the C_1-C_5 alkyl group's hydrogen atoms has been replaced with a —COOH group. Representative examples of an alkylcarboxy group include, but are not limited to, -CH_2COOH, -CH_2CH_2COOH, -$CH_2CH_2CH_2$COOH, -$CH_2CH_2CH_2CH_2$COOH, -CH_2CH(COOH)CH_3, -$CH_2CH_2CH_2CH_2CH_2$COOH, -CH_2CH(COOH)CH_2CH_3, -CH(COOH)CH_2CH_3 and -$C(CH_3)_2CH_2$COOH.

[0049] 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 is optionally substituted. Some cycloalkyl groups include, without limitation, cyclopropyl, cyclobutyl, cyclopentyl, cyclopentenyl, cyclohexyl, cyclohexenyl, cycloheptyl, and cyclooctyl.

[0050] 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 O, N, or S if monocyclic, bicyclic, or tricyclic, respectively), wherein 0, 1, 2, 3, or 4 atoms of each ring are substituted by a substituent. Examples of heteroaryl groups include pyridyl, furyl or furanyl, imidazolyl, benzimidazolyl, pyrimidinyl, thiophenyl or thienyl, quinolinyl, indolyl, thiazolyl, and the like.

[0051] 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.

[0052] 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.

[0053] 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 O, N, or S if monocyclic, bicyclic, or tricyclic, respectively), wherein 0, 1, 2 or 3 atoms of each ring are substituted by a substituent. Examples of heterocyclyl groups include piperazinyl, pyrrolidinyl, dioxanyl, morpholinyl, tetrahydrofuranyl, and the like.

[0054] The term "substituent" refers to a group replacing a second atom or group such as a hydrogen atom on any molecule, compound or moiety. Suitable substituents include, without limitation, halo, hydroxy, mercapto, 0x0, nitro, haloalkyl, alkyl, alkaryl, aryl, aralkyl, alkoxy, thioalkoxy, aryloxy, amino, alkoxycarbonyl, amido, carboxy, alkanesulfonyl, alkylcarbonyl, and cyano groups.

[0055] In some embodiments, the compounds of this invention contain one or more asymmetric centers and thus occur as racemates and racemic mixtures, single enantiomers, individual diastereomers and diastereomeric mixtures. All such isomeric forms of these compounds are included in the present invention unless expressly provided otherwise. In some embodiments, the compounds of this invention are also represented in multiple tautomeric forms, in such instances, the invention includes all tautomeric forms of the compounds described herein *(e.g.*, if alkylation of a ring system results in alkylation at multiple sites, the invention includes all such reaction products). All such isomeric forms of such compounds are included in the present invention unless expressly provided otherwise. All crystal forms of the compounds described herein are included in the present invention unless expressly provided otherwise.

[0056] As used herein, the terms "increase" and "decrease" mean, respectively, to cause a statistically significantly *(i.e.,* $p < 0.1$) increase or decrease of at least 5%.

[0057] As used herein, the recitation of a numerical range for a variable is intended to convey that the invention may be practiced with the variable equal to any of the values within that range. Thus, for a variable which is inherently discrete, the variable is equal to any integer value within the numerical range, including the end-points of the range. Similarly, for a variable which is inherently continuous, the variable is equal to any real value within the numerical range, including the end-points of the range. As an example, and without limitation, a variable which is described as having values between 0 and 2 takes the values 0, 1 or 2 if the variable is inherently discrete, and takes the values 0.0, 0.1, 0.01, 0.001, or any other real values ≥ 0 and ≤ 2 if the variable is inherently continuous.

[0058] As used herein, unless specifically indicated otherwise, the word "or" is used in the inclusive sense of "and/or" and not the exclusive sense of "either/or."

[0059] The term "on average" represents the mean value derived from performing at least three independent replicates for each data point.

Compositions of the Invention

[0060] In one aspect of the invention, compositions are provided comprising a peptidomimetic macrocycle and a biomolecule of interest. For example, the association between peptidomimetic macrocycles and the biomolecules of interest may be non-covalent. In such cases, complex formation takes place based on electrostatic or other non-covalent interactions between the peptidomimetic macrocycles and the biomolecules. For example, a complex may be formed between a peptidomimetic macrocycle carrying a net positive charge at about neutral pH (e.g. 7.4) and a nucleic acid.

[0061] In another aspect of the invention, a composition is provided comprising a peptidomimetic macrocycle conjugated to a biomolecule of interest. Typically, the biomolecule of interest will be conjugated to the peptidomimetic macrocycle via a linker. A variety of linkers may be used for this purpose.

[0062] It is understood that the properties of the linker may be selected based on the desired goals. The size, hydrophobicity, conformational rigidity and stability of the linkers are all parameters which may be adjusted. For example, the length of the linker may be adjusted such that a smaller or larger conjugate is generated, thus allowing tuning of the size of the conjugate. In other cases, it may be desirable to enhance the solubility of the linker by including certain groups such as hydrophilic group. In other embodiments, a linker which is labile *in vivo* may be used. Such a linker could comprise, for example, a disulfide bond which is expected to be reduced in an intracellular environment, separating the biomolecule and the peptidomimetic macrocycle. Alternatively, an ester or amide linker may be employed which is potentially cleaved *in vivo* by cellular proteases. Photolabile linkers may be used for this purpose such that the biomolecule is cleaved from the peptidomimetic macrocycle upon exposure to electromagnetic radiation. Additionally, including more rigid groups may be included such as cyclic structures or groups which increase the conformations constraints on the linker (e.g. double or triple bonds, or tertiary or quaternary centers).

[0063] In some embodiments, the linker is an alkyl linker, unsubstituted or substituted with additional substituents. In other embodiments, the linker is a poly(alkyl ether).

[0064] Biomolecules which may be used in the present invention include polypeptides (natural and unnatural), nucleic acids (including RNA, DNA, or other nucleic acid analogs such as PNA, LNA, or TNA); imaging agents such as fluorescent dyes or quantum dots; metal ions, which may be delivered to a cell as chelates; and small organic molecules, such as therapeutic compounds or other compounds that show binding specificity to cellular targets.

[0065] Compositions of the present invention may include nucleic acid molecules. Nucleic acid molecules may be useful therapeutically for disruption of gene expression, for example, by disruption of mRNA transcript or any other mechanism. Nucleic acid molecules may be composed of, for example, nucleotides, nucleosides, synthetic nucleic acids, or a combination of the aforementioned. The nucleic acid molecules may be single stranded, double stranded or triple stranded. Examples of single strand nucleic acid molecules that have biologic activity to mediate alteration of gene expression include antisense nucleic acid molecules, enzymatic nucleic acid molecules, ribozymes, DNAzymes, and 2'-5'-oligoadenylate nucleic acid molecules. Examples of triple strand nucleic acid molecules that have biologic activity to mediate alteration of gene expression include triplex forming oligonucleotides. Examples of double strand nucleic acid molecules that have biologic activity to mediate alteration of gene expression include multifunctional short interfering nucleic acids (multifunctional siNA), double stranded oligonucleotides, such as double stranded RNA (dsRNA), small interfering RNA (siRNA), micro-RNA (miRNA), aptamers, or oligodeoxynucleotides containing CpG motifs.

[0066] Double stranded oligonucleotides are formed by the assembly of two distinct oligonucleotide sequences where the oligonucleotide sequence of one strand is complementary to the oligonucleotide sequence of the second strand; such double stranded oligonucleotides are generally assembled from two separate oligonucleotides (e.g., siRNA), or from a single molecule that folds on itself to form a double stranded structure (e.g., shRNA or short hairpin RNA). These double stranded oligonucleotides known in the art all have a common feature in that each strand of the duplex has a distinct nucleotide sequence, wherein only one nucleotide sequence region (guide sequence or the antisense sequence) has complementarity to a target nucleic acid sequence and the other strand (sense sequence) comprises nucleotide sequence that is homologous to the target nucleic acid sequence.

[0067] Double stranded RNA induced gene silencing can occur on at least three different levels: (i) transcription inactivation, which refers to RNA guided DNA or histone methylation; (ii) siRNA induced mRNA degradation; and (iii) mRNA induced transcriptional attenuation. It is generally considered that the major mechanism of RNA induced silencing (RNA interference, or RNAi) in mammalian cells is mRNA degradation. RNA interference (RNAi) is a mechanism that inhibits gene expression at the stage of translation or by hindering the transcription of specific genes. Specific RNAi pathway proteins are guided by the dsRNA to the targeted messenger RNA (mRNA), where they "cleave" the target, breaking it down into smaller portions that can no longer be translated into protein. Initial attempts to use RNAi in mammalian cells focused on the use of long strands of dsRNA. However, these attempts to induce RNAi met with limited success, due in part to the induction of the interferon response, which results in a general, as opposed to a target-specific, inhibition of protein synthesis. Thus, long dsRNA is not a viable option for RNAi in mammalian systems. Another outcome is epigenetic changes to a gene - histone modification and DNA methylation - affecting the degree the gene is transcribed.

[0068] More recently it has been shown that when short (18-30 bp) RNA duplexes are introduced into mammalian cells in culture, sequence-specific inhibition of target mRNA can be realized without inducing an interferon response. Certain of these short dsRNAs, referred to as small inhibitory RNAs ("siRNAs"), can act

catalytically at sub-molar concentrations to cleave greater than 95% of the target mRNA in the cell. A description of the mechanisms for siRNA activity, as well as some of its applications are described in Provost et al., Ribonuclease Activity and RNA Binding of Recombinant Human Dicer, E.M.B.O. J., 2002 Nov. 1; 21(21): 5864-5874; Tabara et al., The dsRNA Binding Protein RDE-4 Interacts with RDE-I, DCR-I and a DexH-box Helicase to Direct RNAi in C. elegans, Cell 2002, June 28; 109(7): 861-71; Ketting et al., Dicer Functions in RNA Interference and in Synthesis of Small RNA Involved in Developmental Timing in C. elegans; Martinez et al., Single-Stranded Antisense siRNAs Guide Target RNA Cleavage in RNAi, Cell 2002, September. 6; 110(5):563; Hutvagner & Zamore, A microRNA in a multiple-turnover RNAi enzyme complex, Science 2002, 297:2056.

[0069] From a mechanistic perspective, introduction of long double stranded RNA into plants and invertebrate cells is broken down into siRNA by a Type III endonuclease known as Dicer. Sharp, RNA interference—2001, Genes Dev. 2001, 15:485. Dicer, a ribonuclease-III-like enzyme, processes the dsRNA into 19-23 base pair short interfering RNAs with characteristic two base 3' overhangs. Bernstein, Caudy, Hammond, & Hannon, Role for a bidentate ribonuclease in the initiation step of RNA interference, Nature 2001, 409:363. The siRNAs are then incorporated into an RNA-induced silencing complex (RISC) where one or more helicases unwind the siRNA duplex, enabling the complementary antisense strand to guide target recognition. Nykanen, Haley, & Zamore, ATP requirements and small interfering RNA structure in the RNA interference pathway, Cell 2001, 107:309. Upon binding to the appropriate target mRNA, one or more endonucleases within the RISC cleaves the target to induce silencing. Elbashir, Lendeckel, & Tuschl, RNA interference is mediated by 21- and 22-nucleotide RNAs, Genes Dev 2001, 15:188, FIG. 1.

[0070] Generally, the antisense sequence is retained in the active RISC complex and guides the RISC to the target nucleotide sequence by means of complementary base-pairing of the antisense sequence with the target sequence for mediating sequence-specific RNA interference. It is known in the art that in some cell culture systems, certain types of unmodified siRNAs can exhibit "off target" effects. It is hypothesized that this off-target effect involves the participation of the sense sequence instead of the antisense sequence of the siRNA in the RISC complex (see for example Schwarz et al., 2003, Cell, 115, 199-208). In this instance the sense sequence is believed to direct the RISC complex to a sequence (off-target sequence) that is distinct from the intended target sequence, resulting in the inhibition of the off-target sequence In these double stranded nucleic acid molecules, each strand is complementary to a distinct target nucleic acid sequence. However, the off-targets that are affected by these dsRNAs are not entirely predictable and are non-specific.

[0071] The term "siRNA" refers to small inhibitory RNA duplexes that induce the RNA interference (RNAi) pathway. These molecules can vary in length (generally between 18-30 basepairs) and contain varying degrees of complementarity to their target mRNA in the antisense strand. Some, but not all, siRNA have unpaired overhanging bases on the 5' or 3' end of the sense strand and/or the antisense strand. The term "siRNA" includes duplexes of two separate strands, as well as single strands that can form hairpin structures comprising a duplex region. Small interfering RNA (siRNA), sometimes known as short interfering RNA or silencing RNA, are a class of 20-25 nucleotide-long double-stranded RNA molecules that play a variety of roles in biology.

[0072] While the two RNA strands do not need to be completely complementary, the strands should be sufficiently complementary to hybridize to form a duplex structure. In some instances, the complementary RNA strand may be less than 30 nucleotides, preferably less than 25 nucleotides in length, more preferably 19 to 24 nucleotides in length, more preferably 20-23 nucleotides in length, and even more preferably 22 nucleotides in

length. The dsRNA of the present invention may further comprise at least one single-stranded nucleotide overhang. The dsRNA of the present invention may further comprise a substituted or chemically modified nucleotide. As discussed in detail below, the dsRNA can be synthesized by standard methods known in the art.

[0073] SiRNA may be divided into five (5) groups (non-functional, semi-functional, functional, highly functional, and hyper-functional) based on the level or degree of silencing that they induce in cultured cell lines. As used herein, these definitions are based on a set of conditions where the siRNA is transfected into said cell line at a concentration of 100 nM and the level of silencing is tested at a time of roughly 24 hours after transfection, and not exceeding 72 hours after transfection. In this context, "non-functional siRNA" are defined as those siRNA that induce less than 50% (<50%) target silencing. "Semi-functional siRNA" induce 50-79% target silencing. "Functional siRNA" are molecules that induce 80-95% gene silencing. "Highly-functional siRNA" are molecules that induce greater than 95% gene silencing. "Hyperfunctional siRNA" are a special class of molecules. For purposes of this document, hyperfunctional siRNA are defined as those molecules that: (1) induce greater than 95% silencing of a specific target when they are transfected at subnanomolar concentrations (i.e., less than one nanomolar); and/or (2) induce functional (or better) levels of silencing for greater than 96 hours. These relative functionalities (though not intended to be absolutes) may be used to compare siRNAs to a particular target for applications such as functional genomics, target identification and therapeutics.

[0074] microRNAs (miRNA) are single-stranded RNA molecules of about 21-23 nucleotides in length, which regulate gene expression. miRNAs are encoded by genes that are transcribed from DNA but not translated into protein (non-coding RNA); instead they are processed from primary transcripts known as pri-miRNA to short stem-loop structures called pre-miRNA and finally to functional miRNA. Mature miRNA molecules are partially complementary to one or more messenger RNA (mRNA) molecules, and their main function is to downregulate gene expression.

[0075] Antisense therapy is a form of treatment for genetic disorders or infections. When the genetic sequence of a particular gene is known to be causative of a particular disease, it is possible to synthesize a strand of nucleic acid (DNA, RNA or a chemical analogue) that will bind to the messenger RNA (mRNA) produced by that gene and inactivate it, effectively turning that gene "off. This is because mRNA has to be single stranded for it to be translated. Antisense DNA is single stranded DNA that is complementary to a messenger RNA (mRNA) strand. Antisense DNA is believed to cause a reduction in target RNA levels principally through the action of RNase H, an endonuclease that cleaves the RNA strand of DNA:RNA duplexes. Antisense RNA is single-stranded RNA that is complementary to a messenger RNA (mRNA) strand transcribed within a cell. Both antisense DNA and RNA may be introduced into a cell to inhibit translation of a complementary mRNA by base pairing to it and physically obstructing the translation machinery. Antisense mRNA is an mRNA transcript that is complementary to endogenous mRNA. See for example, US Pat No. 6,433,159, hereby incorporated by reference.

[0076] An aptamer, also referred to herein as a nucleic acid ligand, comprises an isolated nucleic acid molecule having specific binding affinity to a molecule through interactions other than classic Watson-Crick base pairing. Nucleic acid aptamers are single-stranded or double-stranded oligonucleotides that bind to a particular ligand with great affinity and selectivity. In the present invention, nucleic acid aptamer regions can range, for example, from about 15 to about 500 nucleotides, from about 15 to about 200 nucleotides, or from about 15 to about 100 nucleotides. A typical aptamer is 10-15 kDa in size (20-45 nucleotides), binds its target with nanomolar to sub-

nanomolar affinity, and discriminates against closely related targets (e.g., aptamers will typically not bind other proteins from the same gene family).

[0077] For an aptamer to be suitable for use in the present invention, the binding affinity of the aptamer for the ligand must be sufficiently strong and the structure formed by the aptamer when bound to its ligand must be significant enough so as to disrupt translation of the attached transcript. The structure of the aptamer in the absence of the ligand, on the other hand, should be minimal. Whether or not an aptamer meets these criteria can be readily determined by one of ordinary skill in the art.

[0078] The aptamers of the present invention can specifically bind almost any molecular or macromolecular entity as a ligand, such as ions, small organic molecules, nucleic acids, proteins, viruses, fungi and bacteria cells. Aptamers are created and selected using a combination of synthetic chemistry, enzymology and affinity chromatography. A series of structural studies have shown that aptamers are capable of using the same types of binding interactions (e.g., hydrogen bonding, electrostatic complementarities, hydrophobic contacts, steric exclusion) that drive affinity and specificity in antibody-antigen complexes. Aptamers have a number of desirable characteristics for use as therapeutics and diagnostics including high specificity and affinity, biological efficacy, and excellent pharmacokinetic properties. In addition, aptamers are produced by an entirely in vitro process, allowing for the rapid generation of therapeutic candidates. Aptamers as a class have demonstrated therapeutically acceptable toxicity and lack of immunogenicity. It is difficult to elicit antibodies to aptamers most likely because aptamers cannot be presented by T-cells via the MHC and the immune response is generally trained not to recognize nucleic acid fragments. Therapeutic aptamers are chemically robust. They are intrinsically adapted to regain activity following exposure to factors such as heat and denaturants and can be stored for extended periods (>1 yr) at room temperature as lyophilized powders. See, for example, US Pat. App No. 2007/0066551, hereby incorporated by reference.

[0079] Methods of making aptamers are described in, for example, Ellington and Szostak, Nature 346:818 (1990), Tuerk and Gold, Science 249:505 (1990), U.S. Pat. No. 5,582,981, PCT Publication No. WO 00/20040, U.S. Pat. No. 5,270,163, Lorsch and Szostak, Biochemistry, 33:973 (1994), Mannironi et al., Biochemistry 36:9726 (1997), Blind, Proc. Natl Acad. Sci. USA 96:3606-3610 (1999), Huizenga and Szostak, Biochemistry, 34:656-665 (1995), PCT Publication Nos. WO 99/54506, WO 99/27133, WO 97/42317 and U.S. Pat. No. 5,756,291.

[0080] Generally, in their most basic form, in vitro selection techniques for identifying RNA aptamers involve first preparing a large pool of DNA molecules of the desired length that contain at least some region that is randomized or mutagenized. For instance, a common oligonucleotide pool for aptamer selection might contain a region of 20-100 randomized nucleotides flanked on both ends by an about 15-25 nucleotide long region of defined sequence useful for the binding of PCR primers. The oligonucleotide pool is amplified using standard PCR techniques. The DNA pool is then transcribed in vitro. The RNA transcripts are then subjected to affinity chromatography. The transcripts are most typically passed through a column or contacted with magnetic beads or the like on which the target ligand has been immobilized. RNA molecules in the pool which bind to the ligand are retained on the column or bead, while nonbinding sequences are washed away. The RNA molecules which bind the ligand are then reverse transcribed and amplified again by PCR (usually after elution). The selected pool sequences are then put through another round of the same type of selection. Typically, the pool sequences are put through a total of about three to ten iterative rounds of the selection procedure. The cDNA is then amplified,

cloned, and sequenced using standard procedures to identify the sequence of the RNA molecules which are capable of acting as aptamers for the target ligand.

[0081] A ribozyme (from ribonucleic acid enzyme, also called RNA enzyme or catalytic RNA) is an RNA molecule that catalyzes a chemical reaction. RNA-based enzymes (ribozymes) exist in nature, and for the most part they exhibit RNA-cleaving activity (Zhen, B. et al., Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai), 2002, 34(5):635-642). DNA-based enzymes (DNAzymes) that cleave RNA or DNA at specific sequences have also been isolated through selection and amplification. DNAzyme activities in addition to RNA and DNA cleavage include DNA ligation (Soukup, G. A. and Breaker, R. R., Trends Biotechnol., 1999, 17(12):469-476), DNA capping (Hamaguchi, N. et al., Anal. Biochem., 2001, 294(2):126-131), phosphorylation (Soukup, G. A. and Breaker, R. R., Trends Biotechnol., 1999, 17(12):469-476), acyl coenzyme A-transferase activity (Doudna, J. A. and Cech, T. R., Nature, 2002, 418(6894):222-228) and peroxidase activity (Li, Y. and Breaker, R. R., Curr. Opin. Struct. Biol., 1999, 9(3):315-323). Thus, DNAzymes and ribozymes can catalyze several different reactions and they can act as RNA and DNA endonucleases (DNases), kinases, ligases, capping enzymes, promoters of amino acid activation, acyl transfer and the Diels-Alder reaction. Many natural ribozymes catalyze either the hydrolysis of one of their own phosphodiester bonds, or the hydrolysis of bonds in other RNAs, but they have also been found to catalyze the aminotransferase activity of the ribosome.

[0082] Oligodeoxynucleotides containing CpG motifs (CpG ODNs) display a strong immunostimulating activity and drive the immune response toward the ThI (T helper type 1) phenotype. These ODNs have shown promising efficacy in preclinical studies when injected locally in several cancer models. (Carpentier et al. (2006) Neuro Oncol 8(l):60-66).

[0083] Nucleic acid molecules of the present invention may include various substitutions for standard nucleotides. For example, studies have shown that replacing the 3'-terminal nucleotide overhanging segments of a 21-mer siRNA duplex having two-nucleotide 3'-overhangs with deoxyribonucleotides does not have an adverse effect on RNAi activity. Replacing up to four nucleotides on each end of the siRNA with deoxyribonucleotides has been reported to be well tolerated, whereas complete substitution with deoxyribonucleotides results in no RNAi activity (Elbashir et al., 2001, EMBO J., 20, 6877 and Tuschl et al., International PCT Publication No. WO 01/75164). Some examples of some substitutions in the nucleic acid molecules include the use of phosphorothioates, phosphotriesters, methyl phosphonates, chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. Additional examples may be seen, for example, in US Pat No. 6,433,159, hereby incorporated by reference..

[0084] In one embodiment, the biomolecule is an siRNA which is a double-stranded RNA ("dsRNA") molecule. The nucleic acid molecules or constructs of the invention include dsRNA molecules comprising 16-30, e.g., 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in each strand, wherein one of the strands is substantially complementary to, e.g., at least 80% (or more, e.g., 85%, 90%, 95%, or 100%) (for example, having 3, 2, 1, or 0 mismatched nucleotide(s)), to a target region. In this context, it is understood that "double-stranded" includes molecules that have short overhangs or imperfect complementarity. Additionally, siRNA molecules include labeled and/or modified nucleic acid sequences. Any siRNA base or backbone modifications known are encompassed herein.

[0085] In some embodiments, a conjugate of a peptidomimetic macrocycle and a biomolecule has enhanced cell permeability compared to a conjugate of a corresponding non-macrocyclic polypeptide and the biomolecule. The

corresponding non-macrocyclic polypeptide may be, for example, the corresponding natural sequence from which the peptidomimetic macrocycle is derived or may be a peptidomimetic precursor. In other embodiments, endosomal release of a conjugate of a biomolecule and a peptidomimetic macrocycle of the invention is enhanced compared to a conjugate of a corresponding non-macrocyclic polypeptide and the biomolecule.

Methods of Preparing Compositions of the Invention

[0086] Biomolecules of the invention may be prepared as needed based on known methods. For example, the synthesis and purification of nucleic acids may be performed as described in a number of sources. These techniques are well known and are explained in, for example, Current Protocols in Molecular Biology, Volumes I, II, and III, 1997 (F. M. Ausubel ed.); Sambrook et al., 2001, Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; Berger and Kimmel, Guide to Molecular Cloning Techniques Methods in Enzymology volume 152 Academic Press, Inc., San Diego, Calif. (Berger), DNA Cloning: A Practical Approach, Volumes I and II, 1985 (D. N. Glover ed.); Oligonucleotide Synthesis, 1984 (M. L. Gait ed.); Nucleic Acid Hybridization, 1985, (Hames and Higgins); Transcription and Translation, 1984 (Hames and Higgins eds.); Animal Cell Culture, 1986 (R. I. Freshney ed.); Immobilized Cells and Enzymes, 1986 (IRL Press); Perbal, 1984, A Practical Guide to Molecular Cloning; the series, Methods in Enzymology (Academic Press, Inc.); Gene Transfer Vectors for Mammalian Cells, 1987 (J. H. Miller and M. P. Calos eds., Cold Spring Harbor Laboratory); Methods in Enzymology Vol. 154 and Vol. 155 (Wu and Grossman, and Wu, eds., respectively).

[0087] Nucleic acids prepared by solid phase synthesis are a suitable source of nucleic acids for performing the invention. Conventional protection strategies and commercially available reagents for synthesis of both natural and non-natural nucleic acids (as described, for example, in the Glen Research Catalog, Glen Research, Sterling, VA) may be used for this purpose.

[0088] In embodiments in which the biomolecules are double-stranded RNA molecules, dsRNA molecules of the invention can be chemically synthesized, or can be transcribed in vitro from a DNA template, or in vivo from an engineered RNA precursor, e.g., shRNA. The dsRNA molecules can be designed using any method known in the art and can be obtained, for example, from commercial sources such as Dharmacon (Lafayette, Colorado).

[0089] In one aspect of the invention, the peptidomimetic macrocycles are covalently linked to the biomolecule of interest. A variety of linking methods may be used either directly (e.g. with a carbodiimide) or via a linker. See, for example, Wong., S.S., Ed., *Chemistry of Protein Conjugation and Cross-Linking,* CRC Press, Inc., Boca Raton, FIa. (1991) and Langel, U., Ed., *Handbook of Cell-Penetrating Peptides,* CRC Press, In., Boca Raton, FIa. (2006). In particular, carbamate, amide, ester, thioether, disulfide, and hydrazone linkages are generally suitable for preparing conjugates of the invention. If the linker is to be degraded in the intracellular environment, disulfide, ester or amide linkages may be employed. Various functional groups (hydroxyl, amino, halogen etc.) may be used to attach the biomolecules of interest to peptidomimetic macrocycles. Groups which are not known to be part of the biologically active fragment of the biomolecule of interest are generally preferred. For example, if the peptidomimetic macrocyle is to be conjugated to a nucleic acid, a conjugation site at or close to the 5' or 3' end of a strand of said nucleic acid may be chosen such that hybridization between the nucleic acid and an intracellular target sequence is not impeded.

[0090] In one embodiment, the nucleic acids of the invention are conjugated to the N-terminus of the peptidomimetic macrocyles of the invention. For example, the peptidomimetic macrocycles of the invention can be prepared on solid support and are conveniently produced as indicated in more detail below via Fmoc protection. For biomolecules which can survive the conditions used to cleave the reagent from the synthesis resin and deprotect the amino acid side chains, the Fmoc may be cleaved from the N-terminus of the completed resin-bound reagent so that the biomolecule can be linked to the free N-terminal amine. In such cases, the biomolecule to be attached is typically activated to produce, for example, an active ester or carbonate moiety effective to form an amide or carbamate linkage , respectively, with the amino group of the peptidomimetic macrocycle.

[0091] Alternatively, a biomolecule may be synthesized on a solid support and the peptidomimetic macrocycle may be attached after the synthesis has occurred. For example, a nucleic acid may be synthesized on solid phase support modified with a 5' reactive terminal group such as an amine group. A reaction may then be mediated between the reactive terminal group and an activated N-terminus or C-terminus of the peptidomimetic macrocycle.

[0092] Suitable protection and deprotection strategies may be used to ensure that the amino acid side chains of the peptidomimetic macrocycle, the linker, or any part of the biomolecule (such as the backbone, sugar, or bases of a nucleic acid) do not decompose during the preparation of the conjugate.

[0093] Methods of preparing conjugates of nucleic acids such as DNA to polypeptides are disclosed, for example, in U.S. Pat. Nos. 5,169,933; 6,197,513; 6,165,720; 5,547,932; 6,746,868; 6,559,279; and 7,169,814. Coupling of RNA to polypeptides is described, for example, in U.S. Patent Nos. 6,559,279 and 6,762,281. Such technologies may also be applied to the peptidomimetic macrocycles of the invention. Figure 1 discloses several strategies for conjugating peptidomimetic macrocycles to biomolecules such as nucleic acids.

[0094] Additional linking or complex formation methods of nucleic acids to polypeptides are disclosed, for example, in Turner JJ. et al, Blood Cells MoI Dis. 2007 Jan-Feb;38(l): 1-7; U.S. Pat. Application Serial No. 11/676,221, filed on Feb 16, 2007; US Pat. Application Serial No. 10/722,176, filed on Nov 24, 2003; US Pat. Application Serial No. 10/553,659, filed Apr 16, 2004; Lambert et al. (2001), Drug Deliv. Rev., 47(1), 99-1 12; Fattal et al. (1998), J. Control Release, 53(1-3), 137-43; Schwab et al. (1994), Ann. Oncol., 5 Suppl. 4, 55-8; Godard et al. (1995), Eur. J. Biochem., 232(2), 404-10; Leng et al. (2005), J. Gene. Med., 7, 977-986; Meyer et al. (2008), J. Am. Chem. Sci. 130(11), 3273-3273; Albarran et al. (2005), Prot. Eng. Des. Select, 18, 147-152; Chen et al. (2002), Nucl. Acids Res. 30(6), 1338-1345; Venkatesan et al. (2006), Chem. Rev. 106, 3712-3761; and Gierlich, J. et al. (2007), Chem. Eur. J. 13, 9486-9494.

Preparation of Peptidomimetic Macrocycles of the Invention

[0095] Any protein or polypeptide with a known primary amino acid sequence which contains a secondary structure may be used in the present invention. For example, the sequence of a natural polypeptide or a fragment thereof can be analyzed and amino acid analogs containing groups reactive with macrocyclization reagents can be substituted at the appropriate positions. Such determinations are made using methods such as X-ray crystallography of complexes between the secondary structure and a natural binding partner to visualize residues (and surfaces) critical for activity; by sequential mutagenesis of residues in the secondary structure to functionally identify residues (and surfaces) critical for activity; or by other methods. By such determinations, the appropriate amino acids are substituted with the amino acids analogs and macrocycle-forming linkers of the invention. For

example, for an α-helical secondary structure, one surface of the helix *(e.g.,* a molecular surface extending longitudinally along the axis of the helix and radially 45-135° about the axis of the helix) may be required to make contact with another biomolecule *in vivo* or *in vitro* for biological activity. In such a case, a macrocycle-forming linker is designed to link two α-carbons of the helix while extending longitudinally along the surface of the helix in the portion of that surface not directly required for activity.

[0096] In some embodiments of the invention, the peptide sequence is derived from the BCL-2 family of proteins. The BCL-2 family is defined by the presence of up to four conserved BCL-2 homology (BH) domains designated BHl, BH2, BH3, and BH4, all of which include α-helical segments (Chittenden *et al.* (1995), *EMBO* 14:5589; Wang *et al.* (1996), *Genes Dev.* 10:2859). Anti-apoptotic proteins, such as BCL-2 and BCL-X_L, display sequence conservation in all BH domains. Pro-apoptotic proteins are divided into "multidomain" family members (e.g., BAK, BAX), which possess homology in the BHl, BH2, and BH3 domains, and "BH3-domain only" family members (e.g., BID, BAD, BIM, BIK, NOXA, PUMA), that contain sequence homology exclusively in the BH3 amphipathic α-helical segment. BCL-2 family members have the capacity to form homo- and heterodimers, suggesting that competitive binding and the ratio between pro- and anti-apoptotic protein levels dictates susceptibility to death stimuli. Anti-apoptotic proteins function to protect cells from pro-apoptotic excess, *i.e.,* excessive programmed cell death. Additional "security" measures include regulating transcription of pro-apoptotic proteins and maintaining them as inactive conformers, requiring either proteolytic activation, dephosphorylation, or ligand-induced conformational change to activate pro-death functions. In certain cell types, death signals received at the plasma membrane trigger apoptosis via a mitochondrial pathway. The mitochondria can serve as a gatekeeper of cell death by sequestering cytochrome c, a critical component of a cytosolic complex which activates caspase 9, leading to fatal downstream proteolytic events. Multidomain proteins such as BCL-2/BCL-X_L and BAK/BAX play dueling roles of guardian and executioner at the mitochondrial membrane, with their activities further regulated by upstream BH3-only members of the BCL-2 family. For example, BID is a member of the BH3-domain only family of pro-apoptotic proteins, and transmits death signals received at the plasma membrane to effector pro-apoptotic proteins at the mitochondrial membrane. BID has the capability of interacting with both pro- and anti-apoptotic proteins, and upon activation by caspase 8, triggers cytochrome c release and mitochondrial apoptosis. Deletion and mutagenesis studies determined that the amphipathic α-helical BH3 segment of pro-apoptotic family members may function as a death domain and thus may represent a critical structural motif for interacting with multidomain apoptotic proteins. Structural studies have shown that the BH3 helix can interact with anti-apoptotic proteins by inserting into a hydrophobic groove formed by the interface of BHl, 2 and 3 domains. Activated BID can be bound and sequestered by anti-apoptotic proteins (e.g., BCL-2 and BCL-X_L) and can trigger activation of the pro-apoptotic proteins BAX and BAK, leading to cytochrome c release and a mitochondrial apoptosis program. BAD is also a BH3-domain only pro-apoptotic family member whose expression triggers the activation of BAX/BAK. In contrast to BID, however, BAD displays preferential binding to anti-apoptotic family members, BCL-2 and BCL-X_L. Whereas the BAD BH3 domain exhibits high affinity binding to BCL-2, BAD BH3 peptide is unable to activate cytochrome c release from mitochondria *in vitro,* suggesting that BAD is not a direct activator of BAX/BAK. Mitochondria that over-express BCL-2 are resistant to BID-induced cytochrome c release, but co-treatment with BAD can restore BID sensitivity. Induction of mitochondrial apoptosis by BAD appears to result from either: (1) displacement of BAX/BAK activators, such as BID and BID-like proteins, from the BCL-2/BCL-XL binding pocket, or (2) selective occupation of the BCL-

2/BCL-XL binding pocket by BAD to prevent sequestration of BID-like proteins by anti-apoptotic proteins. Thus, two classes of BH3-domain only proteins have emerged, BID-like proteins that directly activate mitochondrial apoptosis, and BAD-like proteins, that have the capacity to sensitize mitochondria to BID-like pro-apoptotics by occupying the binding pockets of multidomain anti-apoptotic proteins. Various α-helical domains of BCL-2 family member proteins amendable to the methodology disclosed herein have been disclosed (Walensky *et al.* (2004), *Science* 305: 1466; and Walensky *et al.*, U.S. Patent Publication No. 2005/0250680, the entire disclosures of which are incorporated herein by reference).

[0097] In other embodiments, the peptide sequence is derived from the tumor suppressor p53 protein which binds to the oncogene protein MDM2. The MDM2 binding site is localized within a region of the p53 tumor suppressor that forms an α helix. In U.S. Pat. No. 7,083,983, the entire contents of which are incorporated herein by reference, Lane *et al.* disclose that the region of p53 responsible for binding to MDM2 is represented approximately by amino acids 13-31 (PLSQETFSDLWKLLPENNV) of mature human P53 protein. Other modified sequences disclosed by Lane are also contemplated in the instant invention. Furthermore, the interaction of p53 and MDM2 has been discussed by Shair *et al.* (1997), *Chem. & Biol.* 4:791, the entire contents of which are incorporated herein by reference, and mutations in the p53 gene have been identified in virtually half of all reported cancer cases. As stresses are imposed on a cell, p53 is believed to orchestrate a response that leads to either cell-cycle arrest and DNA repair, or programmed cell death. As well as mutations in the p53 gene that alter the function of the p53 protein directly, p53 can be altered by changes in MDM2. The MDM2 protein has been shown to bind to p53 and disrupt transcriptional activation by associating with the transactivation domain of p53. For example, an 11 amino-acid peptide derived from the transactivation domain of p53 forms an amphipathic α-helix of 2.5 turns that inserts into the MDM2 crevice. Thus, in some embodiments, novel α-helix structures generated by the method of the present invention are engineered to generate structures that bind tightly to the helix acceptor and disrupt native protein-protein interactions. These structures are then screened using high throughput techniques to identify optimal small molecule peptides. The novel structures that disrupt the MDM2 interaction are useful for many applications, including, but not limited to, control of soft tissue sarcomas (which over-expresses MDM2 in the presence of wild type p53). These cancers are then, in some embodiments, held in check with small molecules that intercept MDM2, thereby preventing suppression of p53. Additionally, in some embodiments, small molecules disrupters of MDM2-p53 interactions are used as adjuvant therapy to help control and modulate the extent of the p53 dependent apoptosis response in conventional chemotherapy.

[0098] A non-limiting exemplary list of suitable peptide sequences for use in the present invention is given below:

TABLE 1

Name	Sequence (bold = critical residues)	Cross-linked Sequence (X = x-link residue)
BH3 peptides		
BID-BH3	QEDIIRNIARH**L**AQV**GD**SMDRSIPP	QEDIIRNIARH**L**A**X**V**GD****X**MDRSIPP
BIM-BH3	DNRPEIWIAQE**L**RRI**GD**EFNAYYAR	DNRPEIWIAQE**L**R**X**I**GD****X**FNAYYAR
BAD-BH3	NLWAAQRYGRE**L**RRM**SD**EFVDSFKK	NLWAAQRYGRE**L**R**X**M**SD****X**FVDSFKK
PUMA-BH3	EEQWAREIGAQ**L**RRMA**D**DLNAQYER	EEQWAREIGAQ**L**R**X**MA**D****X**LNAQYER
Hrk-BH3	RSSAAQLTAAR**L**KAL**GD**ELHQRTM	RSSAAQLTAAR**L**K**X**L**GD****X**LHQRTM
NOXAA-BH3	AELPPEFAAQ**L**RKI**GD**KVYCTW	AELPPEFAAQ**L**R**X**I**GD****X**VYCTW
NOXAB-BH3	VPADLKDECAQ**L**RRI**GD**KVNLRQKL	VPADLKDECAQ**L**R**X**I**GD****X**VNLRQKL
BMF-BH3	QHRAEVQIARK**L**QCIA**D**QFHRLHT	QHRAEVQIARK**L**Q**X**IA**D****X**FHRLHT
BLK-BH3	SSAAQLTAAR**L**KAL**GD**ELHQRT	SSAAQLTAAR**L**K**X**L**GD****X**LHQRT
BIK-BH3	CMEGSDALALR**L**ACI**GD**EMDVSLRA	CMEGSDALALR**L**A**X**I**GD****X**MDVSLRA
Bnip3	DIERRKEVESI**L**KKN**SD**WIWDWSS	DIERRKEVESI**L**K**X**N**SD****X**IWDWSS
BOK-BH3	GRLAEVCAV**L**LRL**GD**ELEMIRP	GRLAEVCAV**L**L**X**L**GD****X**LEMIRP
BAX-BH3	PQDASTKKSEC**L**KRI**GD**ELDSNMEL	PQDASTKKSEC**L**K**X**I**GD****X**LDSNMEL
BAK-BH3	PSSTMGQVGRQ**L**AII**GD**DINRR	PSSTMGQVGRQ**L**A**X**I**GD****X**INRR
BCL2L1-BH3	KQA**L**REA**GD**EFELR	KQA**L**R**X**A**GD****X**FELR
BCL2-BH3	LSPPVVHLALA**L**RQA**GD**DFSRR	LSPPVVHLALA**L**R**X**A**GD****X**FSRR
BCL-XL-BH3	EVIPMAAVKQA**L**REA**GD**EFELRY	EVIPMAAVKQA**L**R**X**A**GD****X**FELRY
BCL-W-BH3	PADPLHQAMRAA**GD**EFETRF	PADPLHQAMR**X**A**GD****X**FETRF
MCL1-BH3	ATSRKLET**L**RRV**GD**GVQRNHETA	ATSRKLET**L**R**X**V**GD****X**VQRNHETA
MTD-BH3	LAEVCTV**L**LRL**GD**ELEQIR	LAEVCTV**L**L**X**L**GD****X**LEQIR
MAP-1-BH3	MTVGELSRA**L**GHENGSLDP	MTVGELSRA**L**G**X**ENG**X**LDP
NIX-BH3	VVEGEKEVEA**L**KKSA**D**WVSDWS	VVEGEKEVEA**L**K**X**SA**D****X**VSDWS
4ICD(ERBB4)-BH3	SMARDPQRY**L**VIQ**GD**DRMKL	SMARDPQRY**L**V**X**Q**GD****X**RMKL

Table 1 lists human sequences which target the BH3 binding site and are implicated in cancers, autoimmune disorders, metabolic diseases and other human disease conditions.

TABLE 2

Name	Sequence (bold = critical residues)	Cross-linked Sequence (X = x-link residue)
BH3 peptides		
BID-BH3	QEDIIRNIARH**L**AQV**GD**SMDRSIPP	QEDIIRNI**X**RH**L****X**QV**GD**SMDRSIPP
BIM-BH3	DNRPEIWIAQE**L**RRI**GD**EFNAYYAR	DNRPEIWI**X**QE**L****X**RI**GD**EFNAYYAR
BAD-BH3	NLWAAQRYGRE**L**RRM**SD**EFVDSFKK	NLWAAQRY**X**RE**L****X**RM**SD**EFVDSFKK
PUMA-BH3	EEQWAREIGAQ**L**RRMA**D**DLNAQYER	EEQWAREI**X**AQ**L****X**RMA**D**DLNAQYER
Hrk-BH3	RSSAAQLTAAR**L**KAL**GD**ELHQRTM	RSSAAQLT**X**AR**L****X**AL**GD**ELHQRTM
NOXAA-BH3	AELPPEFAAQ**L**RKI**GD**KVYCTW	AELPPEF**X**AQ**L****X**KI**GD**KVYCTW
NOXAB-BH3	VPADLKDECAQ**L**RRI**GD**KVNLRQKL	VPADLKDE**X**AQ**L****X**RI**GD**KVNLRQKL
BMF-BH3	QHRAEVQIARK**L**QCIA**D**QFHRLHT	QHRAEVQI**X**RK**L****X**CIA**D**QFHRLHT
BLK-BH3	SSAAQLTAAR**L**KAL**GD**ELHQRT	SSAAQLT**X**AR**L****X**AL**GD**ELHQRT
BIK-BH3	CMEGSDALALR**L**ACI**GD**EMDVSLRA	CMEGSDAL**X**LR**L****X**CI**GD**EMDVSLRA
Bnip3	DIERRKEVESI**L**KKN**SD**WIWDWSS	DIERRKEV**X**SI**L****X**KN**SD**WIWDWSS
BOK-BH3	GRLAEVCAV**L**LRL**GD**ELEMIRP	GRLAEV**X**AV**L****X**RL**GD**ELEMIRP
BAX-BH3	PQDASTKKSEC**L**KRI**GD**ELDSNMEL	PQDASTKK**X**EC**L****X**RI**GD**ELDSNMEL
BAK-BH3	PSSTMGQVGRQ**L**AII**GD**DINRR	PSSTMGQV**X**RQ**L****X**II**GD**DINRR
BCL2L1-BH3	KQA**L**REA**GD**EFELR	**X**QA**L****X**EA**GD**EFELR
BCL2-BH3	LSPPVVHLALA**L**RQA**GD**DFSRR	LSPPVVHL**X**LA**L****X**QA**GD**DFSRR
BCL-XL-BH3	EVIPMAAVKQA**L**REA**GD**EFELRY	EVIPMAAV**X**QA**L****X**EA**GD**EFELRY
BCL-W-BH3	PADPLHQAMRAA**GD**EFETRF	PADPL**X**QAM**X**AA**GD**EFETRF
MCL1-BH3	ATSRKLET**L**RRV**GD**GVQRNHETA	ATSRK**X**ET**L****X**RV**GD**GVQRNHETA
MTD-BH3	LAEVCTV**L**LRL**GD**ELEQIR	LAEV**X**TV**L****X**RL**GD**ELEQIR
MAP-1-BH3	MTVGELSRA**L**GHENGSLDP	MTVGEL**X**RA**L****X**HENGSLDP
NIX-BH3	VVEGEKEVEA**L**KKSA**D**WVSDWS	VVEGEKE**X**EA**L****X**KSA**D**WVSDWS
4ICD(ERBB4)-BH3	SMARDPQRY**L**VIQ**GD**DRMKL	SMARDP**X**RY**L****X**IQ**GD**DRMKL

Table 2 lists human sequences which target the BH3 binding site and are implicated in cancers, autoimmune disorders, metabolic diseases and other human disease conditions.

TABLE 3

Name	Sequence (bold = critical residues)	Cross-linked Sequence (X = x-link residue)
P53 peptides		
hp53 peptide 1	LSQET**FSDLW**KLLPEN	LSQET**FSDXW**KLLPE**X**
hp53 peptide 2	LSQET**FSDLW**KLLPEN	LSQE**X****FSDLW**K**X**LPEN
hp53 peptide 3	LSQET**FSDLW**KLLPEN	LSQ**X**T**FSDLW****X**LLPEN
hp53 peptide 4	LSQET**FSDLW**KLLPEN	LSQET**F****X****DLW**KLL**X**EN
hp53 peptide 5	LSQET**FSDLW**KLLPEN	QSQQT**F****X****NLW**RLL**X**QN

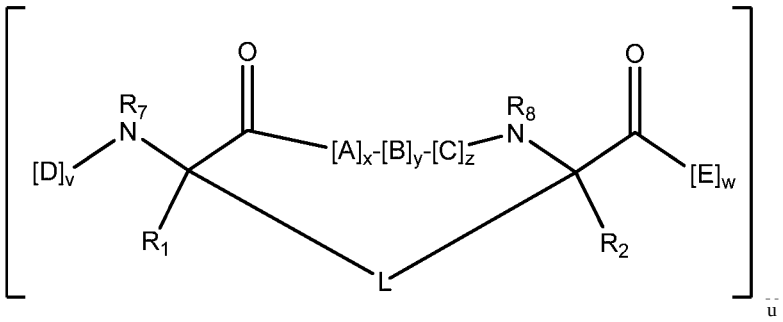
Table 3 lists human sequences which target the p53 binding site of MDM2/X and are implicated in cancers.

TABLE 4

Name	Sequence (bold = critical residues)	Cross-linked Sequence (X = x-link residue)
GPCR peptide ligands		
Angiotensin II	DRV**YI****HPF**	DR**X****Y****X****HPF**
Bombesin	EQRLGNQ**W**AVG**HL**M	EQRLGN**X****W**AVG**HL****X**
Bradykinin	RPPGF**SPFR**	RPP**X**F**SPFR****X**
C5a	ISHKD**MQLGR**	ISHKD**M****X****L**GR**X**
C3a	ARASH**LG****LAR**	ARASH**L****X****LAR****X**
α-melanocyte stimulating hormone	SYSME**HFRW**GKPV	SYSM**X****HFRW****X**KPV

Table 4 lists sequences which target human G protein-coupled receptors and are implicated in numerous human disease conditions (Tyndall *et al.* (2005), *Chem. Rev.* 105:793-826).

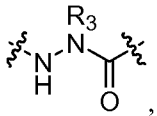
[0099] In some embodiments, the peptidomimetic macrocycles of the invention have the Formula (I):



Formula (I)

wherein:

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

B is a natural or non-natural amino acid, amino acid analog,  , [-NH-L_3-CO-], [-NH-L_3-SCv], or [-NH-L_3-];

R_1 and R_2 are independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo-;

R_3 is hydrogen, alkyl, alkenyl, alkynyl, arylalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, cycloalkylalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R_5;

L is a macrocycle-forming linker of the formula - L_1- L_2- ;

L_1 and L_2 are independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, cycloarylene, heterocycloarylene, or $[-R_4\text{-}K\text{-}R_4\text{-}]_n$, each being optionally substituted with R_5;

each R_4 is alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene;

each K is O, S, SO, SO_2, CO, CO_2, or $CONR_3$;

each R_5 is independently halogen, alkyl, $-OR_6$, $-N(R_6)_2$, $-SR_6$, $-SOR_6$, $-SO_2R_6$, $-CO_2R_6$, a fluorescent moiety, a radioisotope or a therapeutic agent;

each R_6 is independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heterocycloalkyl, a fluorescent moiety, a radioisotope or a therapeutic agent;

R_7 is -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R_5, or part of a cyclic structure with a D residue;

R_8 is -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R_5, or part of a cyclic structure with an E residue;

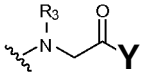
each of v and w is independently an integer from 1-1000;

each of x, y, and z is independently an integer from 0-10; u is an integer from 1-10; and

n is an integer from 1-5.

[00100] In one example, at least one OfR_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 OfR_1 and R_2 is methyl. In other embodiments, R_1 and R_2 are methyl.

[00101] In some embodiments of the invention, x+y+z is at least 3. In other embodiments of the invention, 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 of the invention 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. Gin-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.

[00102] In some embodiments, the peptidomimetic macrocycle of the invention 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 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 

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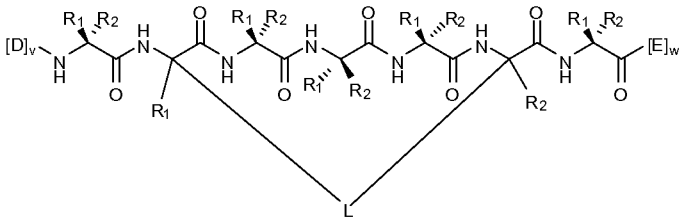
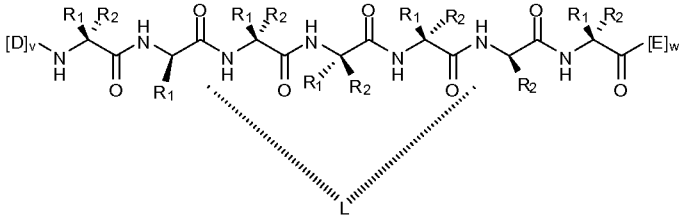
[00103] In other embodiments, the length of the macrocycle-forming linker L as measured from a first Ca to a second Ca is selected to stabilize a desired secondary peptide structure, such as an α-helix formed by residues of the peptidomimetic macrocycle including, but not necessarily limited to, those between the first Ca to a second Ca.

[00104] In one embodiment, the peptidomimetic macrocycle of Formula (I) is:

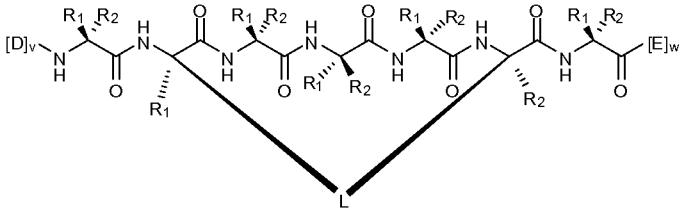


[00105] wherein each R_1 and R_2 is independently independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo-.

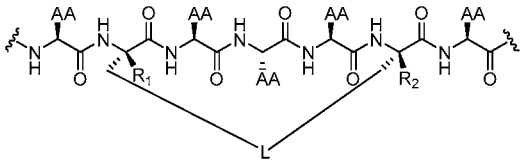
[00106] In related embodiments, the peptidomimetic macrocycle of Formula (I) is:

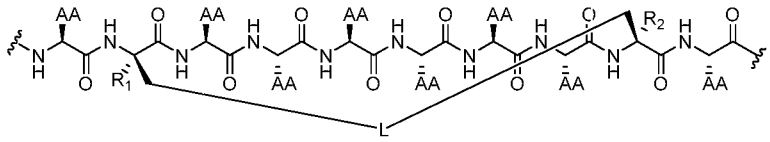


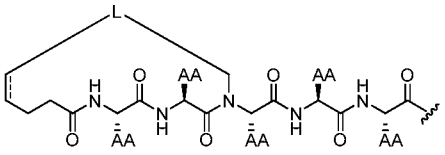
or

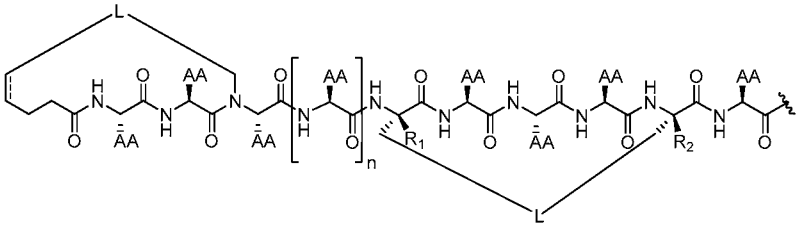


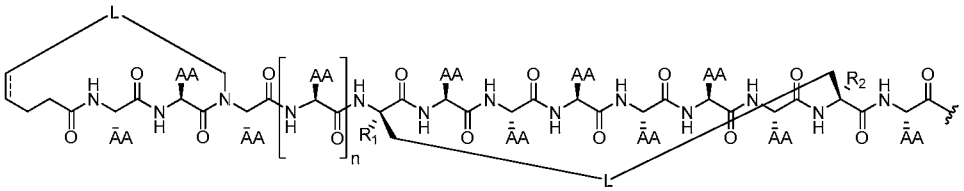
[00107] In other embodiments, the peptidomimetic macrocycle of Formula (I) is a compound of any of the formulas shown below:

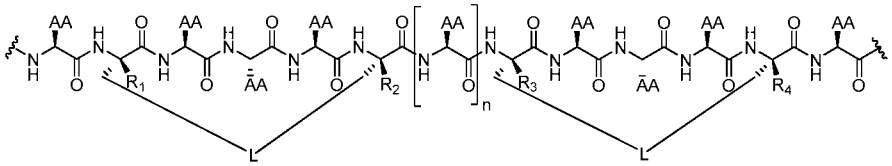


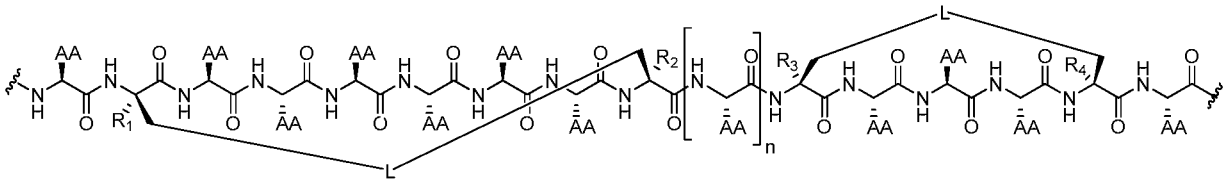


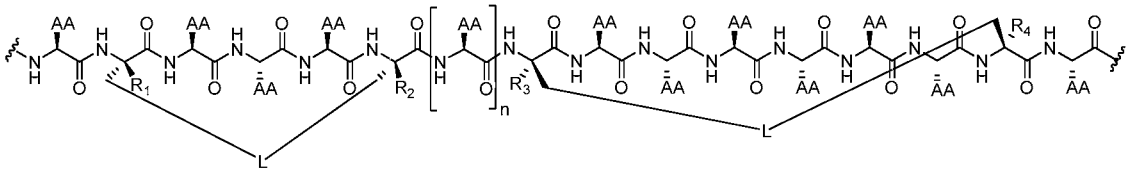


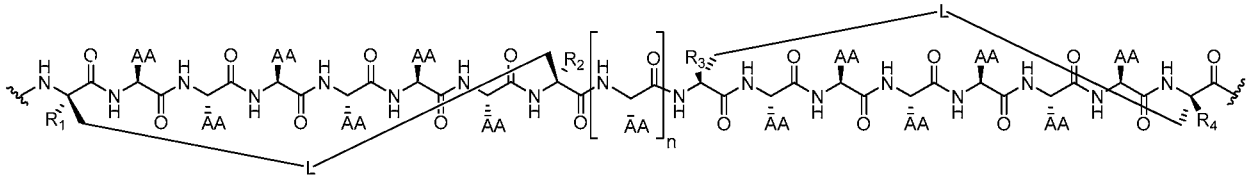


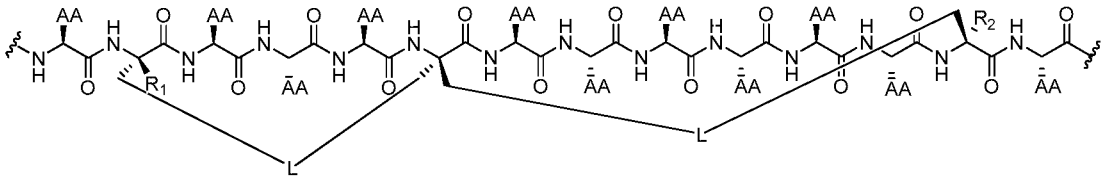


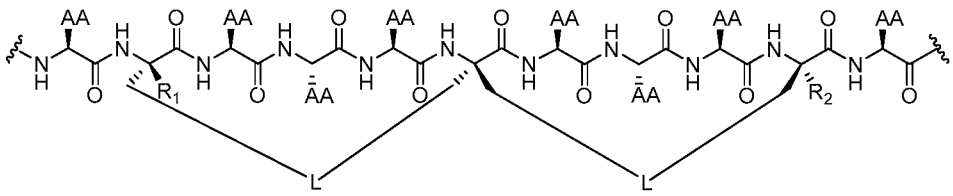


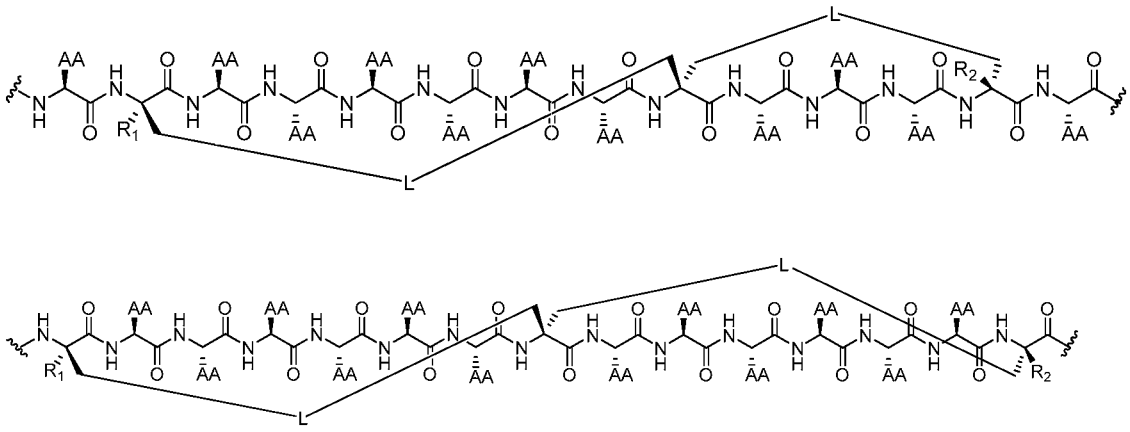






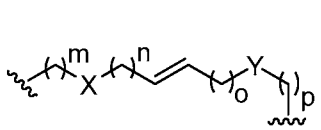




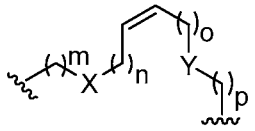


wherein "AA" represents any natural or non-natural amino acid side chain and " " is $[D]_v$, $[E]_w$ as defined above, and n is an integer between 0 and 20, 50, 100, 200, 300, 400 or 500. In some embodiments, n is 0. In other embodiments, n is less than 50.

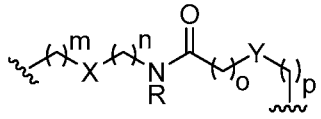
[00108] Exemplary embodiments of the macrocycle-formmg linker L are shown below.



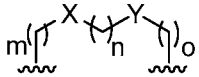
where X, Y = $-CH_2-$, O, S, or NH
m, n, o, p = 0-10



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



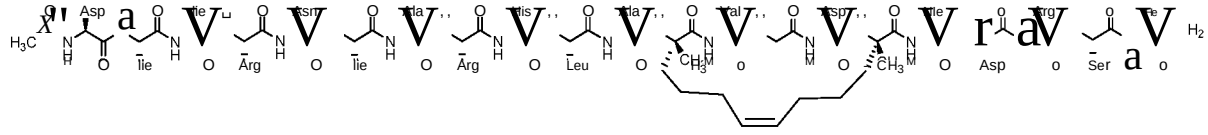
where X, Y = $-CH_2-$, O, S, or NH
m, n, o, p = 0-10
R = H, alkyl, other substituent



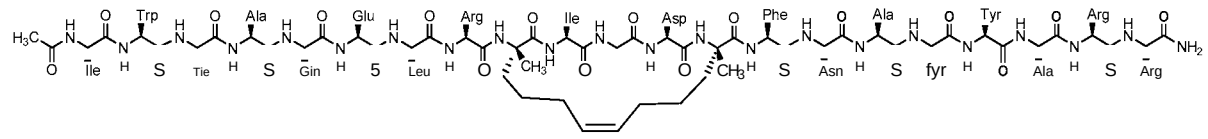
where X, Y = $-CH_2-$, O, S, or NH
m, n, o = 0-10

[00109] Exemplary embodiments of peptidomimetic macrocycles of the invention are shown below:

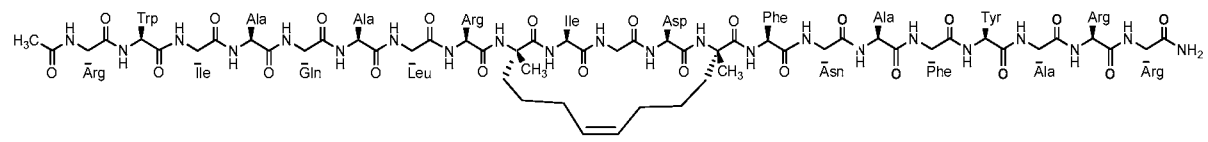
SP-1



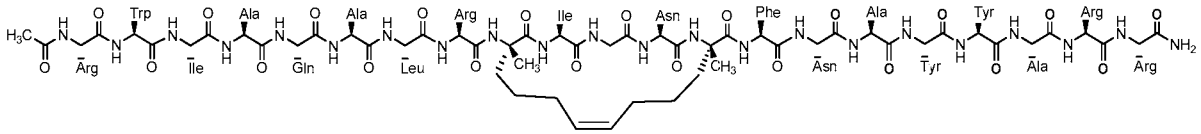
SP-4



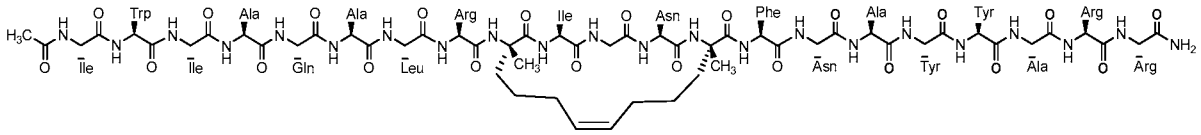
SP-15



SP-23

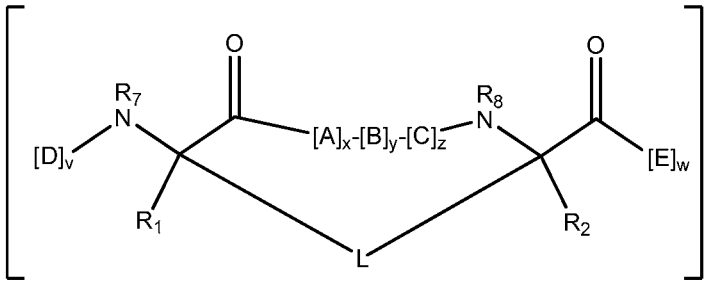


SP-25



Other embodiments of peptidomimetic macrocycles of the invention include analogs of the macrocycles shown above.

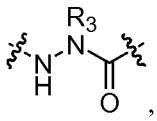
[00110] In some embodiments, the peptidomimetic macrocycles of the invention have the Formula (II):



Formula (II)

wherein:

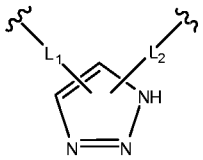
each A, C, D, and E is independently a natural or non-natural amino acid;

B is a natural or non-natural amino acid, amino acid analog,  , [-NH-L_3-CO-], [-NH-L_3-SO_2-], or [-NH-L_3-];

R_1 and R_2 are independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo-;

R_3 is hydrogen, alkyl, alkenyl, alkynyl, arylalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, cycloalkylalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R_5;

L is a macrocycle-formmg linker of the formula



;

L_1, L_2 and L_3 are independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, cycloarylene, heterocycloarylene, or [-R_4-K-R_4-]$_n$, each being optionally substituted with R_5;

each R_4 is alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene;

each K is O, S, SO, SO_2, CO, CO_2, or $CONR_3$;

each R_5 is independently halogen, alkyl, $-OR_6$, $-N(R_6)_2$, $-SR_6$, $-SOR_6$, $-SO_2R_6$, $-CO_2R_6$, a fluorescent moiety, a radioisotope or a therapeutic agent;

each R_6 is independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heterocycloalkyl, a fluorescent moiety, a radioisotope or a therapeutic agent;

R_7 is -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R_5, or part of a cyclic structure with a D residue;

R_8 is -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R_5, or part of a cyclic structure with an E residue;

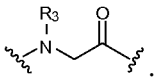
each of v and w is independently an integer from 1-1000;

each of x, y, and z is independently an integer from 0-10; u is an integer from 1-10; and

n is an integer from 1-5.

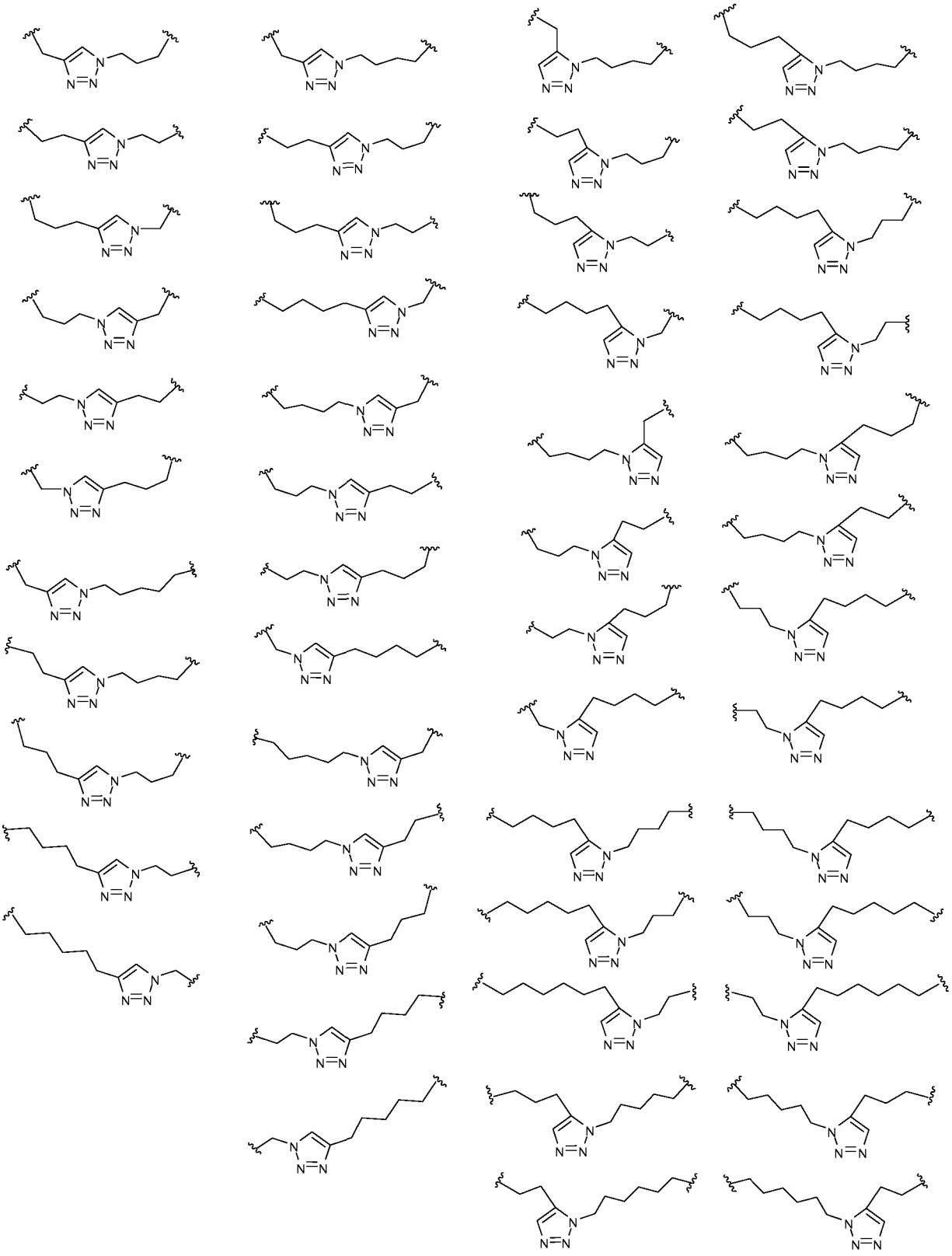
[00111] In one example, at least one OfR_1 and R_2 is alkyl, unsubstituted or substituted with halo-. In another example, both R_1 and R_2 are independently alkyl, unsubstituted or substituted with halo-. In some embodiments, at least one OfR_1 and R_2 is methyl. In other embodiments, R_1 and R_2 are methyl.

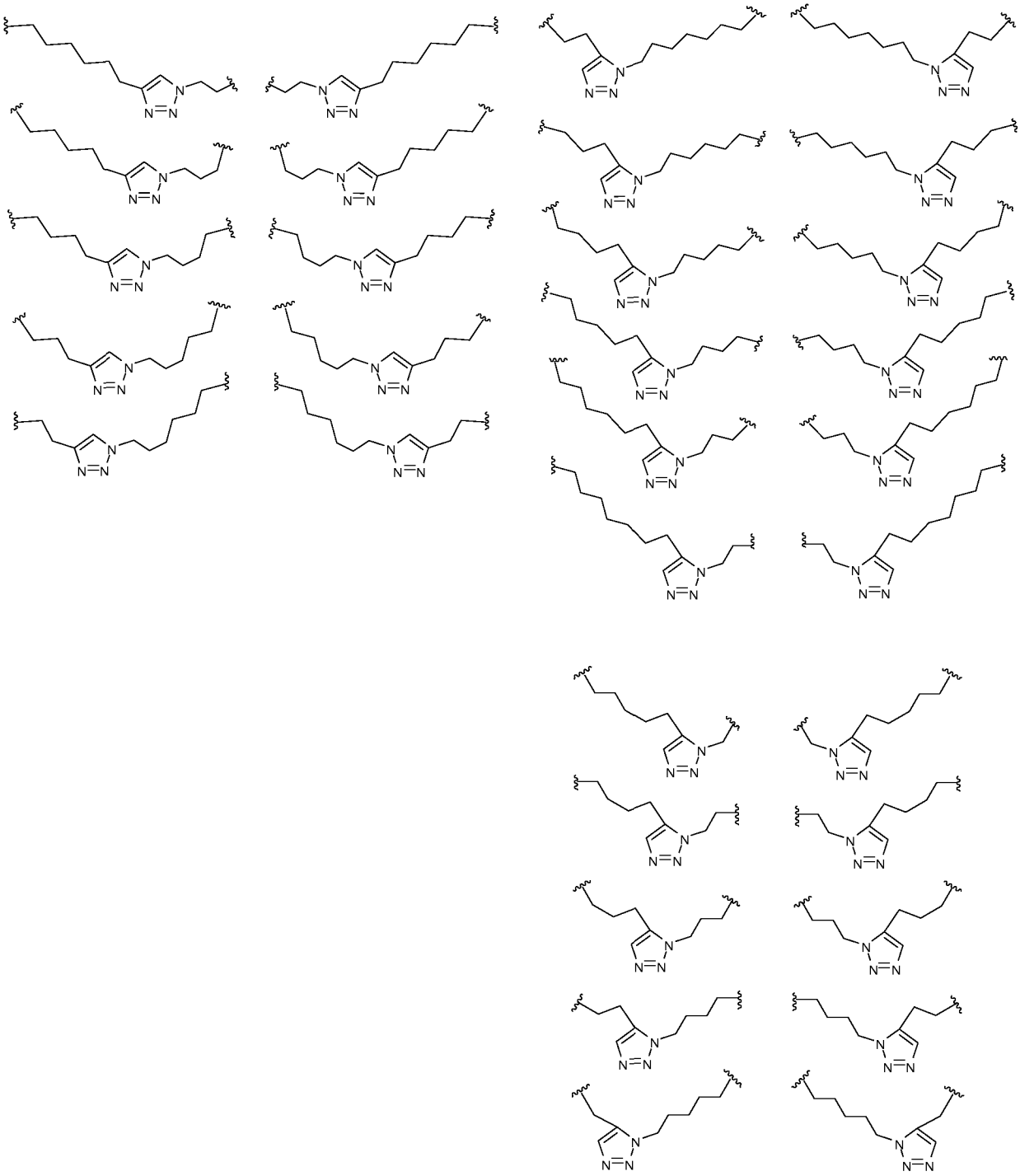
[00112] In some embodiments of the invention, x+y+z is at least 3. In other embodiments of the invention, x+y+z is 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10. Each occurrence of A, B, C, D or E in a macrocycle or macrocycle precursor of the invention is independently selected. For example, a sequence represented by the formula $[A]_x$, when x is 3, encompasses embodiments where the amino acids are not identical, e.g. Gin-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.

[00113] In some embodiments, the peptidomimetic macrocycle of the invention 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 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  .

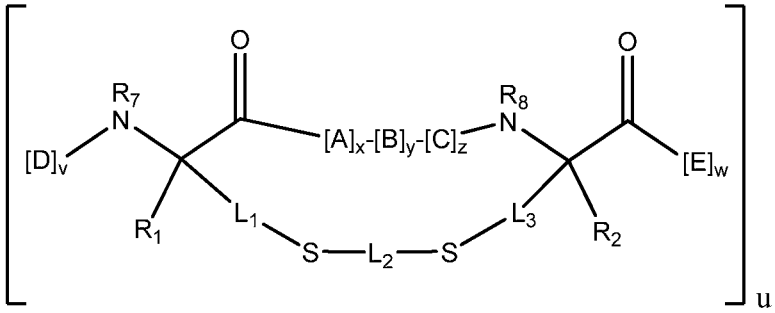
[00114] In other embodiments, the length of the macrocycle-forming linker L as measured from a first Ca to a second Ca is selected to stabilize a desired secondary peptide structure, such as an α-helix formed by residues of the peptidomimetic macrocycle including, but not necessarily limited to, those between the first Ca to a second Ca.

[00115] Exemplary embodiments of the macrocycle-forming linker L are shown below.





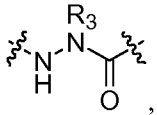
[00116] In other embodiments, the invention provides peptidomimetic macrocycles of Formula (III):



Formula (III)

wherein:

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

B is a natural or non-natural amino acid, amino acid analog,  , [-NH-L_4-CO-], [-NH-L_4-SO_2-], or [-NH-L_4-];

R_1 and R_2 are independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo-;

R_3 is hydrogen, alkyl, alkenyl, alkynyl, arylalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, cycloalkylalkyl, cycloaryl, or heterocycloaryl, unsubstituted or substituted with R_5;

L_1, L_2, L_3 and L_4 are independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, cycloarylene, heterocycloarylene or [-R_4-K-R_4-Jn, each being unsubstituted or substituted with R_5;

K is O, S, SO, SO_2, CO, CO_2, or $CONR_3$;

each R_4 is alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene;

each R_5 is independently halogen, alkyl, -ORg, -$N(R_6)_2$, -SRg, -SORg, -SO_2R_6, -CO_2R_6, a fluorescent moiety, a radioisotope or a therapeutic agent;

each R_6 is independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heterocycloalkyl, a fluorescent moiety, a radioisotope or a therapeutic agent;

R_7 is -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, unsubstituted or substituted with R_5, or part of a cyclic structure with a D residue;

R_8 is -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, unsubstituted or substituted with R_5, or part of a cyclic structure with an E residue;

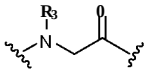
each of v and w is independently an integer from 1-1000;

each of x, y, and z is independently an integer from 0-10; u is an integer from 1-10; and

n is an integer from 1-5.

[00117] In one example, at least one of Ri 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 Ri and R_2 is methyl. In other embodiments, Ri and R_2 are methyl.

[00118] In some embodiments of the invention, x+y+z is at least 3. In other embodiments of the invention, x+y+z is 3, 4, 5, 6, 7, 8, 9 or 10. Each occurrence of A, B, C, D or E in a macrocycle or macrocycle precursor of the invention 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. Gin-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.

[00119] In some embodiments, the peptidomimetic macrocycle of the invention 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 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  .

[00120] In other embodiments, the length of the macrocycle-forming linker [-Li-S-L_2-S-L_3-] as measured from a first Ca to a second Ca is selected to stabilize a desired secondary peptide structure, such as an α-helix formed by residues of the peptidomimetic macrocycle including, but not necessarily limited to, those between the first Ca to a second Ca.

[00121] Macrocycles or macrocycle precursors are synthesized, for example, by solution phase or solid-phase methods, and can contain both naturally-occurring and non-naturally-occurring amino acids. See, for example, Hunt, "The Non-Protein Amino Acids" in Chemistry and Biochemistry of the Amino Acids, edited by G.C. Barrett, Chapman and Hall, 1985. 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.

[00122] In other embodiments, D and/or E in the compound of Formula I, II or III are further modified in order to facilitate cellular uptake. In some embodiments, lipidating or PEGylating a peptidomimetic macrocycle facilitates cellular uptake, increases bioavailability, increases blood circulation, alters pharmacokinetics, decreases immunogenicity and/or decreases the needed frequency of administration.

[00123] In other embodiments, at least one of [D] and [E] in the compound of Formula I, II or III represents a moiety comprising an additional macrocycle-forming linker such that the peptidomimetic macrocycle comprises at least two macrocycle-forming linkers. In a specific embodiment, a peptidomimetic macrocycle comprises two macrocycle-forming linkers.

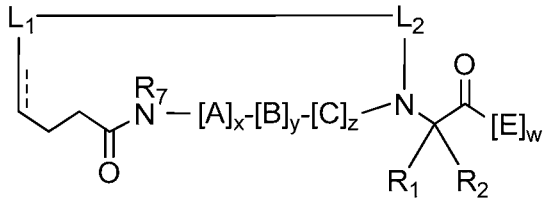
[00124] In the peptidomimetic macrocycles of the invention, any of the macrocycle-forming linkers described herein may be used in any combination with any of the sequences shown in Tables 1-4 and also with any of the R- substituents indicated herein.

[00125] In some embodiments, the peptidomimetic macrocycle comprises at least one α-helix motif. For example, A, B and/or C in the compound of Formula I, II or III include one or more α-helices. As a general

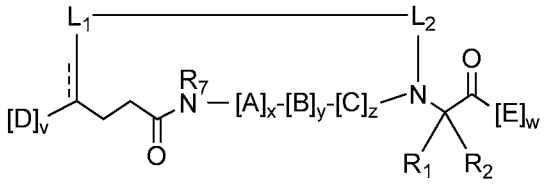
matter, α-helices include between 3 and 4 amino acid residues per turn. In some embodiments, the α-helix of the peptidomimetic macrocycle includes 1 to 5 turns and, therefore, 3 to 20 amino acid residues. In specific embodiments, the α-helix includes 1 turn, 2 turns, 3 turns, 4 turns, or 5 turns. In some embodiments, the macrocycle-forming linker stabilizes an α-helix motif included within the peptidomimetic macrocycle. Thus, in some embodiments, the length of the macrocycle-forming linker L from a first Ca to a second Ca is selected to increase the stability of an α-helix. In some embodiments, the macrocycle-forming linker spans from 1 turn to 5 turns of the α-helix. In some embodiments, the macrocycle-forming linker spans approximately 1 turn, 2 turns, 3 turns, 4 turns, or 5 turns of the α-helix. In some embodiments, the length of the macrocycle-forming linker is approximately 5 A to 9 A per turn of the α-helix, or approximately 6 A to 8 A per turn of the α-helix. Where the macrocycle-forming linker spans approximately 1 turn of an α-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 an α-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 an α-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 an α-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 an α-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 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.

[00126] In other embodiments, the invention provides peptidomimetic macrocycles of Formula (FV) or (IVa):



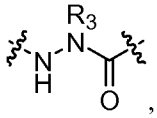
Formula (IV)



Formula (FVa)

wherein:

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

B is a natural or non-natural amino acid, amino acid analog,  , [-NH-L_3-CO-], [-NH-L_3-SO_2-], or [-NH-L_3-];

R_1 and R_2 are independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo-, or part of a cyclic structure with an E residue;

R_3 is hydrogen, alkyl, alkenyl, alkynyl, arylalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, cycloalkylalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R_5;

L is a macrocycle-forming linker of the formula - L_1- L_2- ;

L_1 and L_2 are independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, cycloarylene, heterocycloarylene, or [-R_4-K-R_4-]$_n$, each being optionally substituted with R_5;

each R_4 is alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene;

each K is O, S, SO, SO_2, CO, CO_2, or $CONR_3$;

each R_5 is independently halogen, alkyl, -OR_6, -$N(R_6)_2$, -SR_6, -SOR_6, -SO_2R_6, -CO_2R_6, a fluorescent moiety, a radioisotope or a therapeutic agent;

each R_6 is independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heterocycloalkyl, a fluorescent moiety, a radioisotope or a therapeutic agent;

R_7 is -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R_5;

v is an integer from 1-1000;

w is an integer from 1-1000;

x is an integer from 0-10;

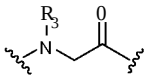
y is an integer from 0-10;

z is an integer from 0-10; and

n is an integer from 1-5.

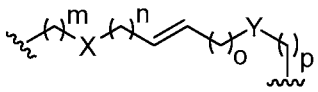
[00127] In one example, at least one of Ri and R_2 is alkyl, unsubstituted or substituted with halo-. In another example, both R_1 and R_2 are independently alkyl, unsubstituted or substituted with halo-. In some embodiments, at least one OfR_1 and R_2 is methyl. In other embodiments, R_1 and R_2 are methyl.

[00128] In some embodiments of the invention, x+y+z is at least 3. In other embodiments of the invention, 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 of the invention 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. Gin-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.

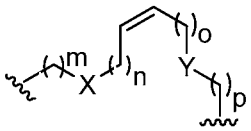
[00129] In some embodiments, the peptidomimetic macrocycle of the invention comprises a secondary structure which is an α-helix and R_8 is -H, allowing intrahelical hydrogen bonding. In some embodiments, at least one of A, B, C, D or E is an α,α-disubstituted amino acid. In one example, B is an α,α-disubstituted amino acid. For 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  .

[00130] In other embodiments, the length of the macrocycle-forming linker L as measured from a first Ca to a second Ca is selected to stabilize a desired secondary peptide structure, such as an α-helix formed by residues of the peptidomimetic macrocycle including, but not necessarily limited to, those between the first Ca to a second Ca.

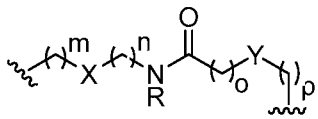
[00131] Exemplary embodiments of the macrocycle-forming linker L are shown below.



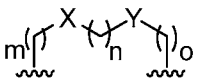
where X, Y = -CH_2-, O, S, or NH
m, n, 0, p = 0-10



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



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



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

Preparation of Peptidomimetic Macrocycles

[00132] Peptidomimetic macrocycles of the invention may be prepared by any of a variety of methods known in the art. For example, any of the residues indicated by "X" in Tables 1, 2, 3 or 4 may be substituted with a residue capable of forming a crosslinker with a second residue in the same molecule or a precursor of such a residue.

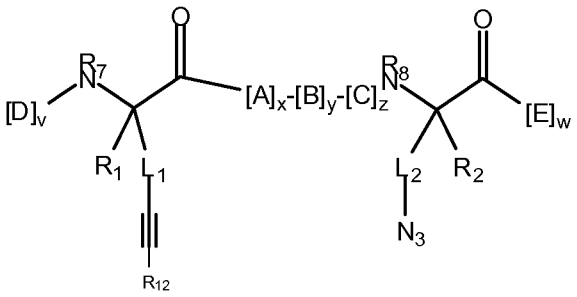
[00133] Various methods to effect formation of peptidomimetic macrocycles are known in the art. For example, the preparation of peptidomimetic macrocycles of Formula I is described in Schafmeister et al., J. Am. Chem. Soc. 122:5891-5892 (2000); Schafmeister & Verdine, J. Am. Chem. Soc. 122:5891 (2005); Walensky et al., Science 305:1466-1470 (2004); US Patent No. 7,192,713; and PCT application WO 2008/121767. The α,α-disubstituted amino acids and amino acid precursors disclosed in the cited references may be employed in synthesis of the peptidomimetic macrocycle precursor polypeptides. Following incorporation of such amino acids into precursor polypeptides, the terminal olefins are reacted with a metathesis catalyst, leading to the formation of the peptidomimetic macrocycle.

[00134] In other embodiments, the peptidomimetic macrocyles of the invention are of Formula IV or IVa. Methods for the preparation of such macrocycles are described, for example, in US Patent No. 7,202,332.

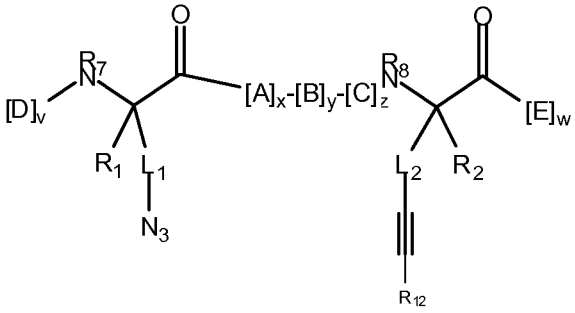
[00135] In some embodiments, the synthesis of these peptidomimetic macrocycles involves a multi-step process that features the synthesis of a peptidomimetic precursor containing an azide moiety and an alkyne moiety; followed by contacting the peptidomimetic precursor with a macrocyclization reagent to generate a triazole-linked peptidomimetic macrocycle. Macrocycles or macrocycle precursors are synthesized, for example, by solution phase or solid-phase methods, and can contain both naturally-occurring and non-naturally-occurring amino acids. See, for example, Hunt, "The Non-Protein Amino Acids" in Chemistry and Biochemistry of the Amino Acids, edited by G.C. Barrett, Chapman and Hall, 1985.

[00136] In some embodiments, an azide is linked to the α-carbon of a residue and an alkyne is attached to the α-carbon of another residue. In some embodiments, the azide moieties are azido-analogs of amino acids L-lysine, D-lysine, alpha-methyl-L-lysine, alpha-methyl-D-lysine, L-ornithine, D-ornithine, alpha-methyl-L-ornithine or alpha-methyl-D-ornithine. In another embodiment, the alkyne moiety is L-propargylglycine. In yet other embodiments, the alkyne moiety is an amino acid selected from the group consisting of L-propargylglycine, D-propargylglycine, (S)-2-amino-2-methyl-4-pentynoic acid, (R)-2-amino-2-methyl-4-pentynoic acid, (S)-2-amino-2-methyl-5-hexynoic acid, (R)-2-amino-2-methyl-5-hexynoic acid, (S)-2-amino-2-methyl-6-heptynoic acid, (R)-2-amino-2-methyl-6-heptynoic acid, (S)-2-amino-2-methyl-7-octynoic acid, (R)-2-amino-2-methyl-7-octynoic acid, (S)-2-amino-2-methyl-8-nonynoic acid and (R)-2-amino-2-methyl-8-nonynoic acid.

[00137] In some embodiments, the invention provides a method for synthesizing a peptidomimetic macrocycle, the method comprising the steps of contacting a peptidomimetic precursor of Formula V or Formula VI:



(Formula V)



(Formula VI)

with a macrocyclization reagent;

wherein v, w, x, y, z, A, B, C, D, E, R_1, R_2, R_7, Rg, L_1 and L_2 are as defined for Formula (II); R_{12} is - H when the macrocyclization reagent is a Cu reagent and R_{12} is - H or alkyl when the macrocyclization reagent is a Ru reagent; and further wherein said contacting step results in a covalent linkage being formed between the alkyne and azide moiety in Formula III or Formula IV. For example, R_{12} may be methyl when the macrocyclization reagent is a Ru reagent.

[00138] In the peptidomimetic macrocycles of the invention, at least one OfR_1 and R_2 is alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo-. In some embodiments, both R_1 and R_2 are independently alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo-. 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.

[00139] For example, at least one OfR_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 OfR_1 and R_2 is methyl. In other embodiments, R_1 and R_2 are methyl. The macrocyclization reagent may be a Cu reagent or a Ru reagent.

[00140] In some embodiments, the peptidomimetic precursor is purified prior to the contacting step. In other embodiments, the peptidomimetic macrocycle is purified after the contacting step. In still other embodiments, the peptidomimetic macrocycle is refolded after the contacting step. The method may be performed in solution, or, alternatively, the method may be performed on a solid support.

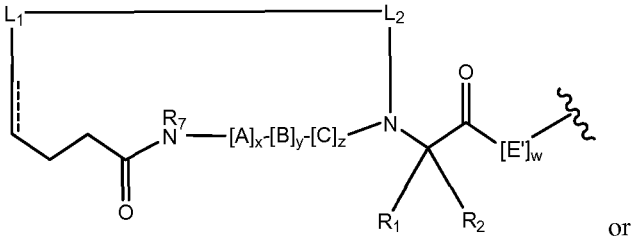
[00141] Also envisioned herein is performing the method of the invention in the presence of a target macromolecule that binds to the peptidomimetic precursor or peptidomimetic macrocycle under conditions that favor said binding. In some embodiments, the method is performed in the presence of a target macromolecule that binds preferentially to the peptidomimetic precursor or peptidomimetic macrocycle under conditions that favor said binding. The method may also be applied to synthesize a library of peptidomimetic macrocycles.

[00142] In some embodiments, the alkyne moiety of the peptidomimetic precursor of Formula V or Formula VI is a sidechain of an amino acid selected from the group consisting of L-propargylglycine, D-propargylglycine, (S)-2-amino-2-methyl-4-pentynoic acid, (R)-2-amino-2-methyl-4-pentynoic acid, (S)-2-amino-2-methyl-5-hexynoic acid, (R)-2-amino-2-methyl-5-hexynoic acid, (S)-2-amino-2-methyl-6-heptynoic acid, (R)-2-amino-2-methyl-6-heptynoic acid, (S)-2-amino-2-methyl-7-octynoic acid, (R)-2-amino-2-methyl-7-octynoic acid, (S)-2-amino-2-methyl-8-nonynoic acid, and (R)-2-amino-2-methyl-8-nonynoic acid. In other embodiments, the azide moiety of the peptidomimetic precursor of Formula V or Formula VI is a sidechain of an amino acid selected from the group

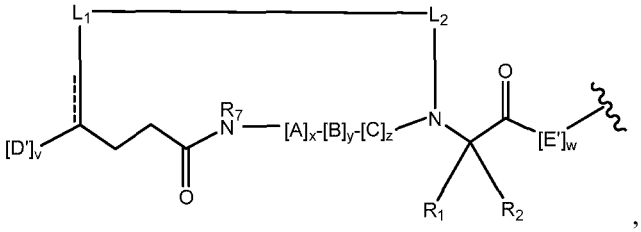
consisting of ε-azido-L-lysine, ε-azido-D-lysine, ε-azido-α-methyl-L-lysine, ε-azido-α-methyl-D-lysine, δ-azido-α-methyl-L-ornithine, and δ-azido-α-methyl-D-ornithine.

[00143] In some embodiments, x+y+z is 3, and and A, B and C are independently natural or non-natural amino acids. In other embodiments, x+y+z is 6, and and A, B and C are independently natural or non-natural amino acids.

[00144] In some embodiments of peptidomimetic macrocycles of the invention, $[D]_v$ and/or $[E]_w$ comprise additional peptidomimetic macrocycles or macrocyclic structures. For example, $[D]_v$ may have the formula:

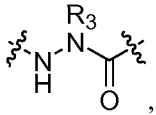


or



,

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

B is a natural or non-natural amino acid, amino acid analog,  , [-NH-L_3-CO-], [-NH-L_3-SO_2-], or [-NH-L_3-];

Ri and R_2 are independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo-, or part of a cyclic structure with an E residue;

R_3 is hydrogen, alkyl, alkenyl, alkynyl, arylalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, cycloalkylalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R_5;

Li and L_2 are independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, cycloarylene, heterocycloarylene, or $[-R_4\text{-K-Rr}]_n$, each being optionally substituted with R_5;

each R_4 is alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene;

each K is O, S, SO, SO_2, CO, CO_2, or $CONR_3$;

each R_5 is independently halogen, alkyl, $-OR_6$, $-N(Re)_2$, $-SR_6$, $-SOR_6$, $-SO_2R_6$, $-CO_2R_6$, a fluorescent moiety, a radioisotope or a therapeutic agent;

each R_6 is independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heterocycloalkyl, a fluorescent moiety, a radioisotope or a therapeutic agent;

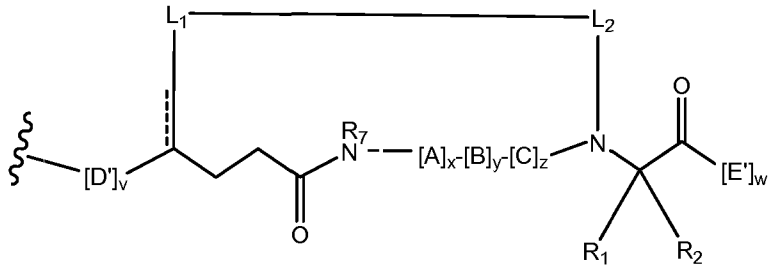
R_7 is -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R_5;

v is an integer from 1-1000;

w is an integer from 1-1000; and

x is an integer from 0-10.

[00145] In another embodiment, $[E]_w$ has the formula:



, wherein the substituents are as defined in the preceding paragraph.

[00146] In some embodiments, the contacting step is performed in a solvent selected from the group consisting of protic solvent, aqueous solvent, organic solvent, and mixtures thereof. For example, the solvent may be chosen from the group consisting OfH_2O, THF, THF/H_2O, tBuOH/H_2O, DMF, DIPEA, CH_3CN or CH_2Cl_2, $ClCH_2CH_2Cl$ or a mixture thereof. The solvent may be a solvent which favors helix formation.

[00147] Alternative but equivalent protecting groups, leaving groups or reagents are substituted, and certain of the synthetic steps are performed in alternative sequences or orders to produce the desired compounds. Synthetic chemistry transformations and protecting group methodologies (protection and deprotection) useful in synthesizing the compounds described herein include, for example, those such as described in Larock, Comprehensive Organic Transformations, VCH Publishers (1989); Greene and Wuts, Protective Groups in Organic Synthesis, 2d. Ed. , John Wiley and Sons (1991); Fieser and Fieser, Fieser and Fieser's Reagents for Organic Synthesis, John Wiley and Sons (1994); and Paquette, ed., Encyclopedia of Reagents for Organic Synthesis, John Wiley and Sons (1995), and subsequent editions thereof.

[00148] The peptidomimetic macrocycles of the invention are made, for example, by chemical synthesis methods, such as described in Fields *et ah,* Chapter 3 in Synthetic Peptides: A User's Guide, ed. Grant, W. H. Freeman & Co., New York, N. Y., 1992, p. 77. Hence, for example, peptides are synthesized using the automated Merrifield techniques of solid phase synthesis with the amine protected by either tBoc or Fmoc chemistry using side chain protected amino acids on, for example, an automated peptide synthesizer (e.g., Applied Biosystems (Foster City, CA), Model 430A, 431, or 433).

[00149] One manner of producing the peptidomimetic precursors and peptidomimetic macrocycles described herein uses 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. Side chain functional groups are protected as necessary with base stable, acid labile groups.

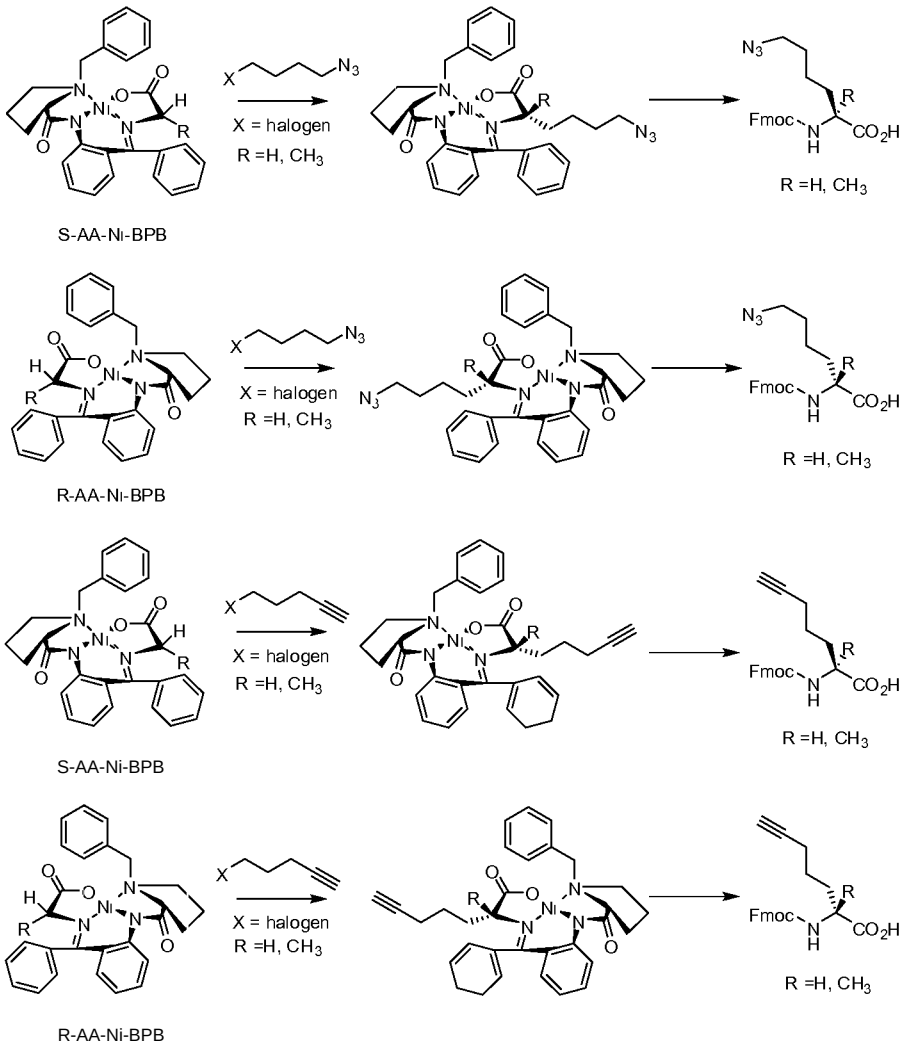
[00150] Longer peptidomimetic precursors are produced, for example, by conjoining individual synthetic peptides using native chemical ligation. Alternatively, the longer synthetic peptides are biosynthesized by well

known recombinant DNA and protein expression techniques. Such techniques are provided in well-known standard manuals with detailed protocols. To construct a gene encoding a peptidomimetic precursor 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.

[00151] The peptidomimetic precursors are made, for example, in a high-throughput, combinatorial fashion using, for example, a high-throughput polychannel combinatorial synthesizer *(e.g.*, Thuramed TETRAS multichannel peptide synthesizer from CreoSalus, Louisville, KY or Model Apex 396 multichannel peptide synthesizer from AAPPTEC, Inc., Louisville, KY).

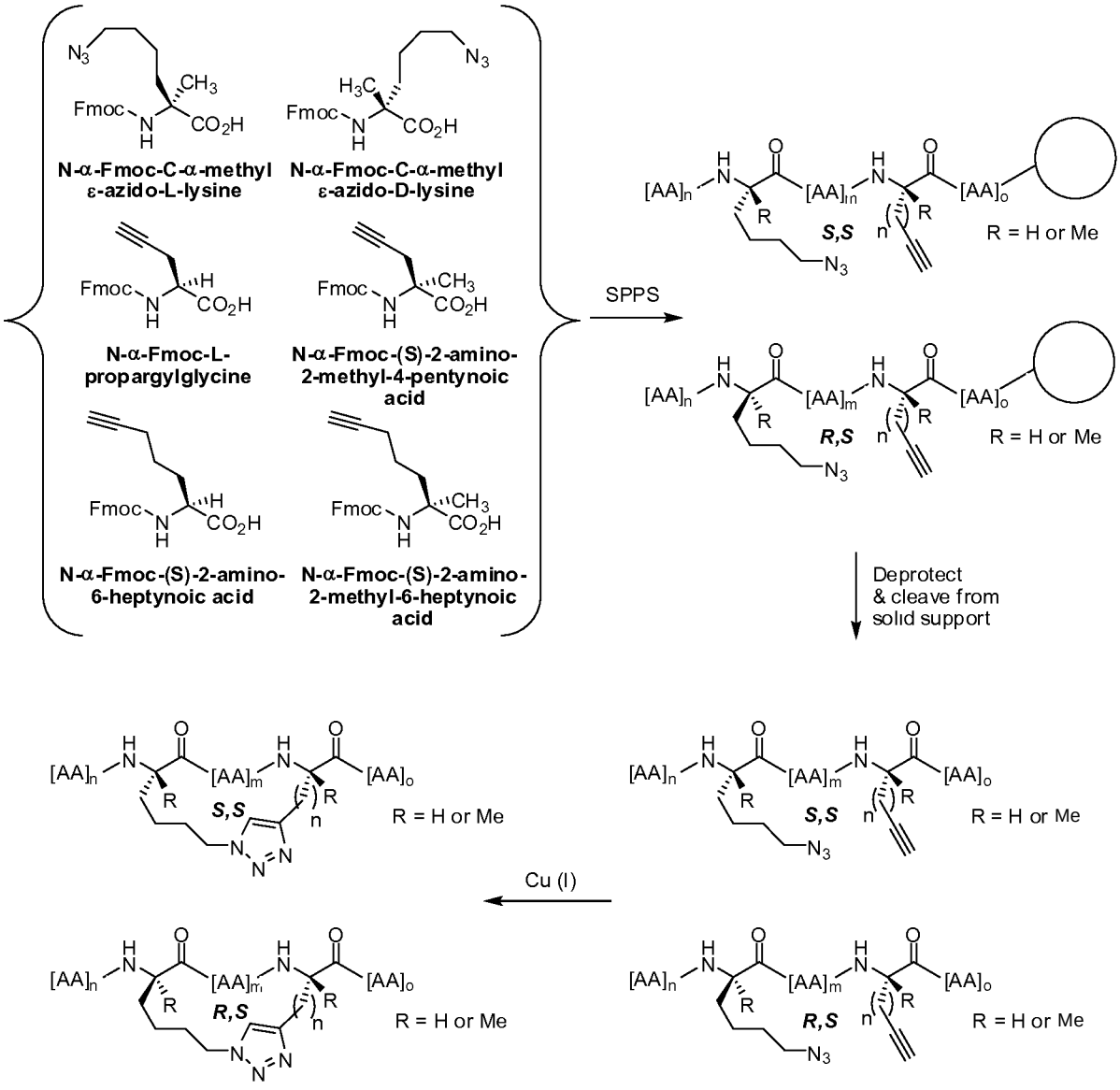
[00152] The following synthetic schemes are provided solely to illustrate the present invention and are not intended to limit the scope of the invention, as described herein. To simplify the drawings, the illustrative schemes depict azido amino acid analogs ε-azido-α-methyl-L-lysine and ε-azido-α-methyl-D-lysine, and alkyne amino acid analogs L-propargylglycine, (S)-2-amino-2-methyl-4-pentynoic acid, and (S)-2-amino-2-methyl-6-heptynoic acid. Thus, in the following synthetic schemes, each R_1, R_2, R_7 and R_8 is -H; each L_1 is $-(CH_2)_4-$; and each L_2 is $-(CH_2)-$. However, as noted throughout the detailed description above, many other amino acid analogs can be employed in which R_1, R_2, R_7, R_8, L_1 and L_2 can be independently selected from the various structures disclosed herein.

[00153] Synthetic Scheme 1:



[00154] Synthetic Scheme 1 describes the preparation of several compounds of the invention. Ni(II) complexes of Schiff bases derived from the chiral auxiliary (S)-2-[N-(N'-benzylprolyl)amino]benzophenone (BPB) and amino acids such as glycine or alanine are prepared as described in Belokon *et al.* (1998), *Tetrahedron Asymm.* 9:4249-4252. The resulting complexes are subsequently reacted with alkylating reagents comprising an azido or alkynyl moiety to yield enantiomerically enriched compounds of the invention. If desired, the resulting compounds can be protected for use in peptide synthesis.

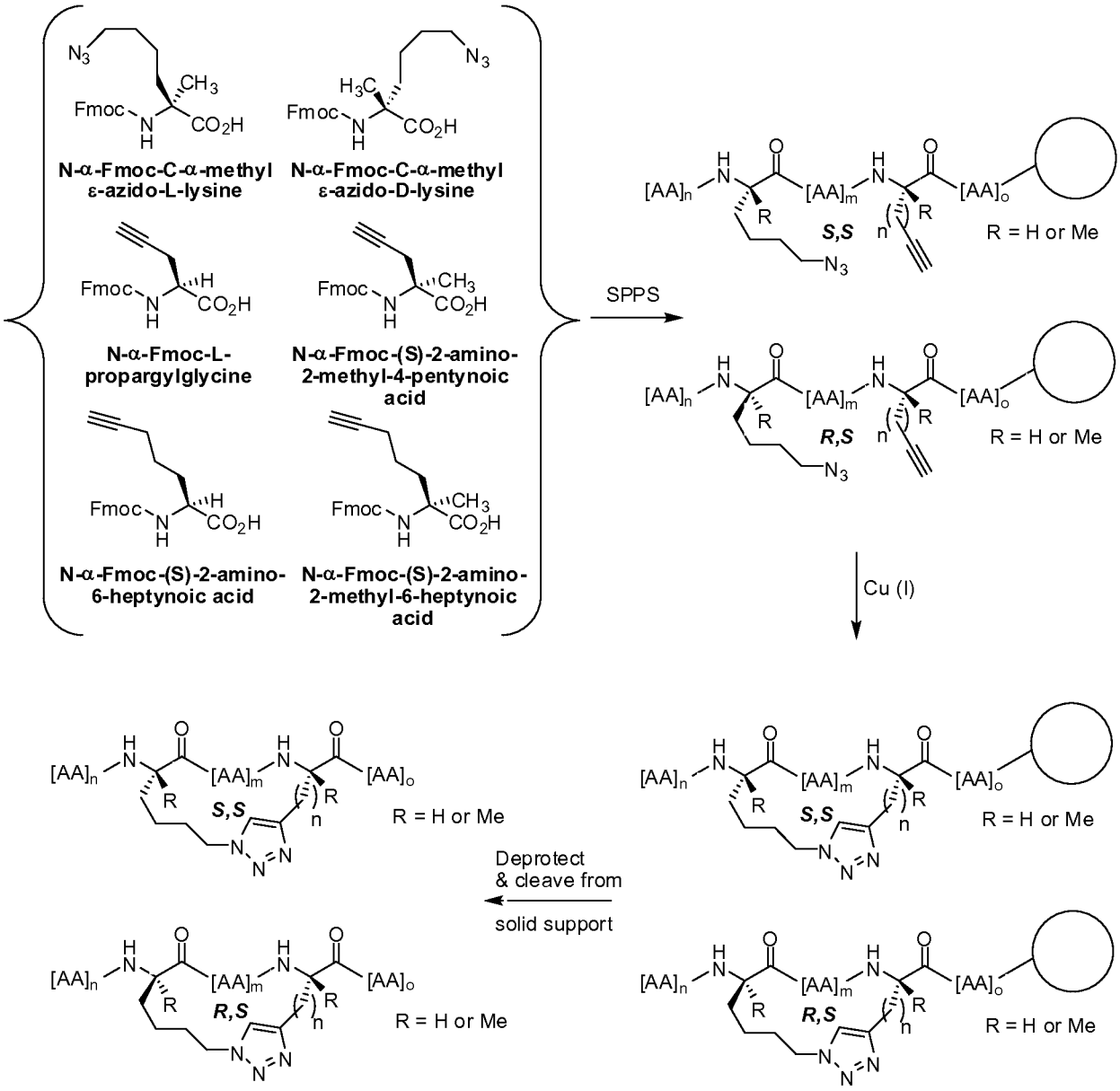
[00155] Synthetic Scheme 2:



[00156] In the general method for the synthesis of peptidomimetic macrocycles shown in Synthetic Scheme 2, the peptidomimetic precursor contains an azide moiety and an alkyne moiety and is synthesized by solution-phase or solid-phase peptide synthesis (SPPS) using the commercially available amino acid N-α-Fmoc-L-propargylglycine and the N-α-Fmoc-protected forms of the amino acids (S)-2-amino-2-methyl-4-pentynoic acid, (S)-2-amino-6-heptynoic acid, (S)-2-amino-2-methyl-6-heptynoic acid, N-methyl-ε-azido-L-lysine, and N-methyl-ε-azido-D-lysine. The peptidomimetic precursor is then deprotected and cleaved from the solid-phase resin by standard conditions *(e.g.,* strong acid such as 95% TFA). The peptidomimetic precursor is reacted as a crude mixture or is purified prior to reaction with a macrocyclization reagent such as a Cu(I) in organic or aqueous solutions (Rostovtsev *etal.* (2002), *Angew. Chem. Int. Ed.* 41:2596-2599; Tornoe *et al.* (2002), *J. Org. Chem.* 67:3057-3064; Deiters *et al.* (2003), *J. Am. Chem. Soc.* 125:11782-11783; Punnae^ *a/.* (2005), *Angew. Chem. Int. Ed.* 44:2215-2220). In one embodiment, the triazole forming reaction is performed under conditions that favor α-

helix formation. In one embodiment, the macrocyclization step is performed in a solvent chosen from the group consisting of H_2O, THF, CH_3CN, DMF , DIPEA, tBuOH or a mixture thereof. In another embodiment, the macrocyclization step is performed in DMF. In some embodiments, the macrocyclization step is performed in a buffered aqueous or partially aqueous solvent.

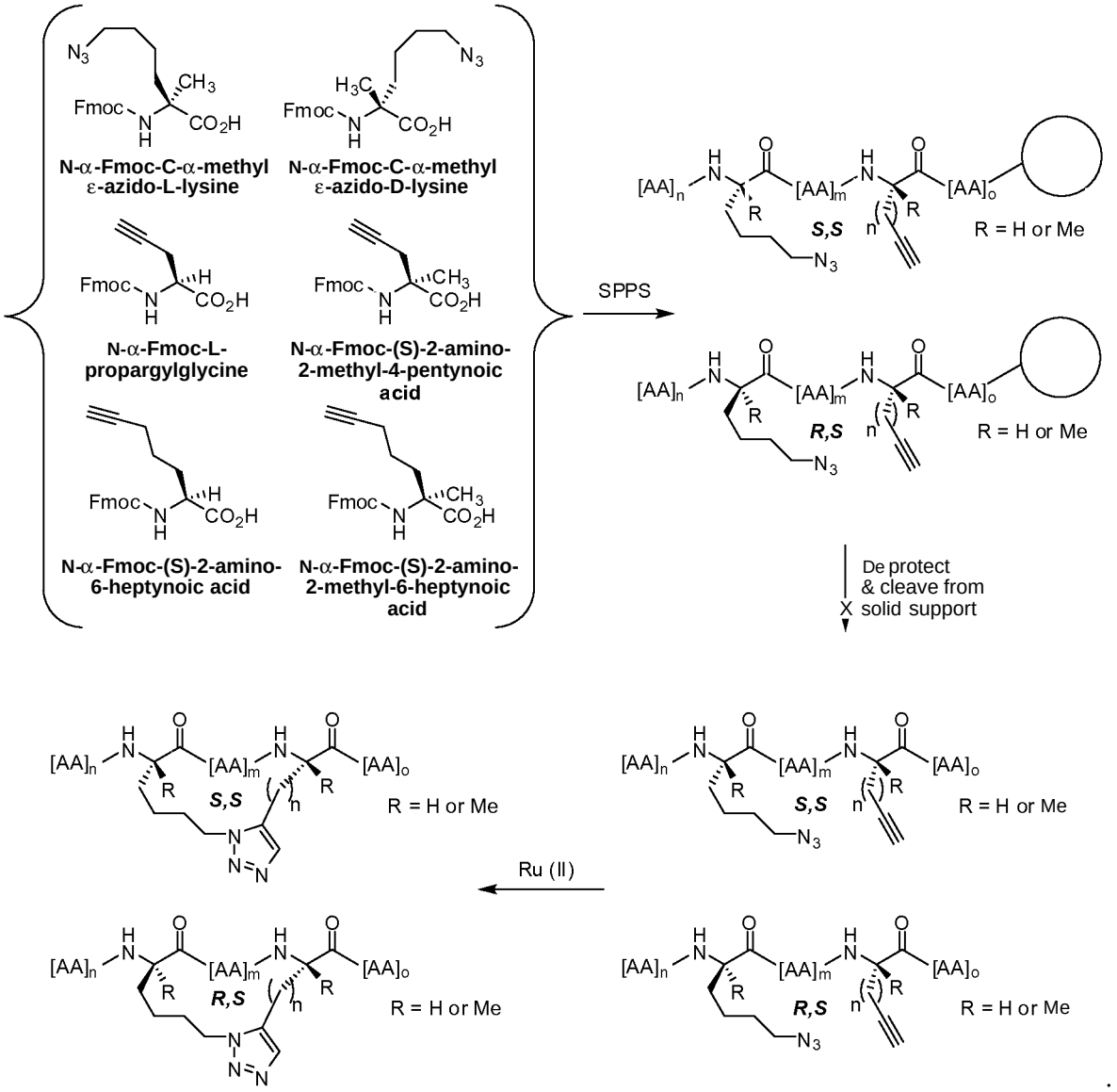
[00157] Synthetic Scheme 3:



[00158] In the general method for the synthesis of peptidomimetic macrocycles shown in Synthetic Scheme 3, the peptidomimetic precursor contains an azide moiety and an alkyne moiety and is synthesized by solid-phase peptide synthesis (SPPS) using the commercially available amino acid N-α-Fmoc-L-propargylglycine and the N-α-Fmoc-protected forms of the amino acids (S)-2-amino-2-methyl-4-pentynoic acid, (S)-2-amino-6-heptynoic acid, (S)-2-amino-2-methyl-6-heptynoic acid, N-methyl-ε-azido-L-lysine, and N-methyl-ε-azido-D-lysine. The

peptidomimetic precursor is reacted with a macrocyclization reagent such as a Cu(I) reagent on the resin as a crude mixture (Rostovtsev *et al.* (2002), *Angew. Chem. Int. Ed.* 41:2596-2599; Tornoe *et al.* (2002), *J. Org. Chem.* 67:3057-3064; Deiters *et al.* (2003), *J. Am. Chem. Soc.* 125:11782-11783; Punnae^ *al.* (2005), *Angew. Chem. Int. Ed.* 44:2215-2220). The resultant triazole-containing peptidomimetic macrocycle is then deprotected and cleaved from the solid-phase resin by standard conditions (e.g., strong acid such as 95% TFA). In some embodiments, the macrocyclization step is performed in a solvent chosen from the group consisting of CH_2CI_2, $ClCH_2CH_2Cl$, DMF, THF, NMP, DIPEA, 2,6-lutidine, pyridine, DMSO, H_2O or a mixture thereof. In some embodiments, the macrocyclization step is performed in a buffered aqueous or partially aqueous solvent.

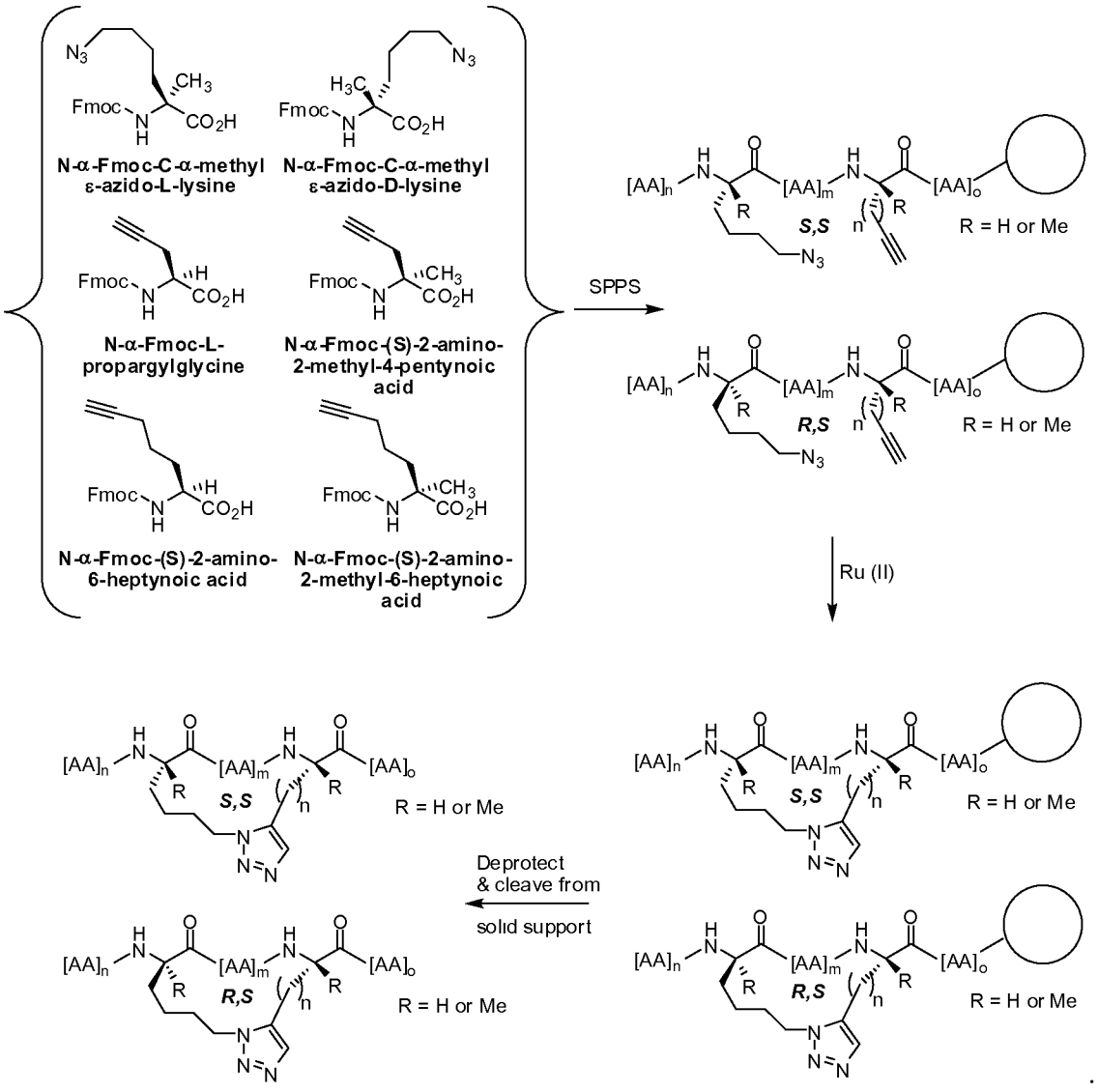
[00159] Synthetic Scheme 4:



[00160] In the general method for the synthesis of peptidomimetic macrocycles shown in Synthetic Scheme 4, the peptidomimetic precursor contains an azide moiety and an alkyne moiety and is synthesized by solution-phase or solid-phase peptide synthesis (SPPS) using the commercially available amino acid N-α-Fmoc-L-propargylglycine

and the N-α-Fmoc-protected forms of the ammo acids (S)-2-ammo-2-methyl-4-pentynoic acid, (S)-2-ammo-6-heptynoic acid, (S)-2-ammo-2-methyl-6-heptynoic acid, N-methyl-ε-azido-L-lysme, and N-methyl-ε-azido-D-lysme. The peptidomimetic precursor is then deprotected and cleaved from the solid-phase resm by standard conditions *(e g* , strong acid such as 95% TFA). The peptidomimetic precursor is reacted as a crude mixture or is purified prior to reaction with a macrocychzation reagent such as a Ru(II) reagents, for example $Cp^*RuCl(PPh_3)_2$ or $[Cp^*RuCl]_4$ (Rasmussen *et al* (2007), *Org Lett* 9:5337'-5339; Zhang *et al* (2005), *J Am Chem Soc* 127:15998-15999). In some embodiments, the macrocychzation step is performed m a solvent chosen from the group consisting of DMF, CH_3CN and THF.

[00161] Synthetic Scheme 5:



[00162] In the general method for the synthesis of peptidomimetic macrocycles shown m Synthetic Scheme 5, the peptidomimetic precursor contains an azide moiety and an alkyne moiety and is synthesized by solid-phase peptide synthesis (SPPS) using the commercially available ammo acidN-α-Fmoc-L-propargylglycme and the N-α-Fmoc-protected forms of the ammo acids (S)-2-ammo-2-methyl-4-pentynoic acid, (S)-2-ammo-6-heptynoic acid,

(S)-2-amino-2-methyl-6-heptynoic acid, N-methyl-ε-azido-L-lysine, and N-methyl-ε-azido-D-lysine. The peptidomimetic precursor is reacted with a macrocyclization reagent such as a Ru(II) reagent on the resin as a crude mixture. For example, the reagent can be $Cp^*RuCl(PPh_3)_2$ or $[Cp^*RuCl]_4$ (Rasmussen *et al.* (2007), *Org. Lett.* 9:5337-5339; Zhang *et al.* (2005), *J. Am. Chem. Soc.* 127:15998-15999). In some embodiments, the macrocyclization step is performed in a solvent chosen from the group consisting of CH_2Cl_2, $ClCH_2CH_2Cl$, CH_3CN, DMF, and THF.

[00163] Several exemplary peptidomimetic macrocycles are shown in Table 5. "NIe" represents norleucine and replaces a methionine residue. It is envisioned that similar linkers are used to synthesize peptidomimetic macrocycles based on the polypeptide sequences disclosed in Table 1 through Table 4.

TABLE 5

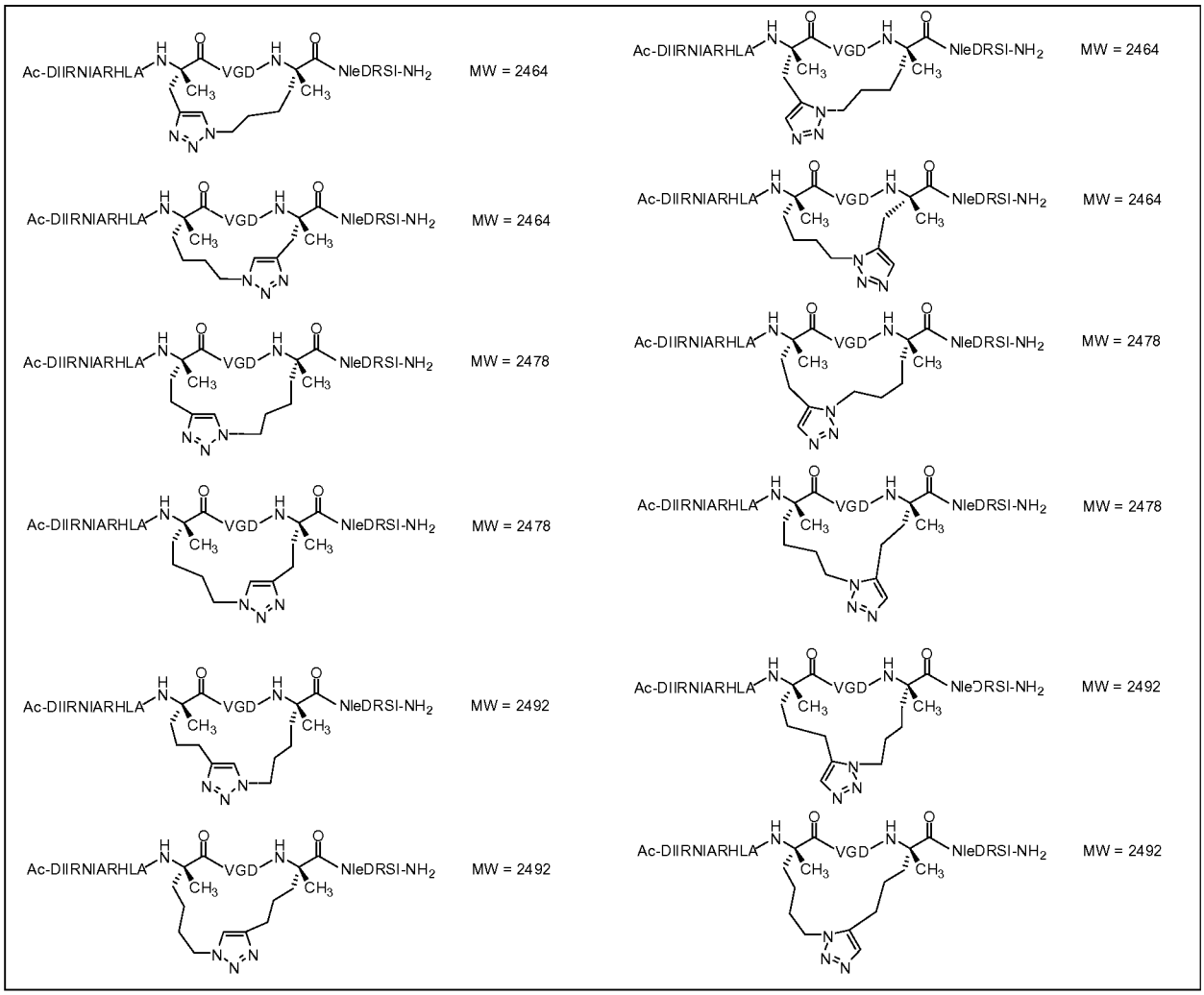


Table 5 shows exemplary peptidommimetic macrocycles of the invention. "NIe" represents norleucine.

[00164] The present invention contemplates the use of non-naturally-occurring amino acids and amino acid analogs in the synthesis of the peptidomimetic macrocycles described herein. Any amino acid or amino acid analog amenable to the synthetic methods employed for the synthesis of stable triazole containing peptidomimetic macrocycles can be used in the present invention. For example, L-propargylglycine is contemplated as a useful amino acid in the present invention. However, other alkyne-containing amino acids that contain a different amino acid side chain are also useful in the invention. For example, L-propargylglycine contains one methylene unit between the α-carbon of the amino acid and the alkyne of the amino acid side chain. The invention also

contemplates the use of amino acids with multiple methylene units between the α-carbon and the alkyne. Also, the azido-analogs of amino acids L-lysine, D-lysine, alpha-methyl-L-lysine, and alpha-methyl-D-lysine are contemplated as useful amino acids in the present invention. However, other terminal azide amino acids that contain a different amino acid side chain are also useful in the invention. For example, the azido-analog of L-lysine contains four methylene units between the α-carbon of the amino acid and the terminal azide of the amino acid side chain. The invention also contemplates the use of amino acids with fewer than or greater than four methylene units between the α-carbon and the terminal azide. Table 6 shows some amino acids useful in the preparation of peptidomimetic macrocycles of the invention.

TABLE 6

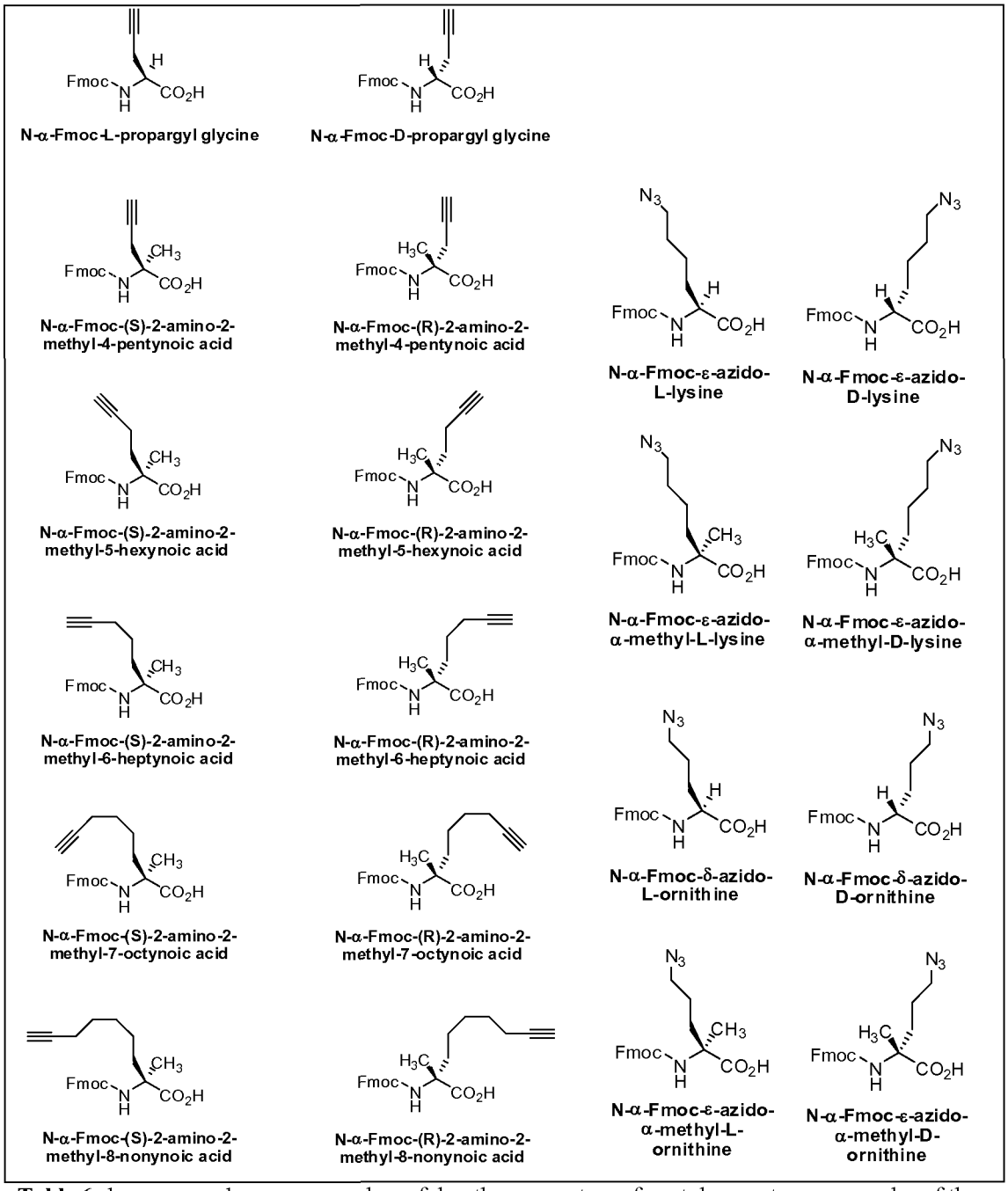


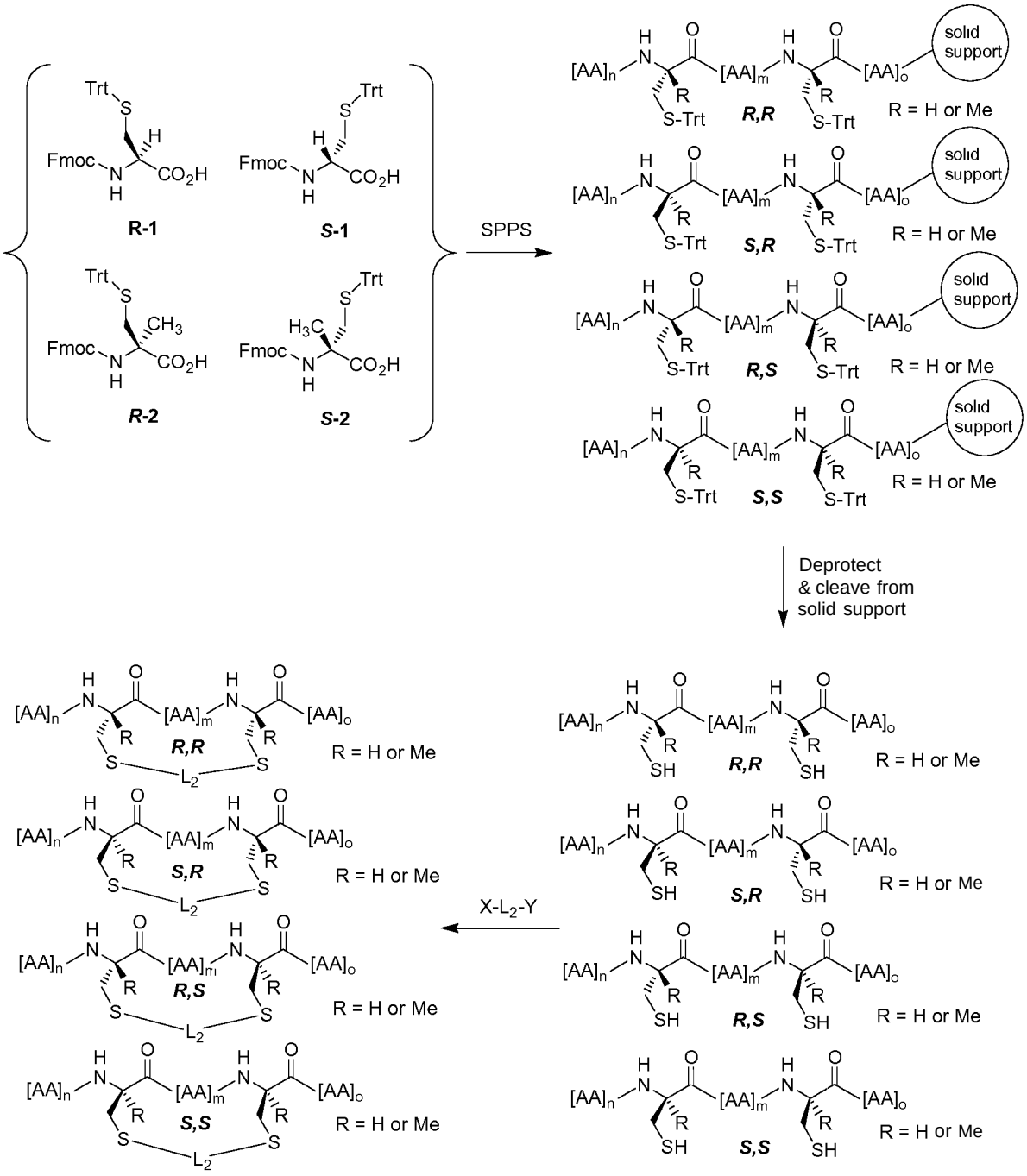
Table 6 shows exemplary amino acids useful in the preparation of peptidomimetic macrocycles of the invention.

[00165] In some embodiments the ammo acids and ammo acid analogs are of the D-configuration. In other embodiments they are of the L-configuration. In some embodiments, some of the ammo acids and ammo acid analogs contained m the peptidomimetic are of the D-configuration while some of the ammo acids and ammo acid analogs are of the L-configuration. In some embodiments the ammo acid analogs are α,α-disubstituted, such as α-methyl-L-propargylglycme, α-methyl-D-propargylglycme, ε-azido-alpha-methyl-L-lysme, and ε-azido-alpha-methyl-D-lysme. In some embodiments the ammo acid analogs are N-alkylated, *e g* , N-methyl-L-propargylglycme, N-methyl-D-propargylglycme, N-methyl-ε-azido-L-lysme, and N-methyl-ε-azido-D-lysme.

[00166] In some embodiments, the -NH moiety of the amino acid is protected using a protecting group, including without limitation -Fmoc and -Boc. In other embodiments, the amino acid is not protected prior to synthesis of the peptidomimetic macrocycle.

[00167] In other embodiments, peptidomimetic macrocycles of Formula III are synthesized. The preparation of such macrocycles is described, for example, in US Application 11/957,325, filed on December 17, 2007. The following synthetic schemes describe the preparation of such compounds. To simplify the drawings, the illustrative schemes depict amino acid analogs derived from L-or D-cysteine, in which L_1 and L_3 are both -(CH_2)-. However, as noted throughout the detailed description above, many other amino acid analogs can be employed in which L_1 and L_3 can be independently selected from the various structures disclosed herein. The symbols "$[AA]_m$", "$[AA]_n$", "$[AA]_0$" represent a sequence of amide bond-linked moieties such as natural or unnatural amino acids. As described previously, each occurrence of "AA" is independent of any other occurrence of "AA", and a formula such as "$[AA]_m$" encompasses, for example, sequences of non-identical amino acids as well as sequences of identical amino acids.

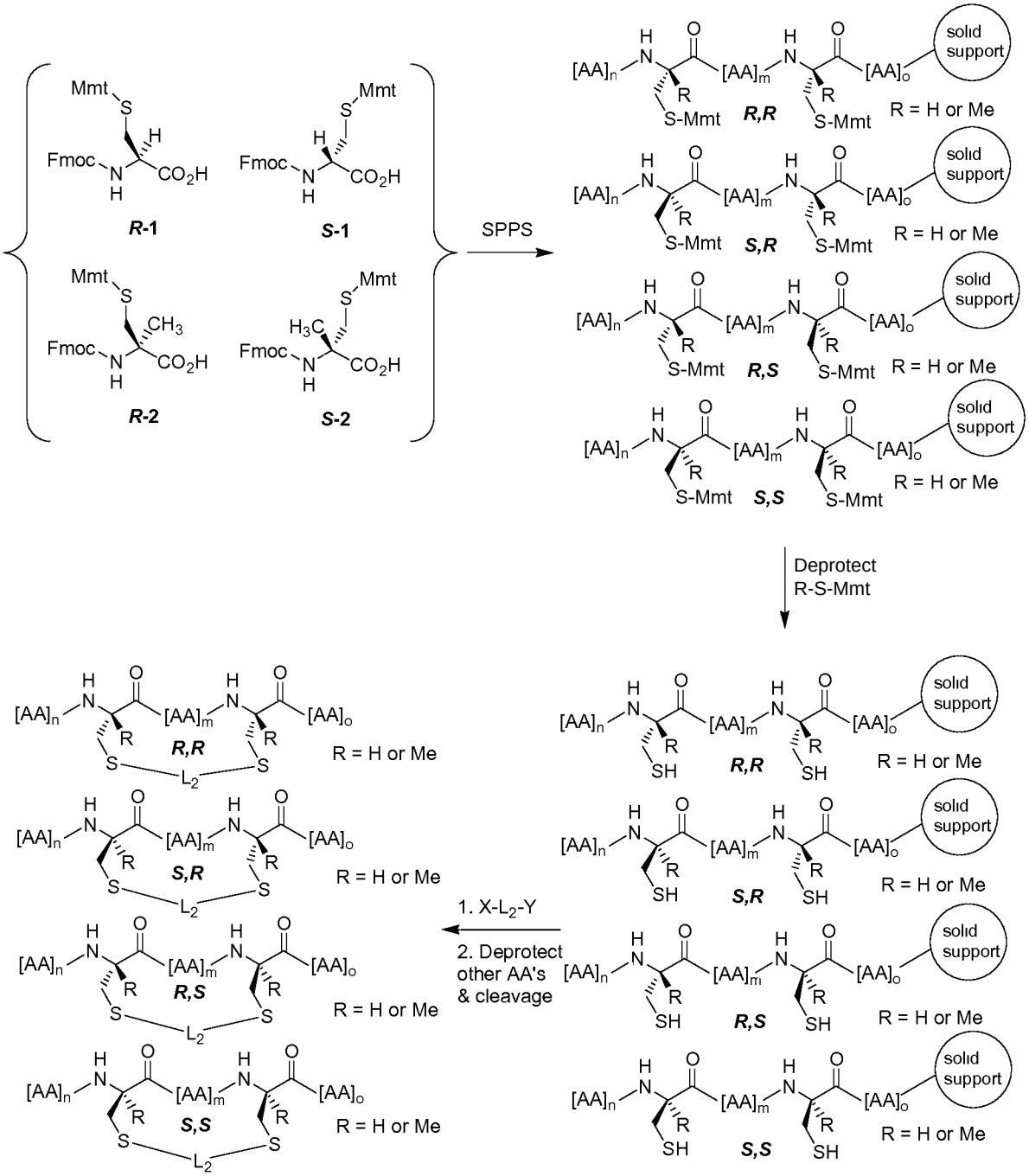
Synthetic Scheme 6:



[00168] In Scheme 6, the peptidomimetic precursor contains two -SH moieties and is synthesized by solid-phase peptide synthesis (SPPS) using commercially available N-α-Fmoc amino acids such as N-α-Fmoc-S-trityl-L-cysteine or N-α-Fmoc-S-trityl-D-cysteine. Alpha-methylated versions of D-cysteine or L-cysteine are generated by known methods (Seebach *et al.* (1996), *Angew. Chem. Int. Ed. Engl.* 35:2708-2748, and references therein) and then converted to the appropriately protected N-α-Fmoc-S-trityl monomers by known methods ("Bioorganic Chemistry: Peptides and Proteins", Oxford University Press, New York: 1998, the entire contents of which are incorporated herein by reference). The precursor peptidomimetic is then deprotected and cleaved from the solid-phase resin by standard conditions (e.g., strong acid such as 95% TFA). The precursor peptidomimetic is reacted

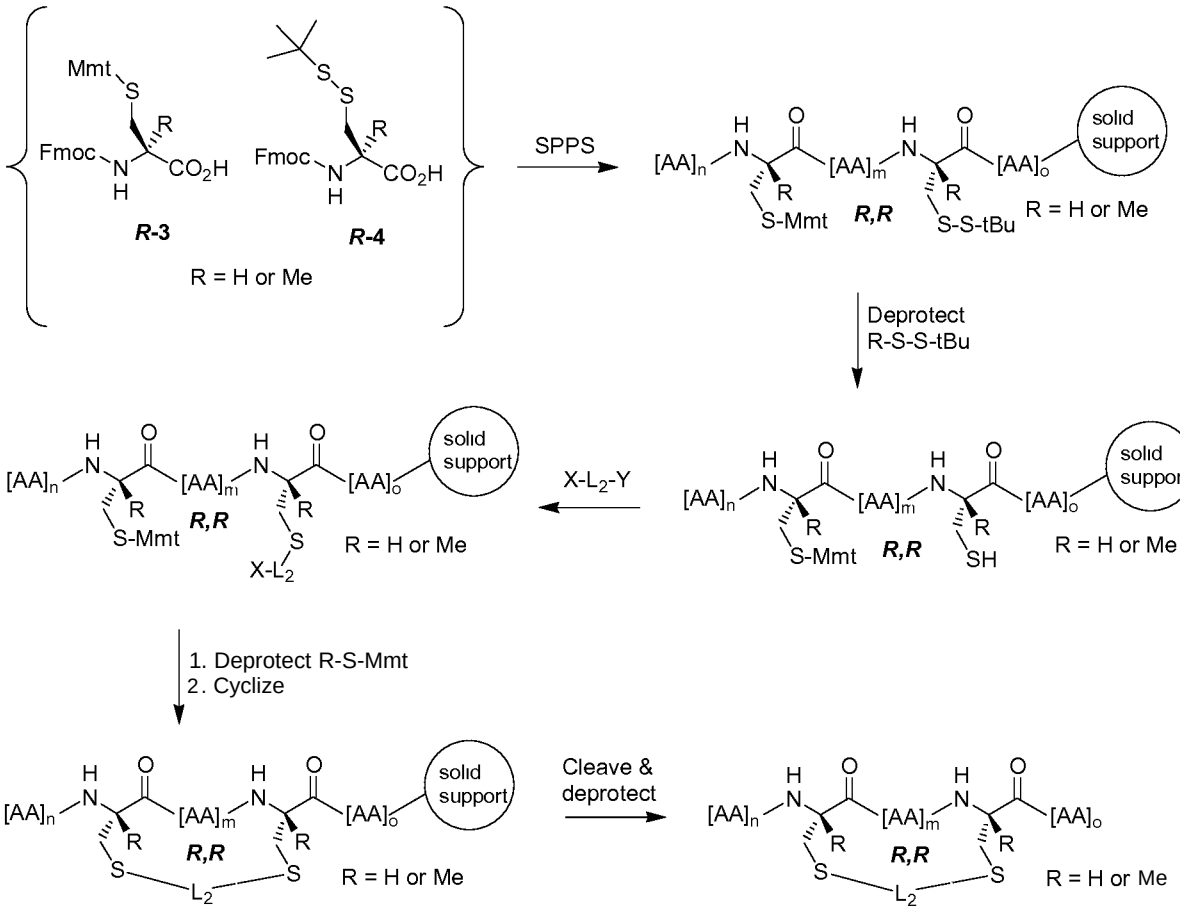
as a crude mixture or is purified prior to reaction with X-L_2-Y in organic or aqueous solutions. In some embodiments the alkylation reaction is performed under dilute conditions (i.e. 0.15 mmol/L) to favor macrocyclization and to avoid polymerization. In some embodiments, the alkylation reaction is performed in organic solutions such as liquid NH_3 (Mosberg et al. (1985), J. Am.Chem. Soc. 107:2986-2987; Szewczuk et al. (1992), Int. J. Peptide Protein Res. 40 :233-242), NH_3MeOH, or NH_3/DMF (Or et al. (1991), J. Org. Chem. 56:3146-3149). In other embodiments, the alkylation is performed in an aqueous solution such as 6M guanidinium HCL, pH 8 (Brunei et al. (2005), Chem. Commun. (20):2552-2554). In other embodiments, the solvent used for the alkylation reaction is DMF or dichloroethane.

Synthetic Scheme 7:



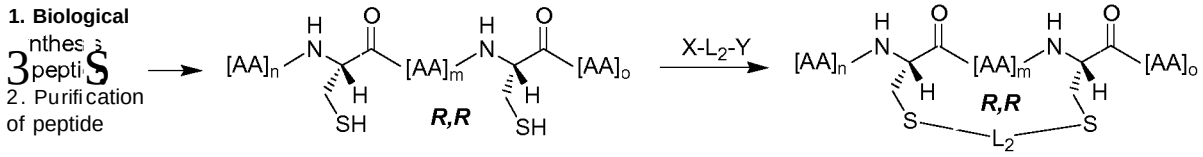
[00169] In Scheme 7, the precursor peptidomimetic contains two or more -SH moieties, of which two are specially protected to allow their selective deprotection and subsequent alkylation for macrocycle formation. The precursor peptidomimetic is synthesized by solid-phase peptide synthesis (SPPS) using commercially available N-α-Fmoc amino acids such as N-α-Fmoc-S-p-methoxytrityl-L-cysteine or N-α-Fmoc-S-p-methoxytrityl-D-cysteine. Alpha-methylated versions of D-cysteine or L-cysteine are generated by known methods (Seebach *et al.* (1996), *Angew. Chem. Int. Ed. Engl.* 35:2708-2748, and references therein) and then converted to the appropriately protected N-α-Fmoc-S-/>-methoxytrityl monomers by known methods (Bioorganic Chemistry: Peptides and Proteins, Oxford University Press, New York: 1998, the entire contents of which are incorporated herein by reference). The Mmt protecting groups of the peptidomimetic precursor are then selectively cleaved by standard conditions (e.g., mild acid such as 1% TFA in DCM). The precursor peptidomimetic is then reacted on the resin with $X\text{-}L_2\text{-}Y$ in an organic solution. For example, the reaction takes place in the presence of a hindered base such as diisopropylethylamine. In some embodiments, the alkylation reaction is performed in organic solutions such as liquid NH_3 (Mosberg *et al.* (1985), *J. Am.Chem. Soc.* 107:2986-2987; Szewczuk *et al.* (1992), *Int. J. Peptide Protein Res.* 40 :233-242), NH_3MeOH orNH $_3$/DMF (Or *et al.* (1991), *J. Org. Chem.* 56:3146-3149). In other embodiments, the alkylation reaction is performed in DMF or dichloroethane. The peptidomimetic macrocycle is then deprotected and cleaved from the solid-phase resin by standard conditions (e.g., strong acid such as 95% TFA).

Synthetic Scheme 8:



[00170] In Scheme 8, the peptidomimetic precursor contains two or more -SH moieties, of which two are specially protected to allow their selective deprotection and subsequent alkylation for macrocycle formation. The peptidomimetic precursor is synthesized by solid-phase peptide synthesis (SPPS) using commercially available N-α-Fmoc amino acids such as N-α-Fmoc-S-p-methoxytrityl-L-cysteine, N-α-Fmoc-S-p-methoxytrityl-D-cysteine, N-α-Fmoc-S-S-t-butyl-L-cysteine, and N-α-Fmoc-S-S-t-butyl-D-cysteine. Alpha-methylated versions of D-cysteine or L-cysteine are generated by known methods (Seebach *et al.* (1996), *Angew. Chem. Int. Ed. Engl.* 35:2708-2748, and references therein) and then converted to the appropriately protected N-α-Fmoc-S-/>-methoxytrityl or N-α-Fmoc-S-S-t-butyl monomers by known methods (Bioorganic Chemistry: Peptides and Proteins, Oxford University Press, New York: 1998, the entire contents of which are incorporated herein by reference). The S-S-tButyl protecting group of the peptidomimetic precursor is selectively cleaved by known conditions (e.g., 20% 2-mercaptoethanol in DMF, reference: Galande *et al.* (2005), *J. Comb. Chem.* 7:174-177). The precursor peptidomimetic is then reacted on the resin with a molar excess OfX-L_2-Y in an organic solution. For example, the reaction takes place in the presence of a hindered base such as diisopropylethylamine. The Mmt protecting group of the peptidomimetic precursor is then selectively cleaved by standard conditions (e.g., mild acid such as 1% TFA in DCM). The peptidomimetic precursor is then cyclized on the resin by treatment with a hindered base in organic solutions. In some embodiments, the alkylation reaction is performed in organic solutions such as NH_3MeOH or NH_3/DMF (Or *et al.* (1991), *J. Org. Chem.* 56:3 146-3 149). The peptidomimetic macrocycle is then deprotected and cleaved from the solid-phase resin by standard conditions (e.g., strong acid such as 95% TFA).

Synthetic Scheme 9:



[00171] In Scheme 9, the peptidomimetic precursor contains two L-cysteine moieties. The peptidomimetic precursor is synthesized by known biological expression systems in living cells or by known *in vitro,* cell-free, expression methods. The precursor peptidomimetic is reacted as a crude mixture or is purified prior to reaction with X-L2-Y in organic or aqueous solutions. In some embodiments the alkylation reaction is performed under dilute conditions (i.e. 0.15 mmol/L) to favor macrocyclization and to avoid polymerization. In some embodiments, the alkylation reaction is performed in organic solutions such as liquid NH_3 (Mosberg et al. (1985), J. Am.Chem. Soc. 107:2986-2987; Szewczuk et al. (1992), Int. J. Peptide Protein Res. 40 :233-242), NH_3MeOH, orNH_3/DMF (Or et al. (1991), J. Org. Chem. 56:3146-3149). In other embodiments, the alkylation is performed in an aqueous solution such as 6M guanidinium HCL, pH 8 (Brunei et al. (2005), Chem. Commun. (20):2552-2554). In other embodiments, the alkylation is performed in DMF or dichloroethane. In another embodiment, the alkylation is performed in non-denaturing aqueous solutions, and in yet another embodiment the alkylation is performed under conditions that favor α-helical structure formation. In yet another embodiment, the alkylation is performed under conditions that favor the binding of the precursor peptidomimetic to another protein, so as to induce the formation of the bound α-helical conformation during the alkylation.

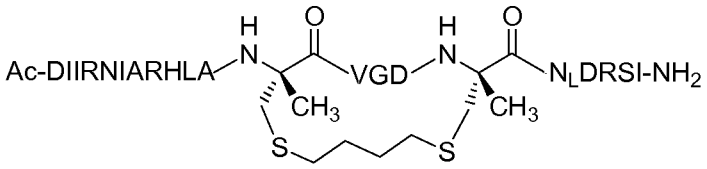
[00172] Various embodiments for X and Y are envisioned which are suitable for reacting with thiol groups. In general, each X or Y is independently be selected from the general category shown in Table 5. For example, X and Y are halides such as -Cl, -Br or -I. Any of the macrocycle-forming linkers described herein may be used in any combination with any of the sequences shown in Tables 1-4 and also with any of the R- substituents indicated herein.

TABLE 7: **Examples of Reactive Groups Capable of Reacting with Thiol Groups and Resulting Linkages**

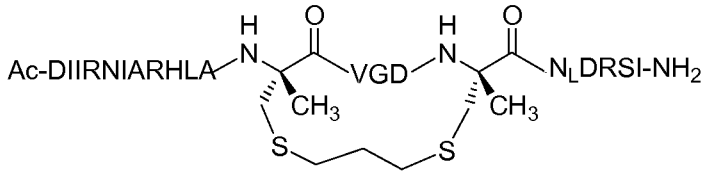
X or Y	Resulting Covalent Linkage
acrylamide	Thioether
halide (e.g. alkyl or aryl halide)	Thioether
sulfonate	Thioether
aziridine	Thioether
epoxide	Thioether
haloacetamide	Thioether
maleimide	Thioether
sulfonate ester	Thioether

[00173] Table 6 shows exemplary macrocycles of the invention. "N_L" represents norleucine and replaces a methionine residue. It is envisioned that similar linkers are used to synthesize peptidomimetic macrocycles based on the polypeptide sequences disclosed in Table 1 through Table 4.

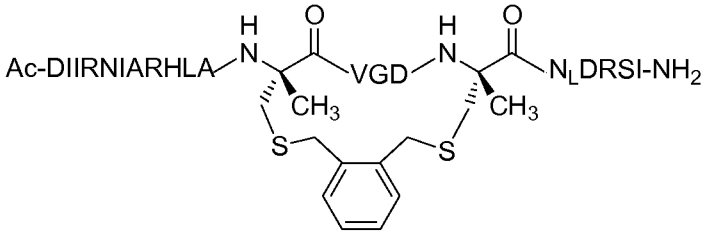
TABLE 8: Examples of Peptidomimetic Macrocycles of the Invention



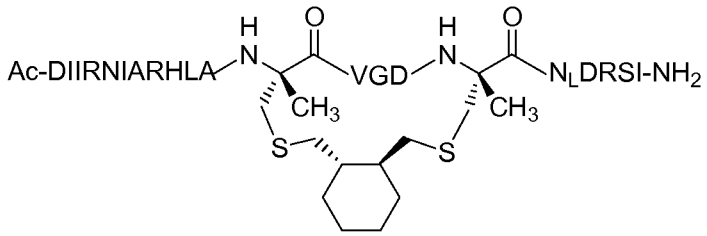
MW = 2477



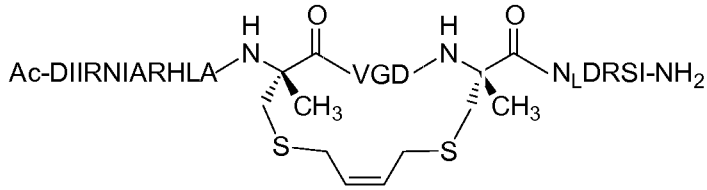
MW = 2463



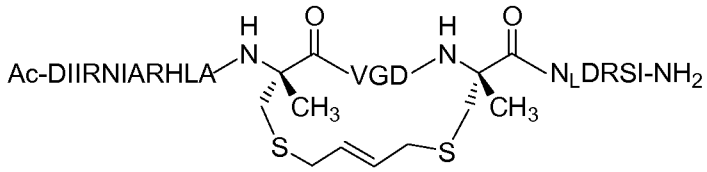
MW = 2525



MW = 2531



MW = 2475



MW = 2475

For the examples shown m this table, "N_L" represents norleucme.

[00174] The present invention contemplates the use of both naturally-occurring and non-naturally-occurrmg ammo acids and ammo acid analogs m the synthesis of the peptidomimetic macrocycles of Formula (III). Any ammo acid or ammo acid analog amenable to the synthetic methods employed for the synthesis of stable bis-sulfhydryl containing peptidomimetic macrocycles can be used m the present invention. For example, cysteine is contemplated as a useful ammo acid m the present invention. However, sulfur containing ammo acids other than cysteine that contain a different ammo acid side chain are also useful. For example, cysteine contains one

methylene unit between the α-carbon of the amino acid and the terminal -SH of the amino acid side chain. The invention also contemplates the use of amino acids with multiple methylene units between the α-carbon and the terminal -SH. Non-limiting examples include α-methyl-L-homocysteine and α-methyl-D-homocysteine. In some embodiments the amino acids and amino acid analogs are of the D- configuration. In other embodiments they are of the L- configuration. In some embodiments, some of the amino acids and amino acid analogs contained in the peptidomimetic are of the D- configuration while some of the amino acids and amino acid analogs are of the L-configuration. In some embodiments the amino acid analogs are α,α-disubstituted, such as α-methyl-L-cysteine and α-methyl-D-cysteine.

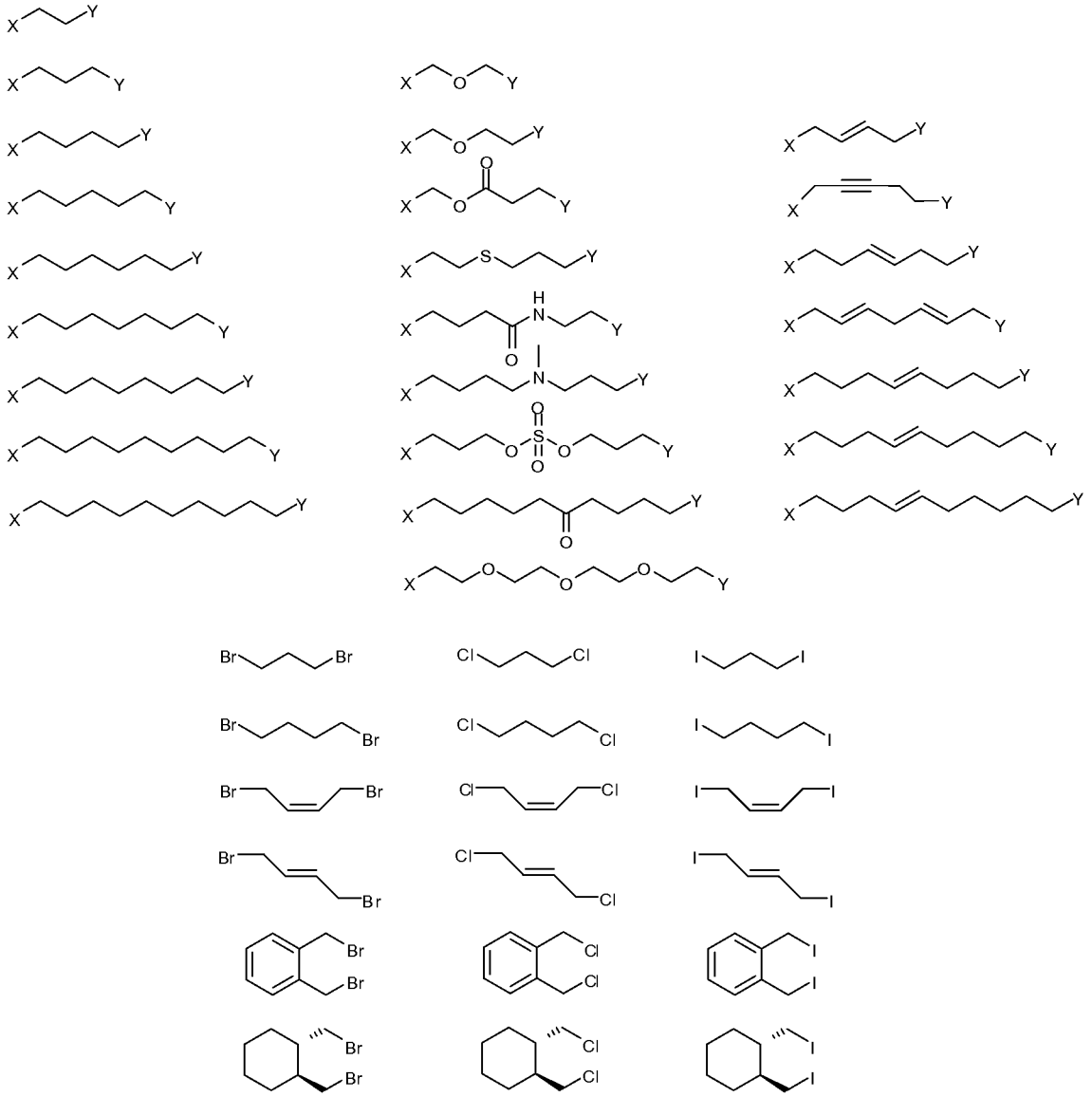
[00175] The invention includes macrocycles in which macrocycle-forming linkers are used to link two or more -SH moieties in the peptidomimetic precursors to form the peptidomimetic macrocycles of the invention. As described above, the macrocycle-forming linkers impart conformational rigidity, increased metabolic stability and/or increased cell penetrability. Furthermore, in some embodiments, the macrocycle-forming linkages stabilize the α-helical secondary structure of the peptidomimetic macrocyles. The macrocycle-forming linkers are of the formula X-L_2-Y, wherein both X and Y are the same or different moieties, as defined above. Both X and Y have the chemical characteristics that allow one macrocycle-forming linker - L_2- to bis alkylate the bis-sulfhydryl containing peptidomimetic precursor. As defined above, the linker - L_2- includes alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, cycloarylene, or heterocycloarylene, or - R_4-K-R_4- , all of which can be optionally substituted with an R_5 group, as defined above. Furthermore, one to three carbon atoms within the macrocycle-forming linkers - L_2-, other than the carbons attached to the -SH of the sulfhydryl containing amino acid, are optionally substituted with a heteroatom such as N, S or O.

[00176] The L_2 component of the macrocycle-forming linker X-L_2-Y may be varied in length depending on, among other things, the distance between the positions of the two amino acid analogs used to form the peptidomimetic macrocycle. Furthermore, as the lengths OfL_1 and/or L_3 components of the macrocycle-forming linker are varied, the length of L_2 can also be varied in order to create a linker of appropriate overall length for forming a stable peptidomimetic macrocycle. For example, if the amino acid analogs used are varied by adding an additional methylene unit to each of L_1 and L_3, the length of L_2 are decreased in length by the equivalent of approximately two methylene units to compensate for the increased lengths OfL_1 and L_3.

[00177] In some embodiments, L_2 is an alkylene group of the formula -$(CH_2)_n$- , where n is an integer between about 1 and about 15. For example, n is 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10. In other embodiments, L_2 is an alkenylene group. In still other embodiments, L_2 is an aryl group.

[00178] Table 9 shows additional embodiments Of X-L_2- Y groups.

TABLE 9. Exemplary X-L $_2$- Y groups of the invention.



Each X and Y m this table, is, for example, independently Cl-, Br- or I-.

[00179] Additional methods of forming peptidomimetic macrocycles which are envisioned as suitable to perform the present invention include those disclosed by Mustapa, M. Firouz Mohd et al., J. Org. Chem (2003), 68, pp. 8193-8198; Yang, Bm et al. Bioorg Med. Chem. Lett. (2004), 14, pp. 1403-1406; U.S. Patent No. 5,364,851; U.S. Patent No. 5,446,128; U.S. Patent No. 5,824,483; U.S. Patent No. 6,713,280; and U.S. Patent No. 7,202,332. In such embodiments, ammoacid precursors are used containing an additional substituent R- at the alpha position. Such ammoacids are incorporated into the macrocycle precursor at the desired positions, which may be at the positions where the crosslinker is substituted or, alternatively, elsewhere m the sequence of the macrocycle precursor. Cyclization of the precursor is then effected according to the indicated method.

[00180] Methods of Use

[00181] In one embodiment, the invention relates to a method for treating a subject having a disease or at risk of developing a disease caused by the expression of a target gene. In this embodiment, the composition of the invention may act as a novel therapeutic agent for controlling one or more of cellular proliferative and/or differentiative disorders, disorders associated with bone metabolism, immune disorders, hematopoietic disorders, cardiovascular disorders, liver disorders, viral diseases, or metabolic disorders. The method comprises administering a pharmaceutical composition of the invention to the subject (e.g., human), such that expression of the target gene is modified, either by upregulation or downregulation.

[00182] In the prevention of disease, the target gene may be one which is required for initiation or maintenance of the disease, or which has been identified as being associated with a higher risk of contracting the disease. In the treatment of disease, the composition of the present invention can be brought into contact with the cells or tissue exhibiting the disease. In a preferred embodiment, the composition of the present invention may enter a cell with a faster rate than a molecule that is not associated with a peptidomimetic macrocycle. For example, a composition of the present invention containing a nucleic acid molecule substantially identical to all or part of a mutated gene associated with cancer, or one expressed at high levels in tumor cells, may be brought into contact with or introduced into a cancerous cell or tumor gene.

[00183] In some embodiments, the compositions of the invention may be used to treat, prevent, and/or diagnose cancers and neoplastic conditions. As used herein, the terms "cancer", "hyperproliferative" and "neoplastic" refer to cells having the capacity for autonomous growth, *i.e.*, an abnormal state or condition characterized by rapidly proliferating cell growth. Hyperproliferative and neoplastic disease states may be categorized as pathologic, *i.e.*, characterizing or constituting a disease state, or may be categorized as non-pathologic, *i.e.*, a deviation from normal but not associated with a disease state. The term is meant to include all types of cancerous growths or oncogenic processes, metastatic tissues or malignantly transformed cells, tissues, or organs, irrespective of histopathologic type or stage of invasiveness. A metastatic tumor can arise from a multitude of primary tumor types, including but not limited to those of breast, lung, liver, colon and ovarian origin. "Pathologic hyperproliferative" cells occur in disease states characterized by malignant tumor growth. Examples of non-pathologic hyperproliferative cells include proliferation of cells associated with wound repair. Examples of cellular proliferative and/or differentiative disorders include cancer, *e.g.*, carcinoma, sarcoma, or metastatic disorders. In some embodiments, the compositions of the present invention are novel therapeutic agents for controlling breast cancer, ovarian cancer, colon cancer, lung cancer, metastasis of such cancers and the like.

[00184] Examples of cancers or neoplastic conditions include, but are not limited to, a fibrosarcoma, myosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, gastric cancer, esophageal cancer, rectal cancer, pancreatic cancer, ovarian cancer, prostate cancer, uterine cancer, cancer of the head and neck, skin cancer, brain cancer, squamous cell carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinoma, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilm's tumor, cervical cancer, testicular cancer, small cell lung carcinoma, non-small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma,

craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma, leukemia, lymphoma, or Kaposi sarcoma.

[00185] Examples of proliferative disorders include hematopoietic neoplastic disorders. As used herein, the term "hematopoietic neoplastic disorders" includes diseases involving hyperplastic/neoplastic cells of hematopoietic origin, *e.g.*, arising from myeloid, lymphoid or erythroid lineages, or precursor cells thereof. Preferably, the diseases arise from poorly differentiated acute leukemias, *e.g.*, erythroblastic leukemia and acute megakaryoblastic leukemia. Additional exemplary myeloid disorders include, but are not limited to, acute promyeloid leukemia (APML), acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML) (reviewed in Vaickus (1991), *Crit Rev. Oncol./Hemotol.* 11:267-97); lymphoid malignancies include, but are not limited to acute lymphoblastic leukemia (ALL) which includes B-lineage ALL and T-lineage ALL, chronic lymphocytic leukemia (CLL), prolymphocytic leukemia (PLL), hairy cell leukemia (HLL) and Waldenstrom's macroglobulinemia (WM). Additional forms of malignant lymphomas include, but are not limited to non-Hodgkin lymphoma and variants thereof, peripheral T cell lymphomas, adult T cell leukemia/lymphoma (ATL), cutaneous T-cell lymphoma (CTCL), large granular lymphocytic leukemia (LGF), Hodgkin's disease and Reed-Stemberg disease.

[00186] Examples of cellular proliferative and/or differentiative disorders of the breast include, but are not limited to, proliferative breast disease including, *e.g.*, epithelial hyperplasia, sclerosing adenosis, and small duct papillomas; tumors, *e.g.*, stromal tumors such as fibroadenoma, phyllodes tumor, and sarcomas, and epithelial tumors such as large duct papilloma; carcinoma of the breast including in situ (noninvasive) carcinoma that includes ductal carcinoma in situ (including Paget's disease) and lobular carcinoma in situ, and invasive (infiltrating) carcinoma including, but not limited to, invasive ductal carcinoma, invasive lobular carcinoma, medullary carcinoma, colloid (mucinous) carcinoma, tubular carcinoma, and invasive papillary carcinoma, and miscellaneous malignant neoplasms. Disorders in the male breast include, but are not limited to, gynecomastia and carcinoma.

[00187] Examples of cellular proliferative and/or differentiative disorders of the lung include, but are not limited to, bronchogenic carcinoma, including paraneoplastic syndromes, bronchioloalveolar carcinoma, neuroendocrine tumors, such as bronchial carcinoid, miscellaneous tumors, and metastatic tumors; pathologies of the pleura, including inflammatory pleural effusions, noninflammatory pleural effusions, pneumothorax, and pleural tumors, including solitary fibrous tumors (pleural fibroma) and malignant mesothelioma.

[00188] Examples of cellular proliferative and/or differentiative disorders of the colon include, but are not limited to, non-neoplastic polyps, adenomas, familial syndromes, colorectal carcinogenesis, colorectal carcinoma, and carcinoid tumors.

[00189] Examples of cellular proliferative and/or differentiative disorders of the liver include, but are not limited to, nodular hyperplasias, adenomas, and malignant tumors, including primary carcinoma of the liver and metastatic tumors.

[00190] Examples of cellular proliferative and/or differentiative disorders of the ovary include, but are not limited to, ovarian tumors such as, tumors of coelomic epithelium, serous tumors, mucinous tumors, endometrioid tumors, clear cell adenocarcinoma, cystadenofibroma, Brenner tumor, surface epithelial tumors; germ cell tumors such as mature (benign) teratomas, monodermal teratomas, immature malignant teratomas, dysgerminoma, endodermal sinus tumor, choriocarcinoma; sex cord-stomal tumors such as, granulosa-theca cell tumors,

thecomafibromas, androblastomas, hill cell tumors, and gonadoblastoma; and metastatic tumors such as Krukenberg tumors.

[00191] One aspect of the invention relates to a method of treating a subject at risk for or afflicted with unwanted cell proliferation, e.g., malignant or nonmalignant cell proliferation. The method comprises providing a composition of the present invention, for example a compositing having a peptidomimetic macrocycle and a nucleic acid molecule, to inhibit a gene which promotes unwanted cell proliferation; and administering a therapeutically effective dose of the composition of the present invention to a subject, preferably a human subject. In one embodiment, the invention features a method for treating or preventing a disease or condition in a subject, wherein the disease or condition is related to angiogenesis or neovascularization, comprising administering to the subject a composition of the present invention under conditions suitable for the treatment or prevention of the disease or condition in the subject, alone or in conjunction with one or more other therapeutic compounds. The invention may treat unwanted cell proliferation by treating or preventing tumor angiogenesis in a subject comprising administering to the subject a composition of the present invention under conditions suitable for the treatment or prevention of tumor angiogenesis in the subject, alone or in conjunction with one or more other therapeutic compounds.

[00192] Additional examples of cancers which the present invention can be used to prevent or treat include solid tumours and leukaemias, including : apudoma, choristoma, branchioma, malignant carcinoid syndrome, carcinoid heart disease, carcinoma (e. g., Walker, basal cell, basosquamous, Brown-Pearce, ductal, Ehrlich tumour, in situ, Krebs 2, Merkel cell, mucinous, non-small cell lung, oat cell, papillary, scirrhous, bronchiolar, bronchogenic, squamous cell, and transitional cell), histiocytic disorders, leukaemia (e. g., B cell, mixed cell, null cell, T cell, T-cell chronic,HTLV-II-associated, lymphocytic acute, lymphocytic chronic, mast cell, and myeloid), histiocytosis malignant, Hodgkin disease, immunoproliferative small, non Hodgkin lymphoma, plasmacytoma, reticuloendotheliosis, melanoma, chondroblastoma, chondroma, chondrosarcoma, fibroma, fibrosarcoma, giant cell tumours, histiocytoma, lipoma, liposarcoma, mesothelioma,myxoma, myxosarcoma, osteoma, osteosarcoma, Ewing sarcoma, synovioma, adenofibroma, adenolymphoma, carcinosarcoma, chordoma, cranio-pharyngioma, dysgerminoma, hamartoma, mesenchymoma, mesonephroma, myosarcoma, ameloblastoma, cementoma, odontoma, teratoma, thymoma, trophoblastic tumour, adeno-carcinoma, adenoma, cholangioma, cholesteatoma, cylindroma, cystadenocarcinoma, cystadenoma, granulosa cell tumour, gynandroblastoma, hepatoma, hidradenoma, islet cell tumour, Leydig cell tumour, papilloma, Sertoli cell tumour, theca cell tumour, leiomyoma, leiomyosarcoma, myoblastoma, mymoma, myosarcoma, rhabdomyoma, rhabdomyosarcoma, ependymoma, ganglioneuroma, glioma, medulloblastoma, meningioma,neurilemmoma, neuroblastoma, neuroepithelioma, neurofibroma, neuroma, paraganglioma, paraganglioma nonchromaffin, angiokeratoma, angiolymphoid hyperplasia with eosinophilia, angioma sclerosing, angiomatosis, glomangioma, hemangioendothelioma, hemangioma,hemangiopericytoma, hemangiosarcoma, lymphangioma, lymphangiomyoma, lymphangiosarcoma, pinealoma, carcinosarcoma, chondrosarcoma, cystosarcoma, phyllodes, fibrosarcoma, hemangiosarcoma,leimyosarcoma, leukosarcoma, liposarcoma, lymphangiosarcoma, myosarcoma,myxosarcoma, ovarian carcinoma, rhabdomyosarcoma, sarcoma (e. g., Ewing, experimental, Kaposi, and mast cell), neoplasms (e. g., bone, breast, digestive system, colorectal, liver, pancreatic, pituitary, testicular, orbital, head and neck, central nervous system, acoustic, pelvic respiratory tract, and urogenital), neurofibromatosis, and cervical dysplasia, and other conditions in which cells have become immortalised or transformed. The invention could be

used in combination with other treatments, such as chemotherapy, cryotherapy, hyperthermia, radiation therapy, and the like.

[00193] In one embodiment, the invention features a method for treating or preventing an ocular disease or condition in a subject, wherein the ocular disease or condition is related to angiogenesis or neovascularization (such as those involving genes in the vascular endothelial growth factor, VEGF pathway or TGF-beta pathway), comprising administering to the subject a multifunctional siNA molecule of the invention under conditions suitable for the treatment or prevention of the disease or condition in the subject, alone or in conjunction with one or more other therapeutic compounds. In another embodiment, the ocular disease or condition comprises macular degeneration, age related macular degeneration, diabetic retinopathy, macular adema, neovascular glaucoma, myopic degeneration, trachoma, scarring of the eye, cataract, ocular inflammation and/or ocular infections.

[00194] The pharmaceutical compositions of the present invention can also be used to treat a variety of immune disorders, in particular those associated with overexpression of a gene or expression of a mutant gene. In one aspect, the invention relates to a method of treating a subject, e.g., a human, at risk for or afflicted with a disease or disorder characterized by an unwanted immune response, e.g., an inflammatory disease or disorder, or an autoimmune disease or disorder. The method comprises providing a composition of the present invention that can inhibit a gene which mediates an unwanted immune response; and administering said composition of the present invention to a subject, preferrably a human subject. Examples of hematopoietic disorders or diseases include, without limitation, autoimmune diseases (including, for example, diabetes mellitus, arthritis (including rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, psoriatic arthritis), multiple sclerosis, encephalomyelitis, myasthenia gravis, systemic lupus erythematosis, autoimmune thyroiditis, dermatitis (including atopic dermatitis and eczematous dermatitis), psoriasis, Sjogren's Syndrome, Crohn's disease, aphthous ulcer, iritis, conjunctivitis, keratoconjunctivitis, ulcerative colitis, asthma, allergic asthma, cutaneous lupus erythematosus, scleroderma, vaginitis, proctitis, drug eruptions, leprosy reversal reactions, erythema nodosum leprosum, autoimmune uveitis, allergic encephalomyelitis, acute necrotizing hemorrhagic encephalopathy, idiopathic bilateral progressive sensorineural hearing, loss, aplastic anemia, pure red cell anemia, idiopathic thrombocytopenia, polychondritis, Wegener's granulomatosis, chronic active hepatitis, Stevens-Johnson syndrome, idiopathic sprue, lichen planus, Graves' disease, sarcoidosis, primary biliary cirrhosis, uveitis posterior, and interstitial lung fibrosis), graft-versus-host disease, cases of transplantation, and allergy.

[00195] Examples of cardiovascular disorders *(e.g.,* inflammatory disorders) that are treated or prevented with the compositions of the invention include, but are not limited to, atherosclerosis, myocardial infarction, stroke, thrombosis, aneurism, heart failure, ischemic heart disease, angina pectoris, sudden cardiac death, hypertensive heart disease; non-coronary vessel disease, such as arteriolosclerosis, small vessel disease, nephropathy, hypertriglyceridemia, hypercholesterolemia, hyperlipidemia, xanthomatosis, asthma, hypertension, emphysema and chronic pulmonary disease; or a cardiovascular condition associated with interventional procedures ("procedural vascular trauma"), such as restenosis following angioplasty, placement of a shunt, stent, synthetic or natural excision grafts, indwelling catheter, valve or other implantable devices. Preferred cardiovascular disorders include atherosclerosis, myocardial infarction, aneurism, and stroke.

[00196] The present invention may also be used in the treatment and prophylaxis of other diseases, especially those associated with expression or overexpression of a particular gene or genes. For example, expression of genes associated with the immune response could be inhibited to treat/prevent autoimmune diseases such as

rheumatoid arthritis, graft-versus-host disease, etc. In such treatment, the compositions of the present invention may be used in conjunction with immunosuppressive drugs. The most commonly used immunosuppressive drugs currently include corticosteroids and more potent inhibitors like, for instance, methotrexate, sulphasalazine, hydroxychloroquine, 6 MP/azathioprine and cyclosporine. All of these treatments have severe side-effects related to toxicity, however, and the need for drugs that would allow their elimination from, or reduction in use is urgent. Other immunosuppressive drugs include the gentler, but less powerful non-steroid treatments such as Aspirin andlbuprofen, and a new class of reagents which are based on more specific immune modulator functions. This latter class includes interleukins, cytokines, recombinant adhesion molecules and monoclonal antibodies. The use of compositions of the present invention to inhibit a gene associated with the immune response in an immunosuppressive treatment protocol could increase the efficiency of immunosuppression, and particularly, may enable the administered amounts of other drugs, which have toxic or other adverse effects to be decreased.

[00197] Another aspect of the invention features a method of treating a subject, e.g., a human, at risk for or afflicted with acute pain or chronic pain. The method comprises providing a composition of the present invention that can inhibit a gene which mediates the processing of pain; and administering a therapeutically effective dose of said composition to a subject, preferrably a human subject. In particularly preferred embodiments the compositions of the present invention silences a component of an ion channel. In particularly preferred embodiments the compositions of the present invention silences a neurotransmitter receptor or ligand.

[00198] Another aspect of the invention relates to a method of treating a subject, e.g., a human, at risk for or afflicted with a neurological disease or disorder. The method comprises providing a composition of the present invention that can inhibit a gene which mediates a neurological disease or disorder; and administering a therapeutically effective dose of said composition to a subject, preferably a human. In a preferred embodiment the disease or disorder is Alzheimer Disease or Parkinson Disease. In particularly preferred embodiments the compositions of the present invention silences an amyloid-family gene, e.g., APP; a presenilin gene, e.g., PSENl and PSEN2, or I-synuclein. In a preferred embodiment the disease or disorder is a neurodegenerative trinucleotide repeat disorder, e.g., Huntington disease, dentatorubral pallidoluysian atrophy or a spinocerebellar ataxia, e.g., SCAl, SCA2, SCA3 (Machado-Joseph disease), SCA7 or SCA8. Some other examples of neurologic disorders that are treated with the compositions of the present invention include ALS, multiple sclerosis, epilepsy, Down's Syndrome, Dutch Type Hereditary Cerebral Hemorrhage Amyloidosis, Reactive Amyloidosis, Familial Amyloid Nephropathy with Urticaria and Deafness, Muckle-Wells Syndrome, Idiopathic Myeloma; Macroglobulinemia-Associated Myeloma, Familial Amyloid Polyneuropathy, Familial Amyloid Cardiomyopathy, Isolated Cardiac Amyloid, Systemic Senile Amyloidosis, Adult Onset Diabetes, Insulinoma, Isolated Atrial Amyloid, Medullary Carcinoma of the Thyroid, Familial Amyloidosis, Hereditary Cerebral Hemorrhage With Amyloidosis, Familial Amyloidotic Polyneuropathy, Scrapie, Creutzfeldt-Jacob Disease, Gerstmann Straussler-Scheinker Syndrome, and Bovine Spongiform Encephalitis, a prion-mediated disease.

[00199] Some examples of endocrinologic disorders that are treated with the compositions of the present invention described herein include but are not limited to diabetes, hypothyroidism, hypopituitarism, hypoparathyroidism, hypogonadism, etc.

[00200] In another embodiment, the invention relates to a method for treating viral diseases, including but not limited to hepatitis C, hepatitis B, hepatitis A, herpes simplex virus (HSV), human papilloma virus (HPV), HIV-AIDS, poliovirus, and smallpox virus. Compositions of the invention are prepared as described herein to target

expressed sequences of a virus, thus ameliorating viral activity and replication. For example, hepatitis C virus (HCV) may be treated using compositions of the present invention having antisense oligonucleotides. Antisense oligonucleotides are useful for the treatment of HCV, as described in US Pat No. 6,433,159, hereby incorporated by reference. The compositions of the present invention can be used in the treatment and/or diagnosis of viral infected tissue, both animal and plant. Also, such compositions can be used in the treatment of virus-associated carcinoma, such as hepatocellular cancer.

[00201] In another aspect the invention features methods of treating a subject infected with a pathogen, e.g., a bacterial, amoebic, parasitic, or fungal pathogen. The method comprises providing a composition of the present invention that can inhibit a pathogen gene; and administering a therapeutically effective dose of said composition to a subject, preferably a human subject.

[00202] Another aspect of the invention relates to a method of treating a subject, e.g., a human, at risk for or afflicted with a metabolic disease or disorder. The method comprises providing a composition of the present invention that can inhibit a gene which mediates a metabolic disease or disorder; and administering a therapeutically effective dose of said composistion to a subject, preferably a human. In a preferred embodiment the disease or disorder is diabetes mellitus or obesity. In particularly preferred embodiments the dsRNA silences PTP-IB, glucose-6-phosphatase, PEPCK, FoxO-1, FoxA-3, Fructose-1,6-biphosphatase, SREBPlC, SCAP, ApoB, SERBP-2, LDLR, Dhcr24, HMG Co-reductase, FAS-fatty acid synthase, caspase 8, TGF-beta 1, TGF-beta 1 receptor 1, collagen, stearoyl-CoA desaturase 1, microsomal trigylceride transfer protein, dipeptidylpeptidase IV, acetyl-CoA-carboxylase-2,ll-hydroxysteroid dehydrogenase 1, APS (adaptor protein with pleckstrin homology and src homology 2 domains), GM3 synthase, acyl CoA:DAG acyltransferase 1, resistin, SHIP-2, hormone sensitive lipase, and PCSK-9.

[00203] In another aspect, the invention provides a method of cleaving or silencing more than one gene with a composition of the present invention. In a further embodiment, the composition of the present invention can be used in combination with other known treatments to treat conditions or diseases discussed above. For example, the described molecules could be used in combination with one or more known therapeutic agents to treat a disease or condition. Non-limiting examples of other therapeutic agents that can be readily combined with the compositions of the present invention are enzymatic nucleic acid molecules, allosteric nucleic acid molecules, antisense, decoy, or aptamer nucleic acid molecules, antibodies such as monoclonal antibodies, small molecules, and other organic and/or inorganic compounds including metals, salts and ions.

[00204] In other or further embodiments, the compositions of the present invention described herein are used to treat, prevent or diagnose conditions characterized by overactive cell death or cellular death due to physiologic insult, etc. Some examples of conditions characterized by premature or unwanted cell death are or alternatively unwanted or excessive cellular proliferation include, but are not limited to hypocellular/hypoplastic, acellular/aplastic, or hypercellular/hyperplastic conditions. Some examples include hematologic disorders including but not limited to fanconi anemia, aplastic anemia, thalaessemia, congenital neutropenia, myelodysplasia.

[00205] In other or further embodiments, the compositions of the invention that act to decrease apoptosis are used to treat disorders associated with an undesirable level of cell death. Thus, in some embodiments, the anti-apoptotic compositions of the invention are used to treat disorders such as those that lead to cell death associated with viral infection, *e.g.*, infection associated with infection with human immunodeficiency virus (HIV). A wide variety of

neurological diseases are characterized by the gradual loss of specific sets of neurons, and the anti-apoptotic compositions of the invention are used, in some embodiments, in the treatment of these disorders. Such disorders include Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS) retinitis pigmentosa, spinal muscular atrophy, and various forms of cerebellar degeneration. The cell loss in these diseases does not induce an inflammatory response, and apoptosis appears to be the mechanism of cell death. In addition, a number of hematologic diseases are associated with a decreased production of blood cells. These disorders include anemia associated with chronic disease, aplastic anemia, chronic neutropenia, and the myelodysplasia syndromes. Disorders of blood cell production, such as myelodysplastic syndrome and some forms of aplastic anemia, are associated with increased apoptotic cell death within the bone marrow. These disorders could result from the activation of genes that promote apoptosis, acquired deficiencies in stromal cells or hematopoietic survival factors, or the direct effects of toxins and mediators of immune responses. Two common disorders associated with cell death are myocardial infarctions and stroke. In both disorders, cells within the central area of ischemia, which is produced in the event of acute loss of blood flow, appear to die rapidly as a result of necrosis. However, outside the central ischemic zone, cells die over a more protracted time period and morphologically appear to die by apoptosis. In other or further embodiments, the anti-apoptotic compositions of the invention are used to treat all such disorders associated with undesirable cell death.

[00206] The following classes of possible target genes are examples of the genes which the present invention may used to inhibit : developmental genes (e. g., adhesion molecules. cyclin kinase inhibitors, Wnt family members, Pax family members, Winged helix family members, Hox family members, cytokines/lymphokines and their receptors, growth/differentiation factors and their receptors, neurotransmitters and their receptors) ; oncogenes (e. g., ABLI, BCLl, BCL2, BCL6, CBFA2, CBL, CSFIR, ERBA, ERBB, EBRB2,ETS1, ETSl, ETV6, FGR, FOS, FYN, HCR, HRAS, JUN, KRAS, LCK, LYN, MDM2, MLL, MYB, MYC,MYCL1, MYCN, NRAS, PIMl, PML, RET, SRC, TALI, TCL3 and YES) ; tumour suppresser genes (e. g., APC, BRCAl, BRCA2, MADH4, MCQNFl, NF2,RB1, TP53 and WTl) ; and enzymes (e. g., ACP desaturases and hydroxylases, ADP-glucose pyrophorylases, ATPases, alcohol dehydrogenases, amylases, amyloglucosidases, catalases, cellulases, cyclooxygenases, decarboxylases, dextrinases, DNA and RNA polymerases, galactosidases, glucanases, glucose oxidases, GTPases, helicases, hemicellulases, integrases, invertases, isomerases, kinases, lactases, lipases, lipoxygenases, lysozymes, pectinesterases, peroxidases, phosphatases, phospholipases, phosphorylases, polygalacturonases, proteinases and peptideases, pullanases, recombinases, reverse transcriptases, topoisomerases, and xylanases).

[00207] Addidtional examples of genes which can be targeted for treatment include, without limitation, an oncogene (Hanahan, D. and R. A. Weinberg, Cell (2000) 100:57; and Yokota, J., Carcinogenesis (2000) 21(3):497-503); a cytokine gene (Rubinstein, M., et al., Cytokine Growth Factor Rev. (1998) 9(2): 175-81); an idiotype (Id) protein gene (Benezra, R., et al., Oncogene (2001) 20(58):8334-41; Norton, J. D., J. Cell Sci. (2000) 113(22):3897-905); a prion gene (Prusiner, S. B., et al., Cell (1998) 93(3):337-48; Safar, J., and S. B. Prusiner, Prog. Brain Res. (1998) 117:421-34); a gene that expresses molecules that induce angiogenesis (Gould, V. E. and B. M. Wagner, Hum. Pathol. (2002) 33(11): 1061-3); adhesion molecules (Chothia, C. and E. Y. Jones, Annu. Rev. Biochem. (1997) 66:823-62; Parise, L. V., et al., Semin. Cancer Biol. (2000) 10(6):407-14); cell surface receptors (Deller, M. C , and Y. E. Jones, Curr. Opin. Struct. Biol. (2000) 10(2):213-9); genes of proteins that are involved in metastasizing and/or invasive processes (Boyd, D., Cancer Metastasis Rev. (1996) 15(l):77-89;

Yokota, J., Carcinogenesis (2000) 21(3):497-503); genes of proteases as well as of molecules that regulate apoptosis and the cell cycle (Matrisian, L. M., Curr. Biol. (1999) 9(20):R776-8; Krepela, E., Neoplasma (2001) 48(5):332-49; Basbaum and Werb, Curr. Opin. Cell Biol. (1996) 8:731-738; Birkedal-Hansen, et al., Crit. Rev. Oral Biol. Med. (1993) 4:197-250; Mignatti and Rifkin, Physiol. Rev. (1993) 73:161-195; Stetler-Stevenson, et al., Annu. Rev. Cell Biol. (1993) 9:541-573; Brinkerhoff, E., and L. M. Matrisan, Nature Reviews (2002) 3:207-214; Strasser, A., et al., Annu. Rev. Biochem. (2000) 69:217-45; Chao, D. T. and S. J. Korsmeyer, Annu. Rev. Immunol. (1998) 16:395-419; Mullauer, L., et al., Mutat. Res. (2001) 488(3):21 1-31; Fotedar, R., et al., Prog. Cell Cycle Res. (1996) 2:147-63; Reed, J. C , Am. J. Pathol. (2000) 157(5):1415-30; D'Ari, R., Bioassays (2001) 23(7):563-5); genes that express the EGF receptor; Mendelsohn, J. and J. Baselga, Oncogene (2000) 19(56):6550-65; Normanno, N., et al., Front. Biosci. (2001) 6:D685-707); and the multi-drug resistance 1 gene, MDRl gene (Childs, S., and V. Ling, Imp. Adv. Oncol. (1994) 21-36).

Pharmaceutical Compositions

[00208] The peptidomimetic macrocycles of the invention also include pharmaceutically acceptable derivatives or prodrugs thereof. A "pharmaceutically acceptable derivative" means any pharmaceutically acceptable salt, ester, salt of an ester, pro-drug or other derivative of a compound of this invention which, upon administration to a recipient, is capable of providing (directly or indirectly) a compound of this invention. Particularly favored pharmaceutically acceptable derivatives are those that increase the bioavailability of the compounds of the invention when administered to a mammal *(e.g.,* by increasing absorption into the blood of an orally administered compound) or which increases delivery of the active compound to a biological compartment *(e.g.,* the brain or lymphatic system) relative to the parent species. Some pharmaceutically acceptable derivatives include a chemical group which increases aqueous solubility or active transport across the gastrointestinal mucosa.

[00209] In some embodiments, the peptidomimetic macrocycles of the invention are modified by covalently or non-covalently joining appropriate functional groups to enhance selective biological properties. Such modifications 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.

[00210] 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 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.

[00211] For preparing pharmaceutical compositions from the compounds of the present invention, pharmaceutically acceptable carriers include either solid or liquid carriers. Solid form preparations include powders, tablets, pills, capsules, cachets, suppositories, and dispersible granules. A solid carrier can be one or more substances, which also acts as diluents, flavoring agents, binders, preservatives, tablet disintegrating agents, or an encapsulating material. Details on techniques for formulation and administration are well described in the

scientific and patent literature, see, *e.g.*, the latest edition of Remington's Pharmaceutical Sciences, Maack Publishing Co, Easton PA.

[00212] In powders, the carrier is a finely divided solid, which is in a mixture with the finely divided active component. In tablets, the active component is mixed with the carrier having the necessary binding properties in suitable proportions and compacted in the shape and size desired.

[00213] Suitable solid excipients are carbohydrate or protein fillers include, but are not limited to sugars, including lactose, sucrose, mannitol, or sorbitol; starch from corn, wheat, rice, potato, or other plants; cellulose such as methyl cellulose, hydroxypropylmethyl-cellulose, or sodium carboxymethylcellulose; and gums including arabic and tragacanth; as well as proteins such as gelatin and collagen. If desired, disintegrating or solubilizing agents are added, such as the cross-linked polyvinyl pyrrolidone, agar, alginic acid, or a salt thereof, such as sodium alginate.

[00214] Liquid form preparations include solutions, suspensions, and emulsions, for example, water or water/propylene glycol solutions. For parenteral injection, liquid preparations can be formulated in solution in aqueous polyethylene glycol solution.

[00215] The pharmaceutical preparation is preferably in unit dosage form. In such form the preparation is subdivided into unit doses containing appropriate quantities of the active component. The unit dosage form can be a packaged preparation, the package containing discrete quantities of preparation, such as packeted tablets, capsules, and powders in vials or ampoules. Also, the unit dosage form can be a capsule, tablet, cachet, or lozenge itself, or it can be the appropriate number of any of these in packaged form.

[00216] When the compositions of this invention comprise a combination of a peptidomimetic macrocycle 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 100%, and more preferably between about 5 to 95% of the dosage normally administered in a monotherapy regimen. In some embodiments, the additional agents are administered separately, as part of a multiple dose regimen, from the compounds of this invention. Alternatively, those agents are part of a single dosage form, mixed together with the compounds of this invention in a single composition.

Examples

Example 1. Preparation of siRNAs for use in the invention

[00217] A set of 21-nucleotide siRNAis designed to downregulate 1) the expression of a gene coding for a fluorescent EGFP protein and 2) the expression of HCV. The siRNA is chemically synthesized as 2' bis(acetoxyethoxy)-methyl ether protected oligos by a commercial manufacturer (Dharmacon). Synthetic oligonucleotides are deprotected, annealed and purified according to the instructions provided by the manufacturer. Successful duplex formation is confirmed by polyacrylamide gel electrophoresis. The sequence of EGFP specific siRNA duplexes is designed following the manufacturer's recommendation and subjected to a BLAST search against the human genome sequence to ensure no genomic gene is targeted. The sequence of the HCV-specific siRNA duplexes is designed following the manufacturer's recommendation and subjected to a BLAST search against the human genome sequence to ensure no genomic gene is targeted. Duplex siRNAs with

5'Cy3 modification at sense strand are used to determine uptake efficiency while duplex siRNAs with 3'amino modification are used in crosslinking with peptidomimetic macrocycle as described below.

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Example 2. Conjugation of siRNA to peptidomimetic macrocycle.

[00218] A set of modified siRNAs (EGFP and HCV) is prepared according to Example 1 containing 3'-amino groups attached to a linker by annealing deprotected 3'-amino modified (Glen Research) single stranded siRNA with its complementary strand sequence. Duplex modified siRNA is then incubated with an excess of a crosslinker such as a sulfosuccinimidyl 4-[p-maleimidophenyl] butyrate crosslinkers (Sulfo-SMPB, PIERCE) in a reaction buffer. After reaction, the mixtures are desalted and the duplex siRNAs are extracted according to manufacturer instructions. The desalted fractions containing malemide-activated siRNA with crosslinker are pooled and incubated with equal molar ratio of a BID-SABH3A peptidomimetic macrocycle analog that contains one reactive cysteine (see U.S. Patent Application No. 10/981,873, filed on Nov. 5, 2004). The resulting conjugate is purified by a method such as HPLC or used as is.

Example 3. Transfection of cells.

[00219] The conjugate resulting from Example 2 is used to transfect cells grown in culture. HeLa cells are grown to 70% confluence on tissue culture plates. The cells are washed and replaced with serum-free medium, and the conjugate is added at appropriate dilutions. The cells are incubated for various periods of time ranging from 1 to 6 hours and are then washed with medium and collected by incubation with trypsin. Total DNA and RNA is isolated via a Qiagen RNA/DNA minikit, and the isolated nucleic acid sequences are prepared for fluorescence uptake analysis in a fluorimeter.

[00220] This experiment may also be performed in a similar methods on HeLa cells grown on microscopy slides. Following incubation with the conjugates of the invention, the cells are washed and prepared for uptake studies by confocal microscopy.

[00221] Suitable controls for this experiment are, for example, siRNA sequences alone at various concentration or siRNA sequences in combination with a commercial transfection reagent such as lipofectamine. siRNA sequences conjugated to a corresponding macrocycle precursor or to a non-macrocyclic corresponding polypeptide sequence may also be used as controls.

Example 4. Uptake measurements.

[00222] The nucleic acid extracts and the transfected cells from Example 3 are examined by fluorescence measurements and confocal microscopy, respectively. Fluorescence measurements indicate the amount of Cy5-labeled siRNA that was taken up into the cells. Confocal microscopy is used to confirm uptake and to determine subcellular localization and distribution of labeled conjugate.

Example 5. Subcellular localization experiments.

[00223] The distribution of conjugate in specific cellular compartments is measured by preparing a conjugate of siRNA sequences and a peptidomimetic macrocycle, where the conjugate is labelled with a pH-sensitive dye such as BCECF or CSNARF. Localization of the dye is examined by measuring the fluorescence of the pH-sensitive dye. High fluorescence compared to a control (e.g. siRNA sequences conjugated to a corresponding macrocycle

precursor or to a non-macrocyclic corresponding polypeptide sequence) indicates endosomal release into the cytosol.

Example 6. Downregulation of EGFP expression by the conjugates of the invention.

[00224] HeLa cells are transfected with EGFP and RFP encoding plasmids. Following transfection, the EGFP siRNA conjugates as prepared in Examples 1 and 2 are incubated with the transfected HeLa cells grown in culture. The cells are then harvested and a clear lysate is prepared which is examined by dual fluorescence measurements at the appropriate excitation and emission wavelengths for the fluorescent dyes. The ratio of fluorescence for the two dyes is measured. This experiment indicates that effective gene silencing can be obtained by using the conjugates of the invention.

Example 7. Downregulation of HCV expression by the conjugates of the invention.

[00225] A HCV siRNA conjugate as prepared in Examples 1 and 2 is incubated with cells expressing HCV grown in culture (according to U.S. Patent No. 6,433,159) at a range of conjugate concentrations. Following incubation, the cells are washed and collected. Extracts are prepared and immunoblotting against the target gene is performed. Controls suitable for this experiment may be, for example, siRNA sequences conjugated to a corresponding macrocycle precursor or to a non-macrocyclic corresponding polypeptide sequence. The decrease in expression of HCV of siRNA conjugate-treated cells indicates effective gene silencing.

[00226] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

CLAIMS

WHAT IS CLAIMED IS:

1. A method of modulating expression of a gene in a cell comprising contacting said cell with a peptidomimetic macrocycle and a nucleic acid.

2. The method of claim 1, wherein said peptidomimetic macrocycle is capable of transporting said nucleic acid into said cell.

3. The method of claim 1, wherein the nucleic acid is double-stranded.

4. The method of claim 1, wherein the nucleic acid is single-stranded.

5. The method of claim 1, wherein the nucleic acid is RNA.

6. The method of claim 1, wherein a strand of the nucleic acid is between 19 and 23 nucleotides long.

7. The method of claim 1, wherein a strand of the nucleic acid is complementary to a fragment of said gene or to a product of said gene.

8. The method of claim 1, wherein a strand of the nucleic acid is identical in sequence to a fragment of said gene or to a product of said gene.

9. The method of claim 1, wherein the peptidomimetic macrocycle forms a non-covalent complex with the nucleic acid.

10. The method of claim 1, wherein the peptidomimetic macrocycle is conjugated to the nucleic acid.

11. The method of claim 1, wherein the nucleic acid is conjugated to an N-terminus of the peptidomimetic macrocycle.

12. The method of claim 1, wherein the nucleic acid is conjugated to a C-terminus of the peptidomimetic macrocycle.

13. The method of claim 1, wherein the nucleic acid is conjugated to an internal amino acid of the peptidomimetic macrocycle.

14. The method of claim 1, wherein the peptidomimetic macrocycle is cell-permeable.

15. The method of claim 1, wherein the peptidomimetic macrocycle comprises a crosslinker connecting a first amino acid to a second amino acid.

16. The method of claim 15, wherein the nucleic acid is conjugated to the crosslinker of the peptidomimetic macrocycle.

17. The method of claim 15, wherein the first amino acid and the second amino acid are separated by three amino acids.

18. The method of claim 15, wherein the crosslinker comprises between 6 and 14 consecutive bonds.

19. The method of claim 15, wherein the crosslinker comprises between 8 and 12 consecutive bonds.

20. The method of claim 15, wherein the macrocycle comprises a ring of about 18 atoms to 26 atoms.

21. The method of claim 15, wherein the first amino acid and the second amino acid are separated by six amino acids.

22. The method of claim 15, wherein the crosslinker comprises between 8 and 16 consecutive bonds.

23. The method of claim 15, wherein the crosslinker comprises between 10 and 13 consecutive bonds.

24. The method of claim 15, wherein the macrocycle comprises a ring of about 29 atoms to 37 atoms.

25. The method of claim 15, wherein the peptidomimetic macrocycle comprises an alpha helix.

26. The method of claim 25, wherein the crosslinker spans from 1 turn to 5 turns of the α-helix.

27. The method of claim 25, wherein the crosslinker spans 1 turn of the alpha helix.

28. The method of claim 25, wherein the crosslinker spans 2 turns of the alpha helix.

29. The method of claim 25, wherein the length of the crosslinker is about 5 A to about 9 A per turn of the α-helix.

30. The method of claim 1, wherein the peptidomimetic macrocycle carries a net neutral charge at pH 7.4.

31. The method of claim 1, wherein the peptidomimetic macrocycle carries a net positive charge at pH 7.4.

32. The method of claim 15, wherein an alpha position of the first amino acid is additionally substituted.

33. The method of claim 15, wherein an alpha position of the second amino acid is additionally substituted.

34. A composition comprising a peptidomimetic macrocycle conjugated to a biomolecule.

35. The composition of claim 34, wherein the peptidomimetic macrocycle comprises a crosslinker connecting a first amino acid to a second amino acid.

36. The composition of claim 35, wherein the first amino acid and the second amino acid are separated by three amino acids.

37. The composition of claim 35, wherein the crosslinker comprises between 6 and 14 consecutive bonds.

38. The composition of claim 35, wherein the crosslinker comprises between 8 and 12 consecutive bonds.

39. The composition of claim 35, wherein the macrocycle comprises a ring of about 18 atoms to 26 atoms.

40. The composition of claim 35, wherein the first amino acid and the second amino acid are separated by six amino acids.

41. The composition of claim 35, wherein the crosslinker comprises between 8 and 16 consecutive bonds.

42. The composition of claim 35, wherein the crosslinker comprises between 10 and 13 consecutive bonds.

43. The composition of claim 35, wherein the macrocycle comprises a ring of about 29 atoms to 37 atoms.

44. The composition of claim 35, wherein the peptidomimetic macrocycle comprises an alpha helix.

45. The composition of claim 44, wherein the crosslinker spans from 1 turn to 5 turns of the α-helix.

46. The composition of claim 44, wherein the crosslinker spans 1 turn of the alpha helix.

47. The composition of claim 44, wherein the crosslinker spans 2 turns of the alpha helix.

48. The composition of claim 44, wherein the length of the crosslinker is about 5 A to about 9 A per turn of the α-helix.

49. The composition of claim 34, wherein the peptidomimetic macrocycle carries a net neutral charge at pH 7.4.

50. The composition of claim 34, wherein the peptidomimetic macrocycle carries a net positive charge at pH 7.4.

51. The composition of claim 35, wherein an alpha position of the first amino acid is additionally substituted.

52. The composition of claim 35, wherein an alpha position of the second amino acid is additionally substituted.

53. The composition of claim 34, wherein the biomolecule is a nucleic acid.

54. The composition of claim 34, wherein the biomolecule is a polypeptide.

55. The composition of claim 34, wherein the biomolecule is an antibody.

56. The composition of claim 34, wherein the biomolecule is an imaging agent.

57. The composition of claim 34, wherein the biomolecule is a fluorescent dye.

58. The composition of claim 34, wherein the biomolecule is a quantum dot.

59. The composition of claim 34, wherein the biomolecule is conjugated to an N-terminus of the peptidomimetic macrocycle.

60. The composition of claim 34, wherein the biomolecule is conjugated to a C-terminus of the peptidomimetic macrocycle.

61. The composition of claim 34, wherein the biomolecule is conjugated to an internal amino acid of the peptidomimetic macrocycle.

62. The composition of claim 35, wherein the biomolecule is conjugated to the crosslinker of the peptidomimetic macrocycle.

63. The composition of claim 34, wherein the peptidomimetic macrocycle is cell-permeable.

64. A method of introducing a biomolecule into a cell comprising contacting said cell with a conjugate comprising a peptidomimetic macrocycle and the biomolecule.

65. The method of claim 64, wherein the biomolecule is a nucleic acid.

66. The method of claim 64, wherein the biomolecule is a polypeptide.

67. The method of claim 64, wherein the biomolecule is an antibody.

68. The method of claim 64, wherein the biomolecule is an imaging agent.

69. The method of claim 64, wherein the biomolecule is a fluorescent dye.

70. The method of claim 64, wherein the biomolecule is a quantum dot.

71. The method of claim 64, wherein the cell is a cancer cell.

72. The method of claim 64, wherein the cell is a mammalian cell.

73. The method of claim 64, wherein the peptidomimetic macrocycle is cell-permeable.

74. The method of claim 64, wherein the peptidomimetic macrocycle comprises a crosslinker connecting a first amino acid to a second amino acid.

75. The method of claim 74, wherein the nucleic acid is conjugated to the crosslinker of the peptidomimetic macrocycle.

76. The method of claim 74, wherein the first amino acid and the second amino acid are separated by three amino acids.

77. The method of claim 74, wherein the crosslinker comprises between 6 and 14 consecutive bonds.

78. The method of claim 74, wherein the crosslinker comprises between 8 and 12 consecutive bonds.

79. The method of claim 74, wherein the macrocycle comprises a ring of about 18 atoms to 26 atoms.

80. The method of claim 74, wherein the first amino acid and the second amino acid are separated by six amino acids.

81. The method of claim 74, wherein the crosslinker comprises between 8 and 16 consecutive bonds.

82. The method of claim 74, wherein the crosslinker comprises between 10 and 13 consecutive bonds.

83. The method of claim 74, wherein the macrocycle comprises a ring of about 29 atoms to 37 atoms.

84. The method of claim 74, wherein the peptidomimetic macrocycle comprises an alpha helix.

85. The method of claim 84, wherein the crosslinker spans from 1 turn to 5 turns of the α-helix.

86. The method of claim 84, wherein the crosslinker spans 1 turn of the alpha helix.

87. The method of claim 84, wherein the crosslinker spans 2 turns of the alpha helix.

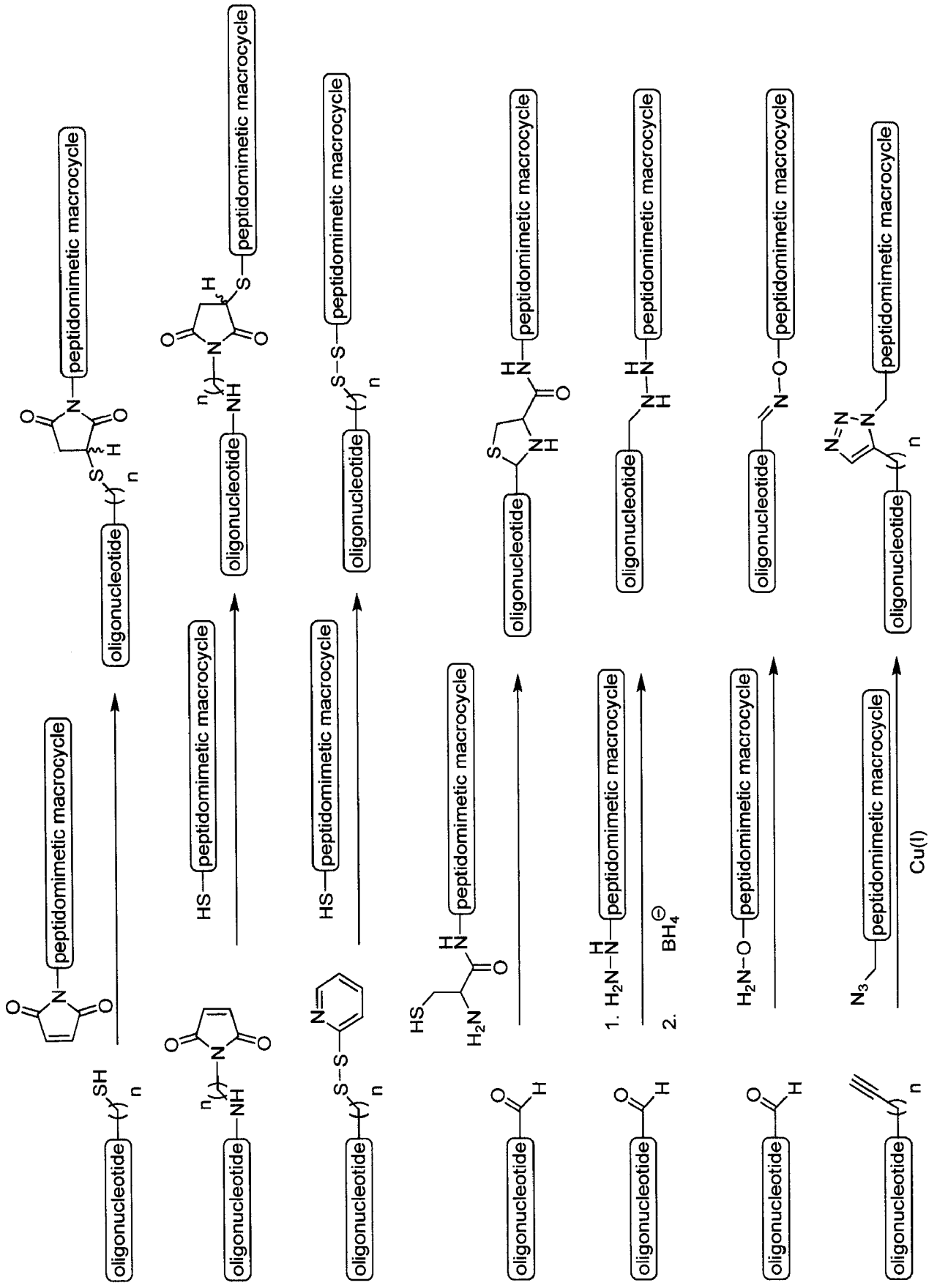
88. The method of claim 84, wherein the length of the crosslinker is about 5 A to about 9 A per turn of the α-helix.

89. The method of claim 64, wherein the peptidomimetic macrocycle carries a net neutral charge at pH 7.4.

90. The method of claim 64, wherein the peptidomimetic macrocycle carries a net positive charge at pH 7.4.

91. The method of claim 74, wherein an alpha position of the first amino acid is additionally substituted.

92. The method of claim 74, wherein an alpha position of the second amino acid is additionally substituted.


(A)
oligonucleotide
SH
n
peptidomimetic macrocycle
(B)
oligonucleotide
NH
n
HS
peptidomimetic macrocycle
(C)
oligonucleotide
n
HS
peptidomimetic macrocycle
(D)
oligonucleotide
H
HS
H2N
peptidomimetic macrocycle
(E)
oligonucleotide
H
1. H2N
2. BH4
peptidomimetic macrocycle
(F)
oligonucleotide
H
H2N
peptidomimetic macrocycle
(G)
oligonucleotide
n
N3
peptidomimetic macrocycle
Cu(I)