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(54) Title: TRIAZOLE-CROSSLINKED AND THIOETHER-CROSSLINKED PEPTIDOMIMETIC MACROCYCLES

(57) Abstract: Provided herein are peptidomimetic macrocycles and methods of using such macrocycles for the treatment of disease.



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TRIAZOLE-CROSSLINKED AND THIOETHER-CROSSLINKED PEPTIDOMIMETIC MACROCYCLES

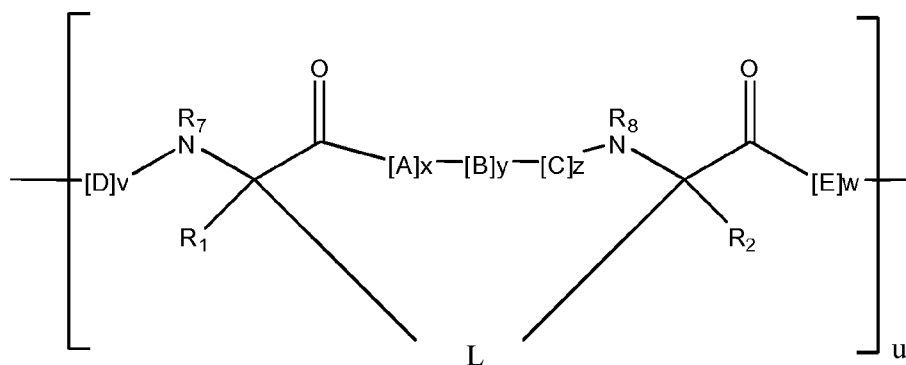
BACKGROUND OF THE INVENTION

- [0001] The human transcription factor protein p53 induces cell cycle arrest and apoptosis in response to DNA damage and cellular stress, and thereby plays a critical role in protecting cells from malignant transformation. The E3 ubiquitin ligase MDM2 (also known as HDM2) negatively regulates p53 function through a direct binding interaction that neutralizes the p53 transactivation activity, leads to export from the nucleus of p53 protein, and targets p53 for degradation via the ubiquitylation-proteasomal pathway. Loss of p53 activity, either by deletion, mutation, or MDM2 overexpression, is the most common defect in human cancers. Tumors that express wild type p53 are vulnerable to pharmacologic agents that stabilize or increase the concentration of active p53. In this context, inhibition of the activities of MDM2 has emerged as a validated approach to restore p53 activity and resensitize cancer cells to apoptosis *in vitro* and *in vivo*. MDMX (MDM4) has more recently been identified as a similar negative regulator of p53, and studies have revealed significant structural homology between the p53 binding interfaces of MDM2 and MDMX. The p53-MDM2 and p53-MDMX protein-protein interactions are mediated by the same 15-residue alpha-helical transactivation domain of p53, which inserts into hydrophobic clefts on the surface of MDM2 and MDMX. Three residues within this domain of p53 (F19, W23, and L26) are essential for binding to MDM2 and MDMX.
- [0002] There remains a considerable need for compounds capable of binding to and modulating the activity of p53, MDM2 and/or MDMX. Provided herein are p53-based peptidomimetic macrocycles that modulate an activity of p53. Also provided herein are p53-based peptidomimetic macrocycles that inhibit the interactions between p53, MDM2 and/or MDMX proteins. Further, provided herein are p53-based peptidomimetic macrocycles that can be used for treating diseases including but not limited to cancer and other hyperproliferative diseases.

SUMMARY OF THE INVENTION

- [0003] Described herein are stably cross-linked peptides related to a portion of human p53 ("p53 peptidomimetic macrocycles"). These cross-linked peptides contain at least two modified amino acids that together form an intramolecular cross-link that can help to stabilize the alpha-helical secondary structure of a portion of p53 that is thought to be important for binding of p53 to MDM2 and for binding of p53 to MDMX. Accordingly, a cross-linked polypeptide described herein can have improved biological activity relative to a corresponding polypeptide that is not cross-linked. The p53 peptidomimetic macrocycles are thought to interfere with binding of p53 to MDM2 and/or of p53 to MDMX, thereby liberating functional p53 and inhibiting its destruction. The p53 peptidomimetic macrocycles described herein can be used therapeutically, for example

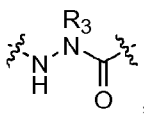
- to treat cancers and other disorders characterized by an undesirably low level or a low activity of p53, and/or to treat cancers and other disorders characterized by an undesirably high level of activity of MDM2 or MDMX. The p53 peptidomimetic macrocycles can also be useful for treatment of any disorder associated with disrupted regulation of the p53 transcriptional pathway, leading to conditions of excess cell survival and proliferation such as cancer and autoimmunity, in addition to conditions of inappropriate cell cycle arrest and apoptosis such as neurodegeneration and immunodeficiencies. In some embodiments, the p53 peptidomimetic macrocycles bind to MDM2 (e.g., GenBank® Accession No.: 228952; GI:228952) and/or MDMX (also referred to as MDM4; GenBank® AccessionNo.: 88702791; GI:88702791).
- [0004] In one aspect, provided herein is a peptidomimetic macrocycle comprising an amino acid sequence which is at least about 60%, 80%, 90%, or 95% identical to an amino acid sequence chosen from the group consisting of the amino acid sequences in Table 4, Table 4a, Table 4b, or Table 5. In some embodiments, the peptidomimetic macrocycle is not a peptide as shown in Table 6, Table 6a, Table 7, Table 7a, or Table 7b. In some embodiments, the peptidomimetic macrocycle has an amino acid sequence chosen from Table 4. In some embodiments, the peptidomimetic macrocycle has an amino acid sequence chosen from Table 4a. In some embodiments, the peptidomimetic macrocycle has an amino acid sequence chosen from Table 4b. In some embodiments, the peptidomimetic macrocycle has an amino acid sequence chosen from Table 5.
- [0005] Alternatively, an amino acid sequence of said peptidomimetic macrocycle is chosen as above, and further wherein the macrocycle does not include an all carbon crosslink or a triazole. In some embodiments, the peptidomimetic macrocycle comprises a helix, such as an α -helix. In other embodiments, the peptidomimetic macrocycle comprises an α,α -disubstituted amino acid. A peptidomimetic macrocycle can comprise a crosslinker linking the α -positions of at least two amino acids. At least one of said two amino acids can be an α,α -disubstituted amino acid.
- [0006] In some embodiments, provided are peptidomimetic macrocycle of the Formula:

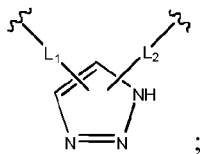


Formula (I)

wherein:

each A, C, D, and E is independently an amino acid;

B is an amino acid, , [-NH-L₃-CO-], [-NH-L₃-SO₂-], or [-NH-L₃-];
each L and L' is independently a macrocycle-forming linker of the formula



L₁, L₂ and L₃ are independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, cycloarylene, heterocycloarylene, or [-R₄-K-R₄]_n, each being optionally substituted with R₅;

each R₃ is independently hydrogen, alkyl, alkenyl, alkynyl, arylalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, cycloalkylalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅;

each R₄ is independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene;

each K is independently O, S, SO, SO₂, CO, CO₂, or CONR₃;

each R₅ is independently halogen, alkyl, -OR₆, -N(R₆)₂, -SR₆, -SOR₆, -SO₂R₆, -CO₂R₆, a fluorescent moiety, a radioisotope or a therapeutic agent;

each R₆ is independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heterocycloalkyl, a fluorescent moiety, a radioisotope or a therapeutic agent;

each R₇ is independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅, or part of a cyclic structure with a D residue;

each R₈ is independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅, or part of a cyclic structure with an E residue;

v and w are independently integers from 1-1000, for example 1-500, 1-200, 1-100, 1-50, 1-30, 1-20 or 1-10;

u is an integer from 1-10, for example 1-5, 1-3 or 1-2;

x, y and z are independently integers from 0-10, for example the sum of x+y+z is 2, 3, or 6; and

n is an integer from 1-5.

[0007] In some embodiments, v and w are integers between 1-30. In some embodiments, w is an integer from 3-1000, for example 3-500, 3-200, 3-100, 3-50, 3-30, 3-20, or 3-10.

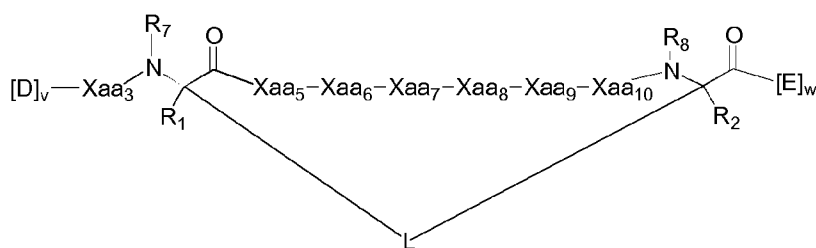
[0008] In some embodiments, the sum of x+y+z is 3 or 6. In some embodiments, the sum of x+y+z is 3. In other embodiments, the sum of x+y+z is 6.

[0009] In some embodiments, the peptidomimetic macrocycles are claimed with the proviso that when u=1 and w=2, the first C-terminal amino acid represented by E is not an Arginine (R) and/or the second C-terminal amino acid represented by E is not a Threonine (T). For instance, when u = 1

and $w = 2$, the first C-terminal amino acid and/or the second C-terminal amino acid represented by E do not comprise a positively charged side chain or a polar uncharged side chain. In some embodiments, when $u = 1$ and $w = 2$, the first C-terminal amino acid and/or the second C-terminal amino acid represented by E comprise a hydrophobic side chain. For example, when $w = 2$, the first C-terminal amino acid and/or the second N-terminal amino acid represented by E comprise a hydrophobic side chain, for example a large hydrophobic side chain.

[0010] In some embodiments, w is between 3 and 1000. For example, the third amino acid represented by E comprises a large hydrophobic side chain.

[0011] Peptidomimetic macrocycles are also provided of the formula:



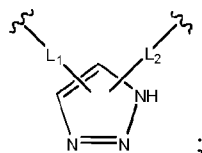
wherein:

each of Xaa_3 , Xaa_5 , Xaa_6 , Xaa_7 , Xaa_8 , Xaa_9 , and Xaa_{10} is individually an amino acid, wherein at least three of Xaa_3 , Xaa_5 , Xaa_6 , Xaa_7 , Xaa_8 , Xaa_9 , and Xaa_{10} are the same amino acid as the amino acid at the corresponding position of the sequence Phe₃-X₄-His₅-Tyr₆-Trp₇-Ala₈-Gln₉-Leu₁₀-X₁₁-Ser₁₂, where each X is an amino acid;

each D and E is independently an amino acid;

R_1 and R_2 are independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo-; or at least one of R_1 and R_2 forms a macrocycle-forming linker L' connected to the alpha position of one of said D or E amino acids;

each L and L' is independently a macrocycle-forming linker of the formula



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_3 is independently hydrogen, alkyl, alkenyl, alkynyl, arylalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, cycloalkylalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R_5 ;

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

each K is independently O, S, SO, SO₂, CO, CO₂, or CONR₃;

each R₅ is independently halogen, alkyl, -OR₆, -N(R₆)₂, -SR₆, -SOR₆, -SO₂R₆, -CO₂R₆, a fluorescent moiety, a radioisotope or a therapeutic agent;

each R₆ is independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heterocycloalkyl, a fluorescent moiety, a radioisotope or a therapeutic agent;

each R₇ is independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅, or part of a cyclic structure with a D residue;

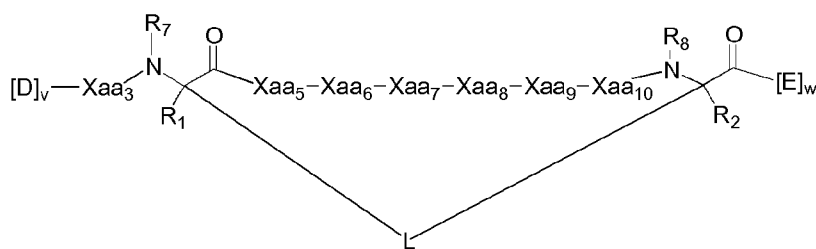
each R₈ is independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅, or part of a cyclic structure with an E residue;

v is an integer from 1-1000, for example 1-500, 1-200, 1-100, 1-50, 1-30, 1-20 or 1-10;

w is an integer from 3-1000, for example 3-500, 3-200, 3-100, 3-50, 3-30, 3-20, or 3-10; and

n is an integer from 1-5.

[0012] In some embodiments, the peptidomimetic macrocycle has the formula:



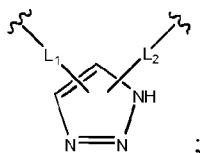
wherein:

each of Xaa₃, Xaa₅, Xaa₆, Xaa₇, Xaa₈, Xaa₉, and Xaa₁₀ is individually an amino acid, wherein at least three of Xaa₃, Xaa₅, Xaa₆, Xaa₇, Xaa₈, Xaa₉, and Xaa₁₀ are the same amino acid as the amino acid at the corresponding position of the sequence Phe₃-X₄-Glu₅-Tyr₆-Trp₇-Ala₈-Gln₉-Leu₁₀/Cba₁₀-X₁₁-Ala₁₂, where each X is an amino acid;

each D and E is independently an amino acid;

R₁ and R₂ are independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo-; or at least one of R₁ and R₂ forms a macrocycle-forming linker L' connected to the alpha position of one of said D or E amino acids;

each L and L' is independently a macrocycle-forming linker of the formula



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 ;

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

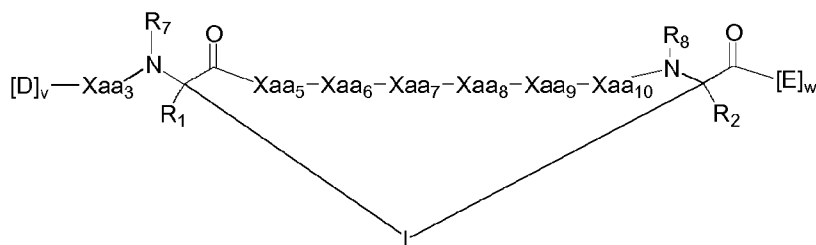
R_8 is $-H$, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R_5 , or part of a cyclic structure with an E residue;

v is an integer from 1-1000, for example 1-500, 1-200, 1-100, 1-50, 1-30, 1-20, or 1-10;

w is an integer from 3-1000, for example 3-500, 3-200, 3-100, 3-50, 3-30, 3-20, or 3-10; and

n is an integer from 1-5.

[0013] In some embodiments, provided are peptidomimetic macrocycles of the Formula I:



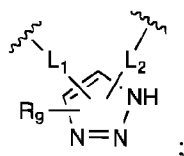
wherein:

each of Xaa_3 , Xaa_5 , Xaa_6 , Xaa_7 , Xaa_8 , Xaa_9 , and Xaa_{10} is individually an amino acid, wherein at least three of Xaa_3 , Xaa_5 , Xaa_6 , Xaa_7 , Xaa_8 , Xaa_9 , and Xaa_{10} are the same amino acid as the amino acid at the corresponding position of the sequence Phe₃-X₄-His₅-Tyr₆-Trp₇-Ala₈-Gln₉-Leu₁₀-X₁₁-Ser₁₂, where each X is an amino acid;

each D and E is independently an amino acid;

R_1 and R_2 are independently $-H$, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo-; or at least one of R_1 and R_2 forms a macrocycle-forming linker L' connected to the alpha position of one of said D or E amino acids;

each L and L' is independently a macrocycle-forming linker of the formula



L₁ and L₂ are independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, cycloarylene, heterocycloarylene, or [-R₄-K-R₄]_n, each being optionally substituted with R₅;

each R₃ is independently hydrogen, alkyl, alkenyl, alkynyl, arylalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, cycloalkylalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅;

each R₄ is independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene;

each K is independently O, S, SO, SO₂, CO, CO₂, or CONR₃;

each R₅ is independently halogen, alkyl, -OR₆, -N(R₆)₂, -SR₆, -SOR₆, -SO₂R₆, -CO₂R₆, a fluorescent moiety, a radioisotope or a therapeutic agent;

each R₆ is independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heterocycloalkyl, a fluorescent moiety, a radioisotope or a therapeutic agent;

each R₇ is independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅, or part of a cyclic structure with a D residue;

each R₈ is independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅, or part of a cyclic structure with an E residue;

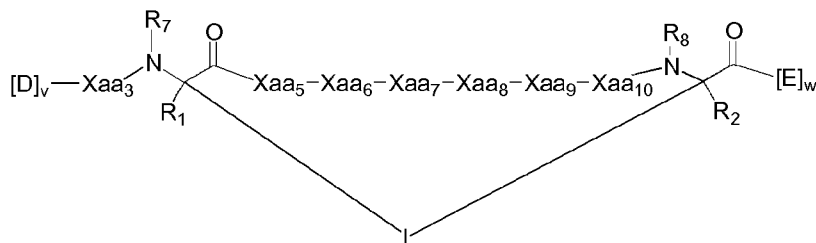
each R₉ is independently alkyl, alkenyl, alkynyl, aryl, cycloalkyl, cycloalkenyl, heteroaryl, or heterocyclyl group, unsubstituted or optionally substituted with R_a and/or R_b;

v is an integer from 1-1000, for example 1-500, 1-200, 1-100, 1-50, 1-30, 1-20 or 1-10;

w is an integer from 3-1000, for example 3-500, 3-200, 3-100, 3-50, 3-30, 3-20, or 3-10; and

n is an integer from 1-5.

[0014] In some embodiments, provided are peptidomimetic macrocycles of the Formula I:



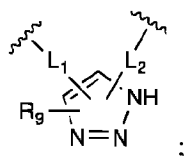
wherein:

each of Xaa₃, Xaa₅, Xaa₆, Xaa₇, Xaa₈, Xaa₉, and Xaa₁₀ is individually an amino acid, wherein at least three of Xaa₃, Xaa₅, Xaa₆, Xaa₇, Xaa₈, Xaa₉, and Xaa₁₀ are the same amino acid as the amino acid at the corresponding position of the sequence Phe₃-X₄-Glu₅-Tyr₆-Trp₇-Ala₈-Gln₉-Leu₁₀/Cba₁₀-X₁₁-Ala₁₂, where each X is an amino acid;

each D and E is independently an amino acid;

R₁ and R₂ are independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo-; or at least one of R₁ and R₂ forms a macrocycle-forming linker L' connected to the alpha position of one of said D or E amino acids;

each L and L' is independently a macrocycle-forming linker of the formula



L₁ and L₂ are independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, cycloarylene, heterocycloarylene, or [-R₄-K-R₄]_n, each being optionally substituted with R₅;

R₃ is hydrogen, alkyl, alkenyl, alkynyl, arylalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, cycloalkylalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅;

each R₄ is alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene;

each K is O, S, SO, SO₂, CO, CO₂, or CONR₃;

each R₅ is independently halogen, alkyl, -OR₆, -N(R₆)₂, -SR₆, -SOR₆, -SO₂R₆, -CO₂R₆, a fluorescent moiety, a radioisotope or a therapeutic agent;

each R₆ is independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heterocycloalkyl, a fluorescent moiety, a radioisotope or a therapeutic agent;

R₇ is -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅, or part of a cyclic structure with a D residue;

R₈ is -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅, or part of a cyclic structure with an E residue;

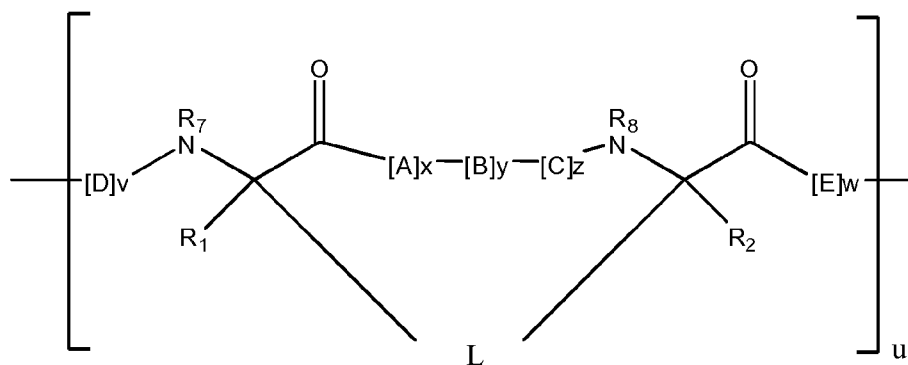
each R₉ is independently alkyl, alkenyl, alkynyl, aryl, cycloalkyl, cycloalkenyl, heteroaryl, or heterocyclyl group, unsubstituted or optionally substituted with R_a and/or R_b;

v is an integer from 1-1000, for example 1-500, 1-200, 1-100, 1-50, 1-30, 1-20, or 1-10;

w is an integer from 3-1000, for example 3-500, 3-200, 3-100, 3-50, 3-30, 3-20, or 3-10; and

n is an integer from 1-5.

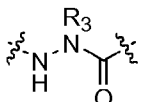
[0015] In some embodiments, provided are peptidomimetic macrocycles of the Formula I, comprising an amino acid sequence which is at least about 60% identical to an amino acid sequence chosen from the group consisting of the amino acid sequences in Tables 4, 4a, or 4b, wherein the peptidomimetic macrocycle has the formula:



Formula (I)

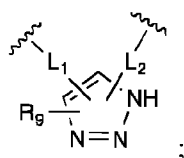
wherein:

each A, C, D, and E is independently an amino acid;

B is an amino acid, , [-NH-L₃-CO-], [-NH-L₃-SO₂-], or [-NH-L₃-];

R₁ and R₂ are independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo-; or at least one of R₁ and R₂ forms a macrocycle-forming linker L' connected to the alpha position of one of said D or E amino acids;

each L and L' is independently a macrocycle-forming linker of the formula



L₁, L₂ and L₃ are independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, cycloarylene, heterocycloarylene, or [-R₄-K-R₄]_n, each being optionally substituted with R₅;

each R₃ is independently hydrogen, alkyl, alkenyl, alkynyl, arylalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, cycloalkylalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅;

each R₄ is independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene;

each K is independently O, S, SO, SO₂, CO, CO₂, or CONR₃;

each R₅ is independently halogen, alkyl, -OR₆, -N(R₆)₂, -SR₆, -SOR₆, -SO₂R₆, -CO₂R₆, 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;

each R_7 is independently $-H$, 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;

each R_8 is independently $-H$, 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 R_9 is independently alkyl, alkenyl, alkynyl, aryl, cycloalkyl, cycloalkenyl, heteroaryl, or heterocyclyl group, unsubstituted or optionally substituted with R_a and/or R_b ;

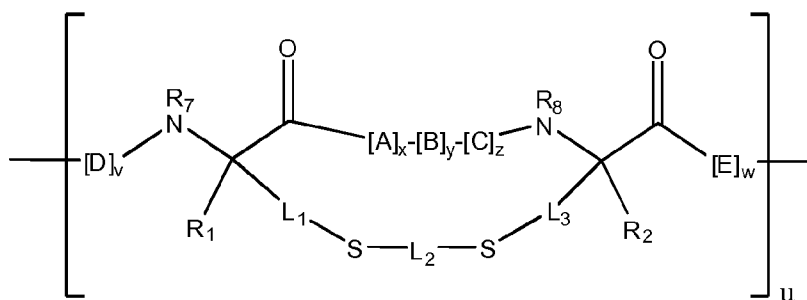
v and w are independently integers from 1-1000, for example 1-500, 1-200, 1-100, 1-50, 1-30, 1-20 or 1-10;

u is an integer from 1-10, for example 1-5, 1-3 or 1-2;

x, y and z are independently integers from 0-10, for example the sum of $x+y+z$ is 2, 3, or 6; and

n is an integer from 1-5.

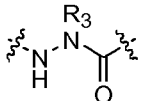
[0016] In some embodiments, provided are peptidomimetic macrocycle of the Formula II:



Formula (II)

wherein:

each A, C, D, and E is independently an amino acid;

B is an amino acid, , $[-NH-L_4-CO-]$, $[-NH-L_4-SO_2-]$, 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-; or at least one of R_1 and R_2 forms a macrocycle-forming linker L' connected to the alpha position of one of said D or E amino acids;

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

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

each K is O, S, SO, SO₂, CO, CO₂, or CONR₃;

each R₄ is alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene;

each R₅ is independently halogen, alkyl, -OR₆, -N(R₆)₂, -SR₆, -SOR₆, -SO₂R₆, -CO₂R₆, a fluorescent moiety, a radioisotope or a therapeutic agent;

each R₆ is independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heterocycloalkyl, a fluorescent moiety, a radioisotope or a therapeutic agent;

R₇ is -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅, or part of a cyclic structure with a D residue;

R₈ is -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅, or part of a cyclic structure with an E residue;

v and w are independently integers from 1-1000, for example 1-500, 1-200, 1-100, 1-50, 1-30, 1-20 or 1-10;

u is an integer from 1-10, for example 1-5, 1-3 or 1-2;

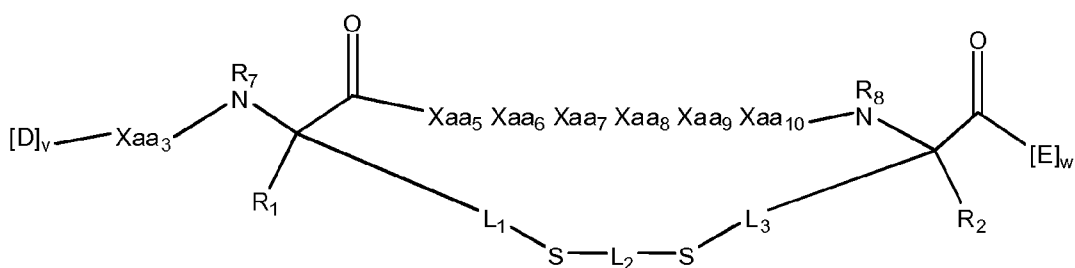
x, y and z are independently integers from 0-10, for example the sum of x+y+z is 2, 3, or 6; and

n is an integer from 1-5.

[0017] In some embodiments, the peptidomimetic macrocycles are claimed with the proviso that when u=1 and w=2, the first C-terminal amino acid represented by E is not an Arginine (R) and/or the second C-terminal amino acid represented by E is not a Threonine (T). For instance, when u = 1 and w= 2, the first C-terminal amino acid and/or the second C-terminal amino acid represented by E do not comprise a positively charged side chain or a polar uncharged side chain. In some embodiments, when u = 1 and w= 2, the first C-terminal amino acid and/or the second C-terminal amino acid represented by E comprise a hydrophobic side chain. For example, when w= 2, the first C-terminal amino acid and/or the second N-terminal amino acid represented by E comprise a hydrophobic side chain, for example a large hydrophobic side chain.

[0018] In some embodiments, w is between 3 and 1000. For example, the third amino acid represented by E comprises a large hydrophobic side chain.

[0019] Peptidomimetic macrocycles are also provided of the formula:



wherein:

each of Xaa₃, Xaa₅, Xaa₆, Xaa₇, Xaa₈, Xaa₉, and Xaa₁₀ is individually an amino acid, wherein at least three of Xaa₃, Xaa₅, Xaa₆, Xaa₇, Xaa₈, Xaa₉, and Xaa₁₀ are the same amino acid as the amino acid at the corresponding position of the sequence Phe₃-X₄-His₅-Tyr₆-Trp₇-Ala₈-Gln₉-Leu₁₀-X₁₁-Ser₁₂, where each X is an amino acid;

each D and E is independently an amino acid;

R₁ and R₂ are independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo-; or at least one of R₁ and R₂ forms a macrocycle-forming linker L' connected to the alpha position of one of said D or E amino acids;

L₁, L₂, L₃ and L₄ are independently alkylene, alkenylene, alkynylene, heteroalkylene,

cycloalkylene, heterocycloalkylene, cycloarylene, heterocycloarylene or [-R₄-K-R₄]_n, each being unsubstituted or substituted with R₅;

each K is O, S, SO, SO₂, CO, CO₂, or CONR₃;

R₃ is hydrogen, alkyl, alkenyl, alkynyl, arylalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, cycloalkylalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅;

each R₄ is alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene;

each R₅ is independently halogen, alkyl, -OR₆, -N(R₆)₂, -SR₆, -SOR₆, -SO₂R₆, -CO₂R₆, a fluorescent moiety, a radioisotope or a therapeutic agent;

each R₆ is independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heterocycloalkyl, a fluorescent moiety, a radioisotope or a therapeutic agent;

R₇ is -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅, or part of a cyclic structure with a D residue;

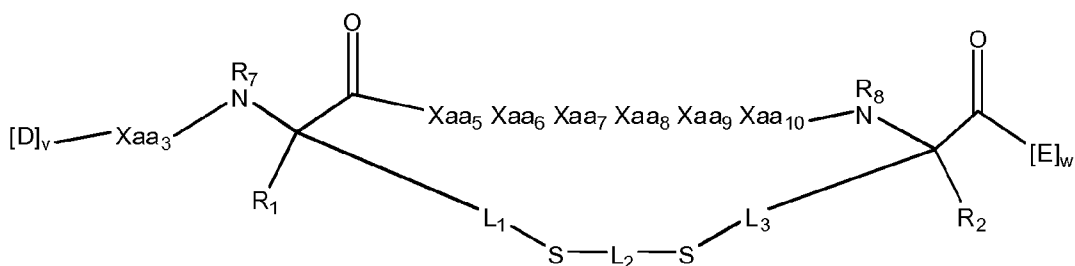
R₈ is -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅, or part of a cyclic structure with an E residue;

v is an integer from 1-1000, for example 1-500, 1-200, 1-100, 1-50, 1-30, 1-20 or 1-10;

w is an integer from 3-1000, for example 3-500, 3-200, 3-100, 3-50, 3-30, 3-20, or 3-10; and

n is an integer from 1-5.

[0020] Peptidomimetic macrocycles are also provided of the formula:



wherein:

each of Xaa₃, Xaa₅, Xaa₆, Xaa₇, Xaa₈, Xaa₉, and Xaa₁₀ is individually an amino acid, wherein at least three of Xaa₃, Xaa₅, Xaa₆, Xaa₇, Xaa₈, Xaa₉, and Xaa₁₀ are the same amino acid as the amino acid at the corresponding position of the sequence Phe₃-X₄-Glu₅-Tyr₆-Trp₇-Ala₈-Gln₉-Leu₁₀/Cba₁₀-X₁₁-Ala₁₂, where each X is an amino acid;

each D and E is independently an amino acid;

R₁ and R₂ are independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo-; or at least one of R₁ and R₂ forms a macrocycle-forming linker L' connected to the alpha position of one of said D or E amino acids;

L₁, L₂, L₃ and L₄ are independently alkylene, alkenylene, alkynylene, heteroalkylene,

cycloalkylene, heterocycloalkylene, cycloarylene, heterocycloarylene or [-R₄-K-R₄]-_n, each being unsubstituted or substituted with R₅;

each K is O, S, SO, SO₂, CO, CO₂, or CONR₃;

R₃ is hydrogen, alkyl, alkenyl, alkynyl, arylalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, cycloalkylalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅;

each R₄ is alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene;

each R₅ is independently halogen, alkyl, -OR₆, -N(R₆)₂, -SR₆, -SOR₆, -SO₂R₆, -CO₂R₆, a fluorescent moiety, a radioisotope or a therapeutic agent;

each R₆ is independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heterocycloalkyl, a fluorescent moiety, a radioisotope or a therapeutic agent;

R₇ is -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅, or part of a cyclic structure with a D residue;

R₈ is -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅, or part of a cyclic structure with an E residue;

v is an integer from 1-1000, for example 1-500, 1-200, 1-100, 1-50, 1-30, 1-20, or 1-10;

w is an integer from 3-1000, for example 3-500, 3-200, 3-100, 3-50, 3-30, 3-20, or 3-10; and

n is an integer from 1-5.

- [0021] In some embodiments, each E is independently an amino acid selected from Ala (alanine), D-Ala (D-alanine), Aib (α -aminoisobutyric acid), Sar (N-methyl glycine), and Ser (serine). In some embodiments, $[D]_v$ is $-\text{Leu}_1\text{-Thr}_2$.
- [0022] In some embodiments, w is an integer from 3-10, for example 3-6, 3-8, 6-8, or 6-10. In some embodiments, w is 3. In other embodiments, w is 6. In some embodiments, v is an integer from 1-10, for example 2-5. In some embodiments, v is 2.
- [0023] In some embodiments, the peptidomimetic macrocycle has improved binding affinity to MDM2 or MDMX relative to a corresponding peptidomimetic macrocycle where w is 0, 1 or 2. In other instances, the peptidomimetic macrocycle has a reduced ratio of binding affinities to MDMX versus MDM2 relative to a corresponding peptidomimetic macrocycle where w is 0, 1 or 2. In still other instances, the peptidomimetic macrocycle has improved *in vitro* anti-tumor efficacy against p53 positive tumor cell lines relative to a corresponding peptidomimetic macrocycle where w is 0, 1 or 2. In some embodiments, the peptidomimetic macrocycle shows improved *in vitro* induction of apoptosis in p53 positive tumor cell lines relative to a corresponding peptidomimetic macrocycle where w is 0, 1 or 2. In other instances, the peptidomimetic macrocycle of claim 1, wherein the peptidomimetic macrocycle has an improved *in vitro* anti-tumor efficacy ratio for p53 positive versus p53 negative or mutant tumor cell lines relative to a corresponding peptidomimetic macrocycle where w is 0, 1 or 2. In some instances the improved efficacy ratio *in vitro*, is 1-29, ≥ 30 -49, or ≥ 50 . In still other instances, the peptidomimetic macrocycle has improved *in vivo* anti-tumor efficacy against p53 positive tumors relative to a corresponding peptidomimetic macrocycle where w is 0, 1 or 2. In some instances the improved efficacy ratio *in vivo* is -29, ≥ 30 -49, or ≥ 50 . In yet other instances, the peptidomimetic macrocycle has improved *in vivo* induction of apoptosis in p53 positive tumors relative to a corresponding peptidomimetic macrocycle where w is 0, 1 or 2. In some embodiments, the peptidomimetic macrocycle has improved cell permeability relative to a corresponding peptidomimetic macrocycle where w is 0, 1 or 2. In other cases, the peptidomimetic macrocycle has improved solubility relative to a corresponding peptidomimetic macrocycle where w is 0, 1 or 2.
- [0024] In some embodiments, Xaa₅ is Glu or an amino acid analog thereof. In some embodiments, Xaa₅ is Glu or an amino acid analog thereof and wherein the peptidomimetic macrocycle has an improved property, such as improved binding affinity, improved solubility, improved cellular efficacy, improved cell permeability, improved *in vivo* or *in vitro* anti-tumor efficacy, or improved induction of apoptosis relative to a corresponding peptidomimetic macrocycle where Xaa₅ is Ala.
- [0025] In some embodiments, the peptidomimetic macrocycle has improved binding affinity to MDM2 or MDMX relative to a corresponding peptidomimetic macrocycle where Xaa₅ is Ala. In other

- embodiments, the peptidomimetic macrocycle has a reduced ratio of binding affinities to MDMX vs MDM2 relative to a corresponding peptidomimetic macrocycle where Xaa₅ is Ala. In some embodiments, the peptidomimetic macrocycle has improved solubility relative to a corresponding peptidomimetic macrocycle where Xaa₅ is Ala, or the peptidomimetic macrocycle has improved cellular efficacy relative to a corresponding peptidomimetic macrocycle where Xaa₅ is Ala.
- [0026] In some embodiments, Xaa₅ is Glu or an amino acid analog thereof and wherein the peptidomimetic macrocycle has improved biological activity, such as improved binding affinity, improved solubility, improved cellular efficacy, improved helicity, improved cell permeability, improved *in vivo* or *in vitro* anti-tumor efficacy, or improved induction of apoptosis relative to a corresponding peptidomimetic macrocycle where Xaa₅ is Ala.
- [0027] In some embodiments, the peptidomimetic macrocycle has an activity against a p53^{+/+} cell line which is at least 2-fold, 3-fold, 5-fold, 10-fold, 20-fold, 30-fold, 50-fold, 70-fold, or 100-fold greater than its binding affinity against a p53^{-/-} cell line. In some embodiments, the peptidomimetic macrocycle has an activity against a p53^{+/+} cell line which is between 1 and 29-fold, between 30 and 49-fold, or ≥ 50 -fold greater than its binding affinity against a p53^{-/-} cell line. Activity can be measured, for example, as an IC₅₀ value. For example, the p53^{+/+} cell line is SJSA-1, RKO, HCT-116, or MCF-7 and the p53^{-/-} cell line is RKO-E6 or SW-480. In some embodiments, the peptide has an IC₅₀ against the p53^{+/+} cell line of less than 1 μ M.
- [0028] In some embodiments, Xaa₅ is Glu or an amino acid analog thereof and the peptidomimetic macrocycle has an activity against a p53^{+/+} cell line which is at least 10-fold greater than its binding affinity against a p53^{-/-} cell line.
- [0029] Additionally, a method is provided of treating cancer in a subject comprising administering to the subject a peptidomimetic macrocycle. Also provided is a method of modulating the activity of p53 or MDM2 or MDMX in a subject comprising administering to the subject a peptidomimetic macrocycle, or a method of antagonizing the interaction between p53 and MDM2 and/or MDMX proteins in a subject comprising administering to the subject such a peptidomimetic macrocycle.

INCORPORATION BY REFERENCE

- [0030] 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.

DETAILED DESCRIPTION OF THE INVENTION

- [0031] 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.
- [0032] 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. A “corresponding uncrosslinked polypeptide” when referred to in the context of a peptidomimetic macrocycle is understood to relate to a polypeptide of the same length as the macrocycle and comprising the equivalent natural amino acids of the wild-type sequence corresponding to the macrocycle.
- [0033] As used herein, the term “stability” refers to the maintenance of a defined secondary structure in solution by a peptidomimetic macrocycle 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 herein are α -helices, 3_{10} helices, β -turns, and β -pleated sheets.
- [0034] As used herein, the term “helical stability” refers to the maintenance of α helical structure by a peptidomimetic macrocycle as measured by circular dichroism or NMR. For example, in some embodiments, a peptidomimetic macrocycle exhibits at least a 1.25, 1.5, 1.75 or 2-fold increase in α -helicity as determined by circular dichroism compared to a corresponding uncrosslinked macrocycle.
- [0035] The term “amino acid” refers to a molecule containing both an amino group and a carboxyl group. 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. The term amino acid, as used herein, includes without limitation α -amino acids, natural amino acids, non-natural amino acids, and amino acid analogs.
- [0036] The term “ α -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.
- [0037] The term “ β -amino acid” refers to a molecule containing both an amino group and a carboxyl group in a β configuration.
- [0038] 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.
- [0039] The following table shows a summary of the properties of natural amino acids:

Amino Acid	3- Letter	1- Letter	Side-chain Polarity	Side-chain charge (pH 7.4)	Hydropathy Index
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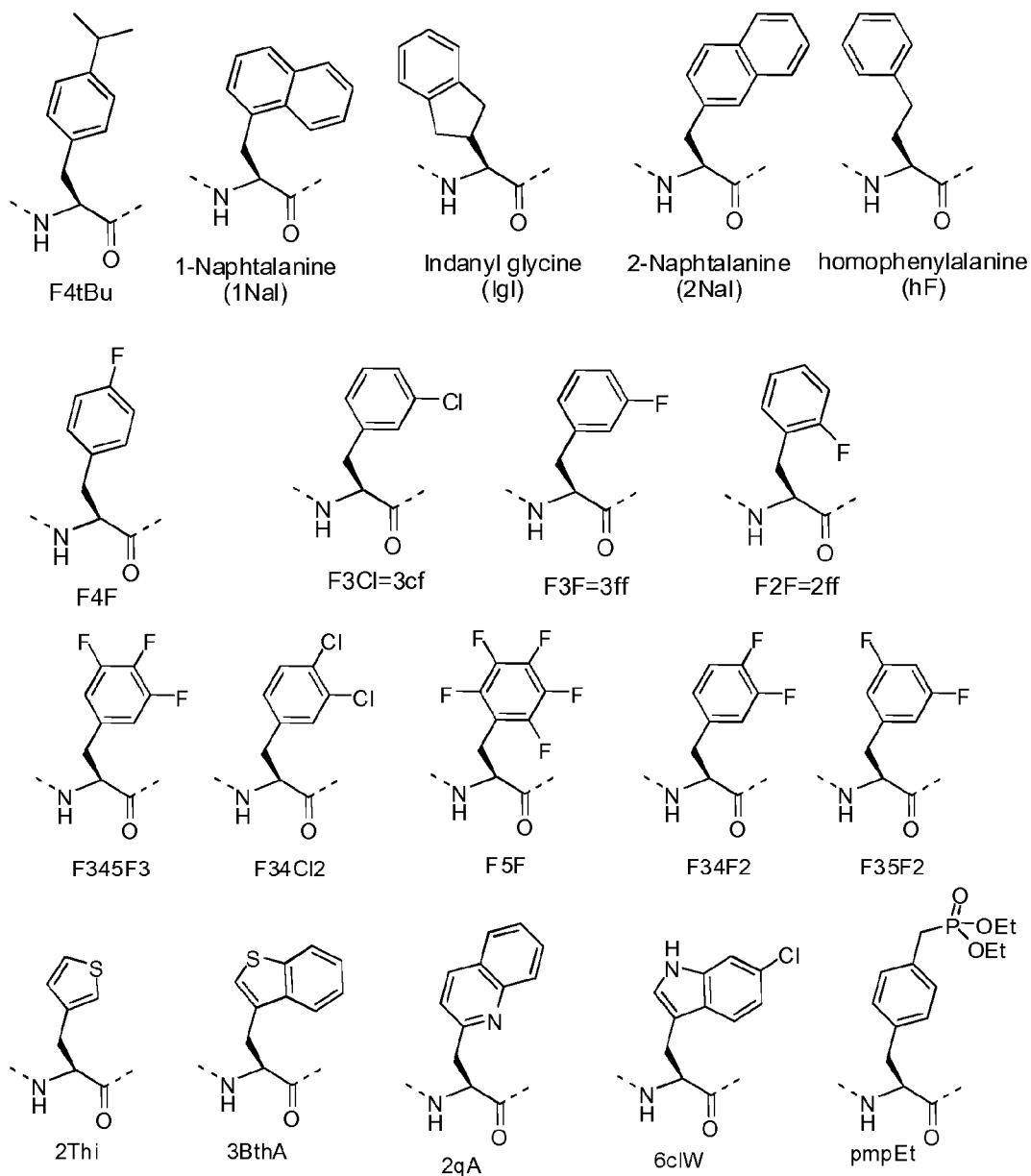
	Code	Code			
Alanine	Ala	A	nonpolar	neutral	1.8
Arginine	Arg	R	polar	positive	-4.5
Asparagine	Asn	N	polar	neutral	-3.5
Aspartic acid	Asp	D	polar	negative	-3.5
Cysteine	Cys	C	polar	neutral	2.5
Glutamic acid	Glu	E	polar	negative	-3.5
Glutamine	Gln	Q	polar	neutral	-3.5
Glycine	Gly	G	nonpolar	neutral	-0.4
Histidine	His	H	polar	positive(10%) neutral(90%)	-3.2
Isoleucine	Ile	I	nonpolar	neutral	4.5
Leucine	Leu	L	nonpolar	neutral	3.8
Lysine	Lys	K	polar	positive	-3.9
Methionine	Met	M	nonpolar	neutral	1.9
Phenylalanine	Phe	F	nonpolar	neutral	2.8
Proline	Pro	P	nonpolar	neutral	-1.6
Serine	Ser	S	polar	neutral	-0.8
Threonine	Thr	T	polar	neutral	-0.7
Tryptophan	Trp	W	nonpolar	neutral	-0.9
Tyrosine	Tyr	Y	polar	neutral	-1.3
Valine	Val	V	nonpolar	neutral	4.2

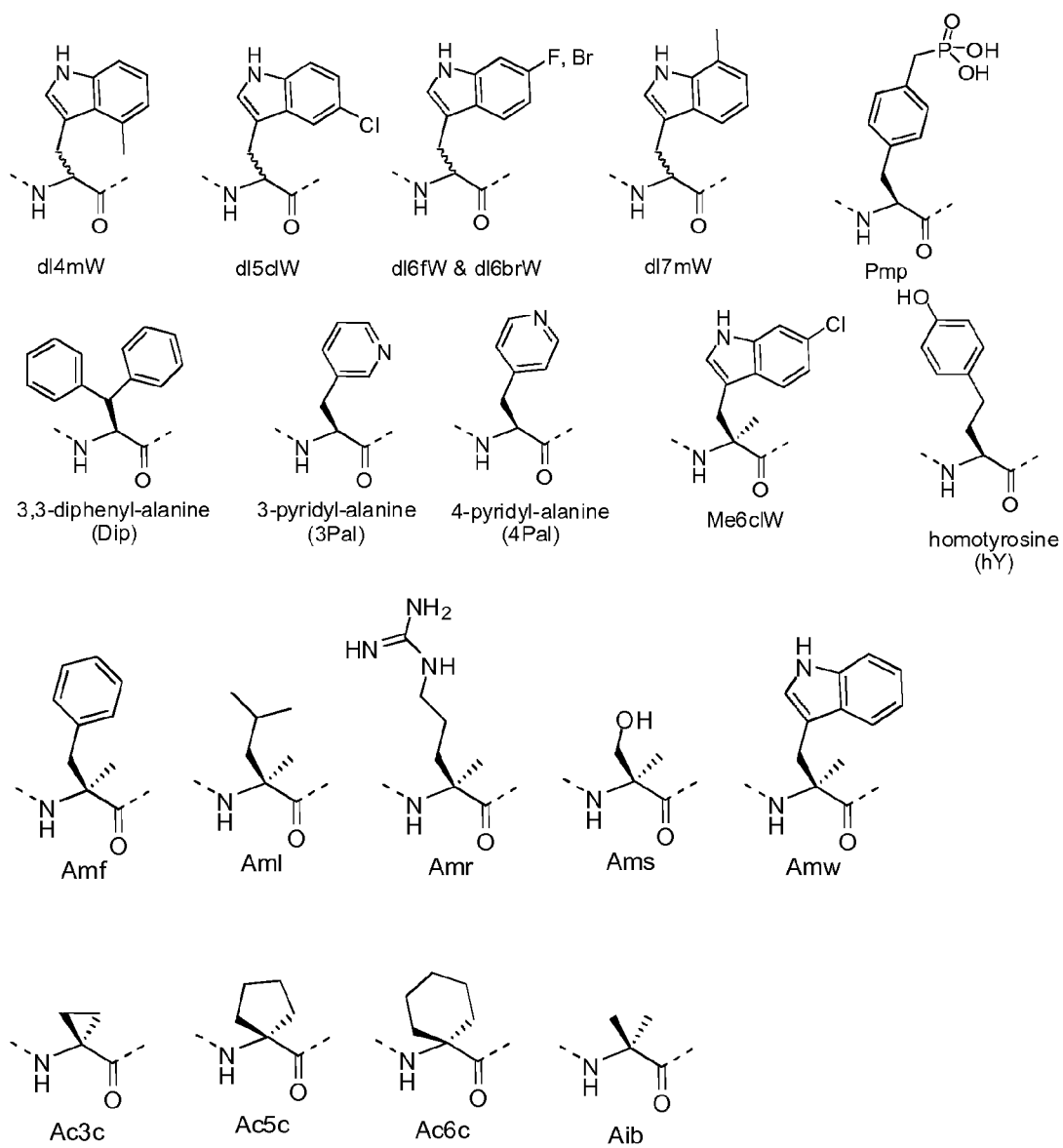
[0040] “Hydrophobic amino acids” include, without limitation, small hydrophobic amino acids and large hydrophobic amino acids. “Small hydrophobic amino acid” are glycine, alanine, proline, and analogs thereof. “Large hydrophobic amino acids” are valine, leucine, isoleucine, phenylalanine, methionine, tryptophan, and analogs thereof. “Polar amino acids” are serine, threonine, asparagine, glutamine, cysteine, tyrosine, and analogs thereof. “Charged amino acids” are lysine, arginine, histidine, aspartate, glutamate, and analogs thereof.

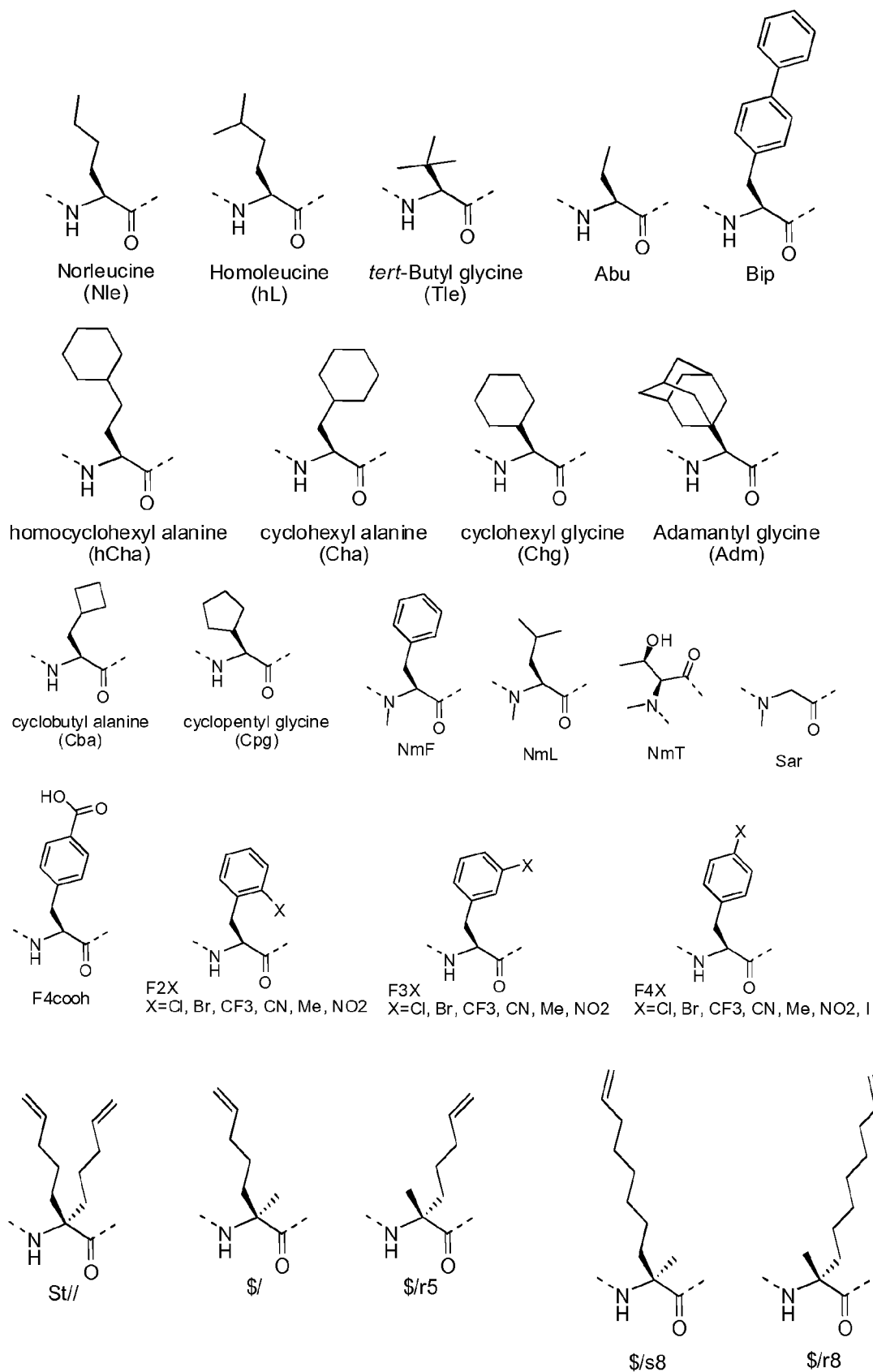
[0041] The term “amino acid analog” refers to a molecule which is structurally similar to an amino acid and which can be substituted for an amino acid in the formation of a peptidomimetic macrocycle. Amino acid analogs include, without limitation, β -amino acids and amino acids where the amino or carboxy group is substituted by a similarly reactive group (*e.g.*, substitution of the primary amine with a secondary or tertiary amine, or substitution of the carboxy group with an ester).

[0042] The term “non-natural amino acid” refers to an amino acid which is not one of the 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. Non-natural amino acids or amino acid analogs include, without limitation, structures according to the following:







[0043] Amino acid analogs include β -amino acid analogs. Examples of β -amino acid analogs include, but are not limited to, the following: cyclic β -amino acid analogs; β - alanine; (R) - β - phenylalanine; (R) - 1,2,3,4 - tetrahydro - isoquinoline - 3 - acetic acid; (R) - 3 - amino - 4 - (1 - naphthyl) - butyric acid; (R) - 3 - amino - 4 - (2,4 - dichlorophenyl)butyric acid; (R) - 3 - amino - 4 - (2 - chlorophenyl) - butyric acid; (R) - 3 - amino - 4 - (2 - cyanophenyl) - butyric acid; (R) - 3 - amino - 4 - (2 - fluorophenyl) - butyric acid; (R) - 3 - amino - 4 - (2 - furyl) - butyric acid; (R) - 3 - amino - 4 - (2 - methylphenyl) - butyric acid; (R) - 3 - amino - 4 - (2 - naphthyl) - butyric acid; (R) - 3 - amino - 4 - (2 - thienyl) - butyric acid; (R) - 3 - amino - 4 - (2 - trifluoromethylphenyl) - butyric acid; (R) - 3 - amino - 4 - (3,4 - dichlorophenyl)butyric acid; (R) - 3 - amino - 4 - (3,4 - difluorophenyl)butyric acid; (R) - 3 - amino - 4 - (3 - benzothienyl) - butyric acid; (R) - 3 - amino - 4 - (3 - chlorophenyl) - butyric acid; (R) - 3 - amino - 4 - (3 - cyanophenyl) - butyric acid; (R) - 3 - amino - 4 - (3 - fluorophenyl) - butyric acid; (R) - 3 - amino - 4 - (3 - methylphenyl) - butyric acid; (R) - 3 - amino - 4 - (3 - pyridyl) - butyric acid; (R) - 3 - amino - 4 - (3 - thienyl) - butyric acid; (R) - 3 - amino - 4 - (3 - trifluoromethylphenyl) - butyric acid; (R) - 3 - amino - 4 - (4 - bromophenyl) - butyric acid; (R) - 3 - amino - 4 - (4 - chlorophenyl) - butyric acid; (R) - 3 - amino - 4 - (4 - cyanophenyl) - butyric acid; (R) - 3 - amino - 4 - (4 - fluorophenyl) - butyric acid; (R) - 3 - amino - 4 - (4 - iodophenyl) - butyric acid; (R) - 3 - amino - 4 - (4 - methylphenyl) - butyric acid; (R) - 3 - amino - 4 - (4 - nitrophenyl) - butyric acid; (R) - 3 - amino - 4 - (4 - pyridyl) - butyric acid; (R) - 3 - amino - 4 - (4 - trifluoromethylphenyl) - butyric acid; (R) - 3 - amino - 4 - pentafluoro - phenylbutyric acid; (R) - 3 - amino - 5 - hexenoic acid; (R) - 3 - amino - 5 - hexynoic acid; (R) - 3 - amino - 5 - phenylpentanoic acid; (R) - 3 - amino - 6 - phenyl - 5 - hexenoic acid; (S) - 1,2,3,4 - tetrahydro - isoquinoline - 3 - acetic acid; (S) - 3 - amino - 4 - (1 - naphthyl) - butyric acid; (S) - 3 - amino - 4 - (2,4 - dichlorophenyl)butyric acid; (S) - 3 - amino - 4 - (2 - chlorophenyl) - butyric acid; (S) - 3 - amino - 4 - (2 - cyanophenyl) - butyric acid; (S) - 3 - amino - 4 - (2 - fluorophenyl) - butyric acid; (S) - 3 - amino - 4 - (2 - furyl) - butyric acid; (S) - 3 - amino - 4 - (2 - methylphenyl) - butyric acid; (S) - 3 - amino - 4 - (2 - naphthyl) - butyric acid; (S) - 3 - amino - 4 - (2 - thienyl) - butyric acid; (S) - 3 - amino - 4 - (2 - trifluoromethylphenyl) - butyric acid; (S) - 3 - amino - 4 - (3,4 - dichlorophenyl)butyric acid; (S) - 3 - amino - 4 - (3,4 - difluorophenyl)butyric acid; (S) - 3 - amino - 4 - (3 - benzothienyl) - butyric acid; (S) - 3 - amino - 4 - (3 - chlorophenyl) - butyric acid; (S) - 3 - amino - 4 - (3 - cyanophenyl) - butyric acid; (S) - 3 - amino - 4 - (3 - fluorophenyl) - butyric acid; (S) - 3 - amino - 4 - (3 - methylphenyl) - butyric acid; (S) - 3 - amino - 4 - (3 - pyridyl) - butyric acid; (S) - 3 - amino - 4 - (3 - thienyl) - butyric acid; (S) - 3 - amino - 4 - (3 - trifluoromethylphenyl) - butyric acid; (S) - 3 - amino - 4 - (4 - bromophenyl) - butyric acid; (S) - 3 - amino - 4 - (4 - chlorophenyl) - butyric acid; (S) - 3 - amino - 4 - (4 - cyanophenyl) - butyric acid; (S) - 3 - amino - 4 - (4 - fluorophenyl) - butyric acid; (S) - 3 - amino - 4 - (4 - iodophenyl) - butyric acid; (S) - 3 - amino - 4 - (4 - methylphenyl) - butyric

acid; (S) - 3 - amino - 4 - (4 - nitrophenyl) - butyric acid; (S) - 3 - amino - 4 - (4 - pyridyl) - butyric acid; (S) - 3 - amino - 4 - (4 - trifluoromethylphenyl) - butyric acid; (S) - 3 - amino - 4 - pentafluoro - phenylbutyric acid; (S) - 3 - amino - 5 - hexenoic acid; (S) - 3 - amino - 5 - hexynoic acid; (S) - 3 - amino - 5 - phenylpentanoic acid; (S) - 3 - amino - 6 - phenyl - 5 - hexenoic acid; 1,2,5,6 - tetrahydropyridine - 3 - carboxylic acid; 1,2,5,6 - tetrahydropyridine - 4 - carboxylic acid; 3 - amino - 3 - (2 - chlorophenyl) - propionic acid; 3 - amino - 3 - (2 - thienyl) - propionic acid; 3 - amino - 3 - (3 - bromophenyl) - propionic acid; 3 - amino - 3 - (4 - chlorophenyl) - propionic acid; 3 - amino - 3 - (4 - methoxyphenyl) - propionic acid; 3 - amino - 4,4,4 - trifluoro - butyric acid; 3 - aminoadipic acid; D- β - phenylalanine; β - leucine; L - β - homoalanine; L - β - homoaspartic acid γ - benzyl ester; L - β - homoglutamic acid δ - benzyl ester; L - β - homoisoleucine; L - β - homoleucine; L - β - homomethionine; L - β - homophenylalanine; L - β - homoproline; L - β - homotryptophan; L - β - homovaline; L - N ω - benzyloxycarbonyl - β - homolysine; N ω - L - β - homoarginine; O - benzyl - L - β - homohydroxyproline; O - benzyl - L - β - homoserine; O - benzyl - L - β - homothreonine; O - benzyl - L - β - homotyrosine; γ - trityl - L - β - homoasparagine; (R) - β - phenylalanine; L - β - homoaspartic acid γ - t - butyl ester; L - β - homoglutamic acid δ - t - butyl ester; L - N ω - β - homolysine; N δ - trityl - L - β - homoglutamine; N ω - 2,2,4,6,7 - pentamethyl - dihydrobenzofuran - 5 - sulfonyl - L - β - homoarginine; O - t - butyl - L - β - homohydroxy - proline; O - t - butyl - L - β - homoserine; O - t - butyl - L - β - homothreonine; O - t - butyl - L - β - homotyrosine; 2 - aminocyclopentane carboxylic acid; and 2 - aminocyclohexane carboxylic acid.

- [0044] Amino acid analogs include analogs of alanine, valine, glycine or leucine. Examples of amino acid analogs of alanine, valine, glycine, and leucine include, but are not limited to, the following: α - methoxyglycine; α - allyl - L - alanine; α - aminoisobutyric acid; α - methyl - leucine; β - (1 - naphthyl) - D - alanine; β - (1 - naphthyl) - L - alanine; β - (2 - naphthyl) - D - alanine; β - (2 - naphthyl) - L - alanine; β - (2 - pyridyl) - D - alanine; β - (2 - pyridyl) - L - alanine; β - (2 - thienyl) - D - alanine; β - (2 - thienyl) - L - alanine; β - (3 - benzothienyl) - D - alanine; β - (3 - benzothienyl) - L - alanine; β - (3 - pyridyl) - D - alanine; β - (3 - pyridyl) - L - alanine; β - (4 - pyridyl) - D - alanine; β - (4 - pyridyl) - L - alanine; β - chloro - L - alanine; β - cyano - L - alanine; β - cyclohexyl - D - alanine; β - cyclohexyl - L - alanine; β - cyclopenten - 1 - yl - alanine; β - cyclopentyl - alanine; β - cyclopropyl - L - Ala - OH • dicyclohexylammonium salt; β - t - butyl - D - alanine; β - t - butyl - L - alanine; γ - aminobutyric acid; L - α,β - diaminopropionic acid; 2,4 - dinitro - phenylglycine; 2,5 - dihydro - D - phenylglycine; 2 - amino - 4,4,4 - trifluorobutyric acid; 2 - fluoro - phenylglycine; 3 - amino - 4,4,4 - trifluoro - butyric acid; 3 - fluoro - valine; 4,4,4 - trifluoro - valine; 4,5 - dehydro - L - leu - OH • dicyclohexylammonium salt; 4 - fluoro - D - phenylglycine; 4 - fluoro - L - phenylglycine; 4 - hydroxy - D - phenylglycine; 5,5,5 - trifluoro - leucine; 6 - aminohexanoic acid; cyclopentyl - D

- Gly – OH • dicyclohexylammonium salt; cyclopentyl - Gly – OH • dicyclohexylammonium salt; D - α,β - diaminopropionic acid; D - α - aminobutyric acid; D - α - t - butylglycine; D - (2 - thienyl)glycine; D - (3 - thienyl)glycine; D - 2 - aminocaproic acid; D - 2 - indanylglycine; D - allylglycine•dicyclohexylammonium salt; D – cyclohexylglycine; D – norvaline; D – phenylglycine; β - aminobutyric acid; β - aminoisobutyric acid; (2 - bromophenyl)glycine; (2 - methoxyphenyl)glycine; (2 - methylphenyl)glycine; (2 - thiazoyl)glycine; (2 - thienyl)glycine; 2 - amino - 3 - (dimethylamino) - propionic acid; L - α,β - diaminopropionic acid; L - α - aminobutyric acid; L - α - t - butylglycine; L - (3 - thienyl)glycine; L - 2 - amino - 3 - (dimethylamino) - propionic acid; L - 2 - aminocaproic acid dicyclohexyl - ammonium salt; L - 2 - indanylglycine; L - allylglycine•dicyclohexyl ammonium salt; L – cyclohexylglycine; L – phenylglycine; L – propargylglycine; L – norvaline; N - α - aminomethyl - L – alanine; D - α,γ - diaminobutyric acid; L - α,γ - diaminobutyric acid; β - cyclopropyl - L – alanine; (N - β - (2,4 - dinitrophenyl)) - L - α,β - diaminopropionic acid; (N - β - 1 - (4,4 - dimethyl - 2,6 - dioxocyclohex - 1 - ylidene)ethyl) - D - α,β - diaminopropionic acid; (N - β - 1 - (4,4 - dimethyl - 2,6 - dioxocyclohex - 1 - ylidene)ethyl) - L - α,β - diaminopropionic acid; (N - β - 4 - methyltrityl) - L - α,β - diaminopropionic acid; (N - β - allyloxycarbonyl) - L - α,β - diaminopropionic acid; (N - γ - 1 - (4,4 - dimethyl - 2,6 - dioxocyclohex - 1 - ylidene)ethyl) - D - α,γ - diaminobutyric acid; (N - γ - 1 - (4,4 - dimethyl - 2,6 - dioxocyclohex - 1 - ylidene)ethyl) - L - α,γ - diaminobutyric acid; (N - γ - 4 - methyltrityl) - D - α,γ - diaminobutyric acid; (N - γ - 4 - methyltrityl) - L - α,γ - diaminobutyric acid; (N - γ - allyloxycarbonyl) - L - α,γ - diaminobutyric acid; D - α,γ - diaminobutyric acid; 4,5 - dehydro - L – leucine; cyclopentyl - D - Gly – OH; cyclopentyl - Gly – OH; D – allylglycine; D – homocyclohexylalanine; L - 1 - pyrenylalanine; L - 2 - aminocaproic acid; L – allylglycine; L – homocyclohexylalanine; and N - (2 - hydroxy - 4 - methoxy - Bzl) - Gly – OH.

[0045] Amino acid analogs further include analogs of arginine or lysine. Examples of amino acid analogs of arginine and lysine include, but are not limited to, the following: citrulline; L - 2 - amino - 3 - guanidinopropionic acid; L - 2 - amino - 3 - ureidopropionic acid; L – citrulline; Lys(Me)₂ – OH; Lys(N₃) – OH; N δ - benzyloxycarbonyl - L – ornithine; N ω - nitro - D – arginine; N ω - nitro - L – arginine; α - methyl – ornithine; 2,6 - diaminoheptanedioic acid; L – ornithine; (N δ - 1 - (4,4 - dimethyl - 2,6 - dioxo - cyclohex - 1 - ylidene)ethyl) - D – ornithine; (N δ - 1 - (4,4 - dimethyl - 2,6 - dioxo - cyclohex - 1 - ylidene)ethyl) - L – ornithine; (N δ - 4 - methyltrityl) - D – ornithine; (N δ - 4 - methyltrityl) - L – ornithine; D – ornithine; L – ornithine; Arg(Me)(Pbf) – OH; Arg(Me)₂ – OH (asymmetrical); Arg(Me)₂ - OH (symmetrical); Lys(ivDde) – OH; Lys(Me)₂ - OH • HCl; Lys(Me₃) - OH chloride; N ω - nitro - D – arginine; and N ω - nitro - L – arginine.

[0046] Amino acid analogs include analogs of aspartic or glutamic acids. Examples of amino acid analogs of aspartic and glutamic acids include, but are not limited to, the following: α - methyl -

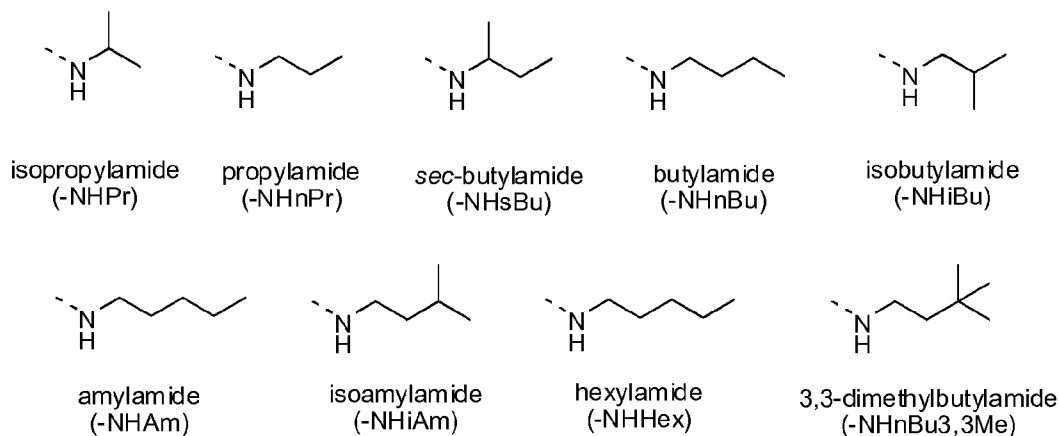
- D - aspartic acid; α - methyl - glutamic acid; α - methyl - L - aspartic acid; γ - methylene - glutamic acid; (N - γ - ethyl) - L - glutamine; [N - α - (4 - aminobenzoyl)] - L - glutamic acid; 2,6 - diaminopimelic acid; L - α - aminosuberic acid; D - 2 - aminoadipic acid; D - α - aminosuberic acid; α - aminopimelic acid; iminodiacetic acid; L - 2 - aminoadipic acid; threo - β - methyl - aspartic acid; γ - carboxy - D - glutamic acid γ,γ - di - t - butyl ester; γ - carboxy - L - glutamic acid γ,γ - di - t - butyl ester; Glu(OAll) - OH; L - Asu(OtBu) - OH; and pyroglutamic acid.
- [0047] Amino acid analogs include analogs of cysteine and methionine. Examples of amino acid analogs of cysteine and methionine include, but are not limited to, Cys(farnesyl) - OH, Cys(farnesyl) - OMe, α - methyl - methionine, Cys(2 - hydroxyethyl) - OH, Cys(3 - aminopropyl) - OH, 2 - amino - 4 - (ethylthio)butyric acid, buthionine, buthioninesulfoximine, ethionine, methionine methylsulfonium chloride, selenomethionine, cysteic acid, [2 - (4 - pyridyl)ethyl] - DL - penicillamine, [2 - (4 - pyridyl)ethyl] - L - cysteine, 4 - methoxybenzyl - D - penicillamine, 4 - methoxybenzyl - L - penicillamine, 4 - methylbenzyl - D - penicillamine, 4 - methylbenzyl - L - penicillamine, benzyl-D-cysteine, benzyl - L - cysteine, benzyl - DL - homocysteine, carbamoyl - L - cysteine, carboxyethyl - L - cysteine, carboxymethyl - L - cysteine, diphenylmethyl - L - cysteine, ethyl - L - cysteine, methyl - L - cysteine, t-butyl - D - cysteine, trityl - L - homocysteine, trityl - D - penicillamine, cystathionine, homocystine, L-homocystine, (2-aminoethyl) - L - cysteine, seleno - L - cystine, cystathionine, Cys(StBu) - OH, and acetamidomethyl - D - penicillamine.
- [0048] Amino acid analogs include analogs of phenylalanine and tyrosine. Examples of amino acid analogs of phenylalanine and tyrosine include β - methyl - phenylalanine, β - hydroxyphenylalanine, α - methyl - 3 - methoxy - DL - phenylalanine, α - methyl - D - phenylalanine, α - methyl - L - phenylalanine, 1,2,3,4 - tetrahydroisoquinoline - 3 - carboxylic acid, 2,4 - dichloro - phenylalanine, 2 - (trifluoromethyl) - D - phenylalanine, 2 - (trifluoromethyl) - L - phenylalanine, 2 - bromo - D - phenylalanine, 2 - bromo - L - phenylalanine, 2 - chloro - D - phenylalanine, 2 - chloro - L - phenylalanine, 2 - cyano - D - phenylalanine, 2 - cyano - L - phenylalanine, 2 - fluoro - D - phenylalanine, 2 - fluoro - L - phenylalanine, 2 - methyl - D - phenylalanine, 2 - methyl - L - phenylalanine, 2 - nitro - D - phenylalanine, 2 - nitro - L - phenylalanine, 2,4,5 - trihydroxy - phenylalanine, 3,4,5 - trifluoro - D - phenylalanine, 3,4,5 - trifluoro - L - phenylalanine, 3,4 - dichloro - D - phenylalanine, 3,4 - dichloro - L - phenylalanine, 3,4 - difluoro - D - phenylalanine, 3,4 - difluoro - L - phenylalanine, 3,4 - dihydroxy - L - phenylalanine, 3,4 - dimethoxy - L - phenylalanine, 3,5,3' - triiodo - L - thyronine, 3,5 - diiodo - D - tyrosine, 3,5 - diiodo - L - tyrosine, 3,5 - diiodo - L - thyronine, 3 - (trifluoromethyl) - D - phenylalanine, 3 - (trifluoromethyl) - L - phenylalanine, 3 - amino - L - tyrosine, 3 - bromo - D - phenylalanine, 3 - bromo - L - phenylalanine, 3 - chloro - D - phenylalanine, 3 - chloro - L - phenylalanine, 3 - chloro - L - tyrosine, 3 - cyano - D - phenylalanine, 3 - cyano - L - phenylalanine, 3 - fluoro - D - phenylalanine, 3 - fluoro - L -

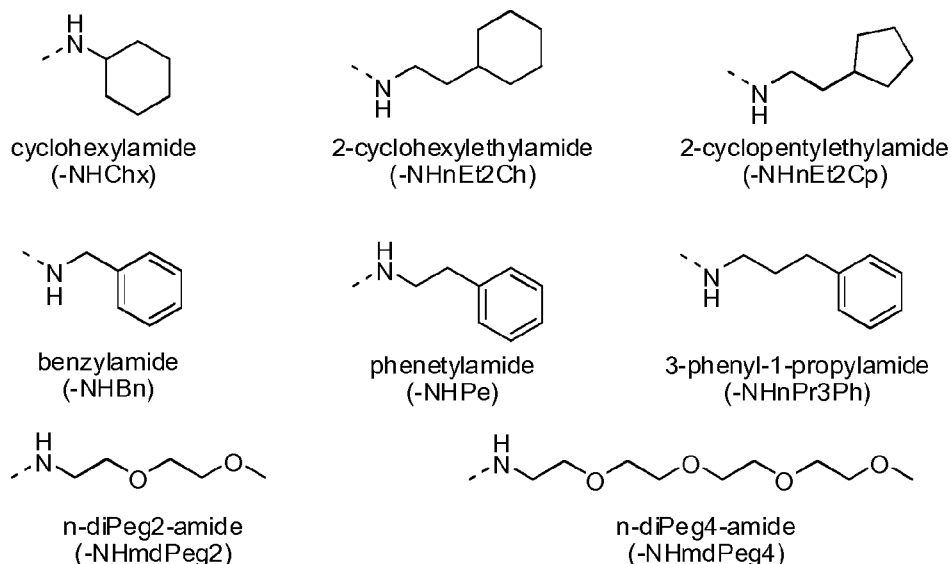
phenylalanine, 3 - fluoro - tyrosine, 3 - iodo - D - phenylalanine, 3 - iodo - L - phenylalanine, 3 - iodo - L - tyrosine, 3 - methoxy - L - tyrosine, 3 - methyl - D - phenylalanine, 3 - methyl - L - phenylalanine, 3 - nitro - D - phenylalanine, 3 - nitro - L - phenylalanine, 3 - nitro - L - tyrosine, 4 - (trifluoromethyl) - D - phenylalanine, 4 - (trifluoromethyl) - L - phenylalanine, 4 - amino - D - phenylalanine, 4 - amino - L - phenylalanine, 4 - benzoyl - D - phenylalanine, 4 - benzoyl - L - phenylalanine, 4 - bis(2 - chloroethyl)amino - L - phenylalanine, 4 - bromo - D - phenylalanine, 4 - bromo - L - phenylalanine, 4 - chloro - D - phenylalanine, 4 - chloro - L - phenylalanine, 4 - cyano - D - phenylalanine, 4 - cyano - L - phenylalanine, 4 - fluoro - D - phenylalanine, 4 - fluoro - L - phenylalanine, 4 - iodo - D - phenylalanine, 4 - iodo - L - phenylalanine, homophenylalanine, thyroxine, 3,3 - diphenylalanine, thyronine, ethyl-tyrosine, and methyl-tyrosine.

- [0049] Amino acid analogs include analogs of proline. Examples of amino acid analogs of proline include, but are not limited to, 3,4-dehydro-proline, 4-fluoro-proline, cis-4-hydroxy-proline, thiazolidine-2-carboxylic acid, and trans-4-fluoro-proline.
- [0050] Amino acid analogs include analogs of serine and threonine. Examples of amino acid analogs of serine and threonine include, but are not limited to, 3 - amino - 2 - hydroxy - 5 - methylhexanoic acid, 2 - amino - 3 - hydroxy - 4 - methylpentanoic acid, 2 - amino - 3 - ethoxybutanoic acid, 2 - amino - 3 - methoxybutanoic acid, 4 - amino - 3 - hydroxy - 6 - methylheptanoic acid, 2 - amino - 3 - benzyloxypropionic acid, 2 - amino - 3 - benzyloxypropionic acid, 2 - amino - 3 - ethoxypropionic acid, 4 - amino - 3 - hydroxybutanoic acid, and α -methylserine.
- [0051] Amino acid analogs include analogs of tryptophan. Examples of amino acid analogs of tryptophan include, but are not limited to, the following: α - methyl - tryptophan; β - (3 - benzothienyl) - D - alanine; β - (3 - benzothienyl) - L - alanine; 1 - methyl - tryptophan; 4 - methyl - tryptophan; 5 - benzyloxy - tryptophan; 5 - bromo - tryptophan; 5 - chloro - tryptophan; 5 - fluoro - tryptophan; 5 - hydroxy - tryptophan; 5 - hydroxy - L - tryptophan; 5 - methoxy - tryptophan; 5 - methoxy - L - tryptophan; 5 - methyl - tryptophan; 6 - bromo - tryptophan; 6 - chloro - D - tryptophan; 6 - chloro - tryptophan; 6 - fluoro - tryptophan; 6 - methyl - tryptophan; 7 - benzyloxy - tryptophan; 7 - bromo - tryptophan; 7 - methyl - tryptophan; D - 1,2,3,4 - tetrahydro - norharman - 3 - carboxylic acid; 6 - methoxy - 1,2,3,4 - tetrahydronorharman - 1 - carboxylic acid; 7 - azatryptophan; L - 1,2,3,4 - tetrahydro - norharman - 3 - carboxylic acid; 5 - methoxy - 2 - methyl - tryptophan; and 6 - chloro - L - tryptophan.
- [0052] In some embodiments, amino acid analogs are racemic. In some embodiments, the D isomer of the amino acid analog is used. In some embodiments, the L isomer of the amino acid analog is used. In other embodiments, the amino acid analog comprises chiral centers that are in the R or S configuration. In still other embodiments, the amino group(s) of a β -amino acid analog is substituted with a protecting group, *e.g.*, tert-butyloxycarbonyl (BOC group), 9-fluorenylmethyloxycarbonyl (Fmoc), tosyl, and the like. In yet other embodiments, the

carboxylic acid functional group of a β -amino acid analog is protected, *e.g.*, as its ester derivative. In some embodiments the salt of the amino acid analog is used.

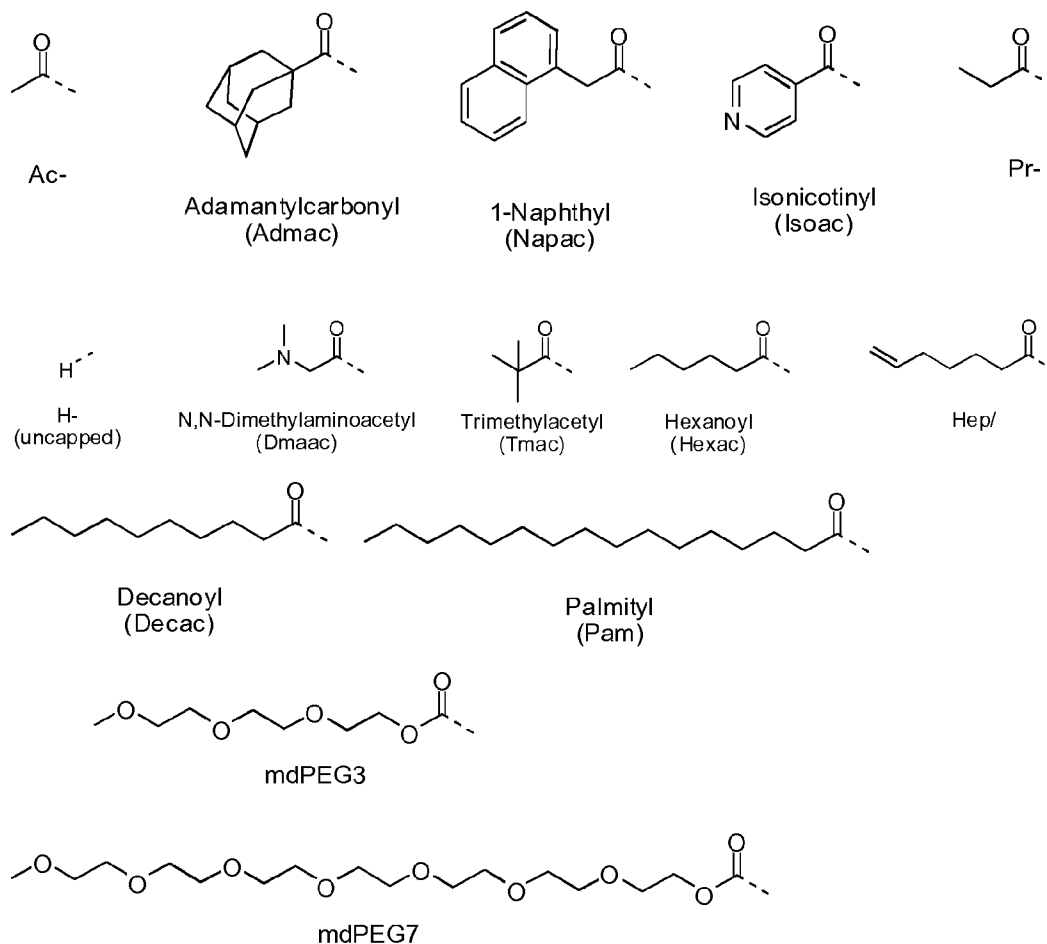
- [0053] A “non-essential” amino acid residue, as used herein, is an amino acid residue present in a wild-type sequence of a polypeptide that can be altered without abolishing or substantially altering essential biological or biochemical activity (*e.g.*, receptor binding or activation) of the polypeptide.
- [0054] An “essential” amino acid residue, as used herein, is an amino acid residue present in a wild-type sequence of a polypeptide that, when altered, results in abolishing or a substantial reduction in the polypeptide's essential biological or biochemical activity(*e.g.*, receptor binding or activation).
- [0055] A “conservative amino acid substitution” is one in which an amino acid residue is replaced with a different 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 polypeptide, for example, is 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, or 6-Cl-tryptophan for tryptophan).
- [0056] The term “capping group” refers to the chemical moiety occurring at either the carboxy or amino terminus of the polypeptide chain of the subject peptidomimetic macrocycle. The capping group of a carboxy terminus includes an unmodified carboxylic acid (ie $-\text{COOH}$) or a carboxylic acid with a substituent. For example, the carboxy terminus can be substituted with an amino group to yield a carboxamide at the C-terminus. Various substituents include but are not limited to primary and secondary amines, including pegylated secondary amines. Representative secondary amine capping groups for the C-terminus include:





[0057] The capping group of an amino terminus includes an unmodified amine (ie -NH_2) or an amine with a substituent. For example, the amino terminus can be substituted with an acyl group to yield a carboxamide at the N-terminus. Various substituents include but are not limited to substituted acyl groups, including C_1 - C_6 carbonyls, C_7 - C_{30} carbonyls, and pegylated carbamates.

Representative capping groups for the N-terminus include:



- [0058] 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-difluoro-decane 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.
- [0059] The symbol “” when used as part of a molecular structure refers to a single bond or a *trans* or *cis* double bond.
- [0060] The term “amino acid side chain” refers to a moiety attached to the α -carbon (or another backbone atom) 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).
- [0061] 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.
- [0062] 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).
- [0063] The term “macrocyclization reagent” or “macrocycle-forming reagent” as used herein refers to any reagent which can be used to prepare a peptidomimetic macrocycle by mediating the reaction between two reactive groups. Reactive groups can 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(CO₂CH₃)₂, CuSO₄, and CuCl₂ 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 can additionally include, for example, Ru reagents known in the art such as Cp*RuCl(PPh₃)₂, [Cp*RuCl]₄ or other Ru reagents which can 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. In other examples, catalysts have W or Mo centers. Various catalysts are disclosed in Grubbs et al., “Ring Closing Metathesis and Related Processes in Organic Synthesis” Acc. Chem. Res. 1995, 28, 446-452,

- U.S. Pat. No. 5,811,515; U.S. Pat. No. 7,932,397; U.S. Application No. 2011/0065915; U.S. Application No. 2011/0245477; Yu et al., "Synthesis of Macrocyclic Natural Products by Catalyst-Controlled Stereoselective Ring-Closing Metathesis," *Nature* 2011, 479, 88; and Peryshkov et al., "Z-Selective Olefin Metathesis Reactions Promoted by Tungsten Oxo Alkylidene Complexes," *J. Am. Chem. Soc.* 2011, 133, 20754. 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.
- [0064] The term "halo" or "halogen" refers to fluorine, chlorine, bromine or iodine or a radical thereof.
- [0065] 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, C₁-C₁₀ 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.
- [0066] The term "alkylene" refers to a divalent alkyl (*i.e.*, -R-).
- [0067] 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, C₂-C₁₀ indicates that the group has from 2 to 10 (inclusive) carbon atoms in it. The term "lower alkenyl" refers to a C₂-C₆ 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.
- [0068] 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, C₂-C₁₀ indicates that the group has from 2 to 10 (inclusive) carbon atoms in it. The term "lower alkynyl" refers to a C₂-C₆ 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.
- [0069] 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 "arylalkoxy" refers to an alkoxy substituted with aryl.
- [0070] "Arylalkyl" refers to an aryl group, as defined above, wherein one of the aryl group's hydrogen atoms has been replaced with a C₁-C₅ 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, 4-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.

- [0071] "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- $C(O)NH_2$ -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,
- [0072] "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.
- [0073] "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, $-CH_2-C(O)NH_2$, $-CH_2CH_2-C(O)NH_2$, $-CH_2CH_2CH_2C(O)NH_2$, $-CH_2CH_2CH_2CH_2C(O)NH_2$, $-CH_2CH_2CH_2CH_2CH_2C(O)NH_2$, $-CH_2CH(C(O)NH_2)CH_3$, $-CH_2CH(C(O)NH_2)CH_2CH_3$, $-CH(C(O)NH_2)CH_2CH_3$, $-C(CH_3)_2CH_2C(O)NH_2$, $-CH_2-CH_2-NH-C(O)-CH_3$, $-CH_2-CH_2-NH-C(O)-CH_3-CH_3$, and $-CH_2-CH_2-NH-C(O)-CH=CH_2$.
- [0074] "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 $-C(CH_3)_2CH_2OH$.
- [0075] "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, $-CH_2COOH$, $-CH_2CH_2COOH$, $-CH_2CH_2CH_2COOH$, $-CH_2CH_2CH_2CH_2COOH$, $-CH_2CH(COOH)CH_3$, $-CH_2CH_2CH_2CH_2CH_2COOH$, $-CH_2CH(COOH)CH_2CH_3$, $-CH(COOH)CH_2CH_3$ and $-C(CH_3)_2CH_2COOH$.
- [0076] 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.
- [0077] 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.
- [0078] 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.
- [0079] 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.
- [0080] 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.
- [0081] 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, oxo, nitro, haloalkyl, alkyl, alkaryl, aryl, aralkyl, alkoxy, thioalkoxy, aryloxy, amino, alkoxycarbonyl, amido, carboxy, alkanesulfonyl, alkylcarbonyl, and cyano groups.
- [0082] In some embodiments, one or more compounds disclosed herein contain one or more asymmetric centers and thus occur as racemates and racemic mixtures, single enantiomers, individual diastereomers and diastereomeric mixtures. In one embodiment isomeric forms of these compounds are included in the present invention unless expressly provided otherwise. In some embodiments, one or more compounds disclosed herein are also represented in multiple tautomeric forms, in such instances, the one or more compounds includes all tautomeric forms of the compounds described herein (*e.g.*, if alkylation of a ring system results in alkylation at multiple sites, the one or more compounds 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.
- [0083] 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%.
- [0084] As used herein, the recitation of a numerical range for a variable is intended to convey that the invention can 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

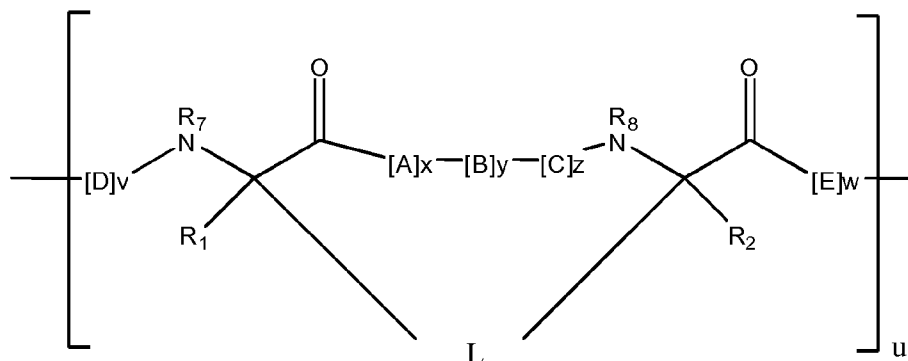
described as having values between 0 and 2 takes the values 0, 1 or 2 if the variable is inherently discrete, and takes the values 0.0, 0.1, 0.01, 0.001, or any other real values ≥ 0 and ≤ 2 if the variable is inherently continuous.

- [0085] 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.”
- [0086] The term “on average” represents the mean value derived from performing at least three independent replicates for each data point.
- [0087] The term “biological activity” encompasses structural and functional properties of a macrocycle. Biological activity is, for example, structural stability, alpha-helicity, affinity for a target, resistance to proteolytic degradation, cell penetrability, intracellular stability, *in vivo* stability, or any combination thereof.
- [0088] The term “binding affinity” refers to the strength of a binding interaction, for example between a peptidomimetic macrocycle and a target. Binding affinity can be expressed, for example, as an equilibrium dissociation constant (“ K_D ”), which is expressed in units which are a measure of concentration (e.g. M, mM, μ M, nM etc). Numerically, binding affinity and K_D values vary inversely, such that a lower binding affinity corresponds to a higher K_D value, while a higher binding affinity corresponds to a lower K_D value. Where high binding affinity is desirable, “improved” binding affinity refers to higher binding affinity and therefore lower K_D values.
- [0089] The term “ratio of binding affinities” refers to the ratio of dissociation constants (K_D values) of a first binding interaction (the numerator), versus a second binding interaction (denominator). Consequently, a “reduced ratio of binding affinities” to Target 1 versus Target 2 refers to a lower value for the ratio expressed as $K_D(\text{Target 1})/K_D(\text{Target 2})$. This concept can also be characterized as “improved selectivity” for Target 1 versus Target 2, which can be due either to a decrease in the K_D value for Target 1 or an increase in the value for the K_D value for Target 2.
- [0090] The term “in vitro efficacy” refers to the extent to which a test compound, such as a peptidomimetic macrocycle, produces a beneficial result in an in vitro test system or assay. In vitro efficacy can be measured, for example, as an “ IC_{50} ” or “ EC_{50} ” value, which represents the concentration of the test compound which produces 50% of the maximal effect in the test system.
- [0091] The term “ratio of in vitro efficacies” or “in vitro efficacy ratio” refers to the ratio of IC_{50} or EC_{50} values from a first assay (the numerator) versus a second assay (the denominator). Consequently, an improved in vitro efficacy ratio for Assay 1 versus Assay 2 refers to a lower value for the ratio expressed as $IC_{50}(\text{Assay 1})/IC_{50}(\text{Assay 2})$ or alternatively as $EC_{50}(\text{Assay 1})/EC_{50}(\text{Assay 2})$. This concept can also be characterized as “improved selectivity” in Assay 1 versus Assay 2, which can be due either to a decrease in the IC_{50} or EC_{50} value for Target 1 or an increase in the value for the IC_{50} or EC_{50} value for Target 2.

[0092] The details of one or more particular embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

Peptidomimetic Macrocycles

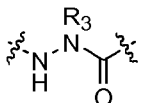
[0093] In some embodiments, a peptidomimetic macrocycle has the Formula (I):



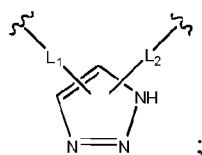
Formula (I)

wherein:

each A, C, D, and E is independently an amino acid;

B is an amino acid, , $[-NH-L_3-CO-]$, $[-NH-L_3-SO_2-]$, or $[-NH-L_3-]$;

each L and L' is independently a macrocycle-forming 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_3 is independently hydrogen, alkyl, alkenyl, alkynyl, arylalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, cycloalkylalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R_5 ;

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

each K is independently O, S, SO, SO₂, CO, CO₂, or CONR₃;

each R_5 is independently halogen, alkyl, -OR₆, -N(R₆)₂, -SR₆, -SOR₆, -SO₂R₆, -CO₂R₆, 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;

each R_7 is independently $-H$, 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;

each R_8 is independently $-H$, 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;

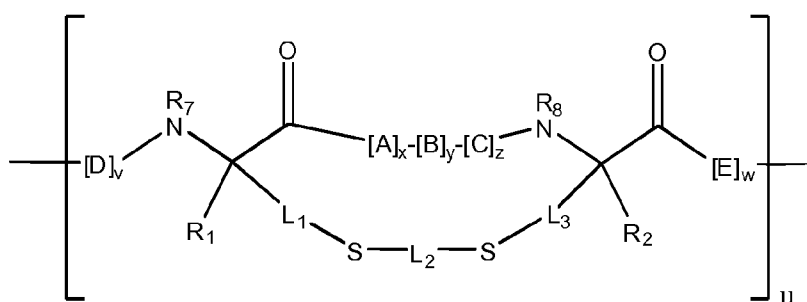
v and w are independently integers from 1-1000, for example 1-500, 1-200, 1-100, 1-50, 1-30, 1-20 or 1-10;

u is an integer from 1-10, for example 1-5, 1-3 or 1-2;

x , y and z are independently integers from 0-10, for example the sum of $x+y+z$ is 2, 3, or 6; and

n is an integer from 1-5.

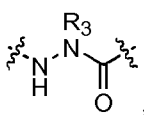
[0094] In some embodiments, a peptidomimetic macrocycle has the Formula:



Formula (II)

wherein:

each A, C, D, and E is independently an amino acid;

B is an amino acid, , $[-NH-L_4-CO-]$, $[-NH-L_4-SO_2-]$, 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-; or at least one of R_1 and R_2 forms a macrocycle-forming linker L' connected to the alpha position of one of said D or E amino acids;

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

L_1 , L_2 , L_3 and L_4 are independently alkylene, alkenylene, alkynylene, heteroalkylene,

cycloalkylene, heterocycloalkylene, cycloarylene, heterocycloarylene or $[-R_4-K-R_4-]_n$, each being unsubstituted or substituted with R_5 ;

each K is O, S, SO, SO₂, CO, CO₂, or CONR₃;

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

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;

v and w are independently integers from 1-1000, for example 1-500, 1-200, 1-100, 1-50, 1-30, 1-20 or 1-10;

u is an integer from 1-10, for example 1-5, 1-3 or 1-2;

x, y and z are independently integers from 0-10, for example the sum of $x+y+z$ is 2, 3, or 6; and

n is an integer from 1-5.

[0095] In some embodiments, v and w are integers between 1-30. In some embodiments, w is an integer from 3-1000, for example 3-500, 3-200, 3-100, 3-50, 3-30, 3-20, or 3-10.

[0096] In some embodiments, the peptidomimetic macrocycles are claimed with the proviso that when $u=1$ and $w=2$, the first C-terminal amino acid represented by E is not an Arginine (R) and/or the second C-terminal amino acid represented by E is not a Threonine (T). For instance, when $u=1$ and $w=2$, the first C-terminal amino acid and/or the second C-terminal amino acid represented by E do not comprise a positively charged side chain or a polar uncharged side chain. In some embodiments, when $u=1$ and $w=2$, the first C-terminal amino acid and/or the second C-terminal amino acid represented by E comprise a hydrophobic side chain. For example, when $w=2$, the first C-terminal amino acid and/or the second N-terminal amino acid represented by E comprise a hydrophobic side chain, for example a large hydrophobic side chain.

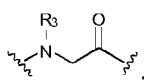
[0097] In some embodiments, w is between 3 and 1000. For example, the third amino acid represented by E comprises a large hydrophobic side chain.

[0098] In some embodiments of a peptidomimetic macrocycle of Formula I, L_1 and L_2 , either alone or in combination, do not form an all hydrocarbon chain or a thioether. In other embodiments of a peptidomimetic macrocycle of Formula II, L_1 and L_2 , either alone or in combination, do not form an all hydrocarbon chain or a triazole.

[0099] In one example, at least one of R_1 and R_2 is alkyl, unsubstituted or substituted with halo-. In another example, both R_1 and R_2 are independently alkyl, unsubstituted or substituted with halo-. In some embodiments, at least one of R_1 and R_2 is methyl. In other embodiments, R_1 and R_2 are methyl.

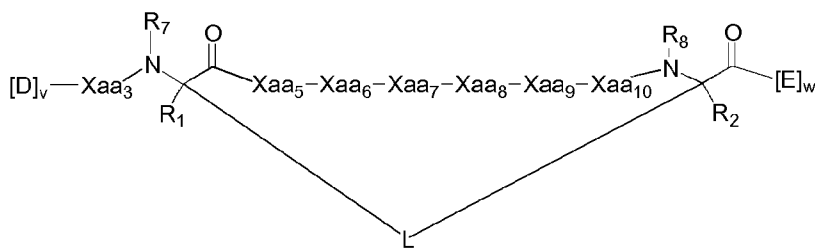
[00100] In some embodiments, $x+y+z$ is at least 3. In other embodiments, $x+y+z$ is 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10. In some embodiments, the sum of $x+y+z$ is 3 or 6. In some embodiments, the sum of $x+y+z$ is 3. In other embodiments, the sum of $x+y+z$ is 6. Each occurrence of A, B, C, D or E in a macrocycle or macrocycle precursor is independently selected. For example, a sequence represented by the formula $[A]_x$, when x is 3, encompasses embodiments where the amino acids are not identical, e.g. Gln-Asp-Ala as well as embodiments where the amino acids are identical, e.g. Gln-Gln-Gln. This applies for any value of x , y , or z in the indicated ranges. Similarly, when u is greater than 1, each compound can encompass peptidomimetic macrocycles which are the same or different. For example, a compound can comprise peptidomimetic macrocycles comprising different linker lengths or chemical compositions.

[00101] In some embodiments, the peptidomimetic macrocycle 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 .

[00102] In other embodiments, the length of the macrocycle-forming linker L as measured from a first $C\alpha$ to a second $C\alpha$ 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 $C\alpha$ to a second $C\alpha$.

[00103] Peptidomimetic macrocycles are also provided of the formula:



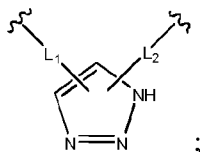
wherein:

each of Xaa_3 , Xaa_5 , Xaa_6 , Xaa_7 , Xaa_8 , Xaa_9 , and Xaa_{10} is individually an amino acid, wherein at least three of Xaa_3 , Xaa_5 , Xaa_6 , Xaa_7 , Xaa_8 , Xaa_9 , and Xaa_{10} are the same amino acid as the amino acid at the corresponding position of the sequence Phe₃-X₄-His₅-Tyr₆-Trp₇-Ala₈-Gln₉-Leu₁₀-X₁₁-Ser₁₂, where each X is an amino acid;

each D and E is independently an amino acid;

R_1 and R_2 are independently $-H$, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo-; or at least one of R_1 and R_2 forms a macrocycle-forming linker L' connected to the alpha position of one of said D or E amino acids;

each L and L' is independently a macrocycle-forming linker of the formula



L₁ and L₂ are independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, cycloarylene, heterocycloarylene, or [-R₄-K-R₄]_n, each being optionally substituted with R₅;

each R₃ is independently hydrogen, alkyl, alkenyl, alkynyl, arylalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, cycloalkylalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅;

each R₄ is independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene;

each K is independently O, S, SO, SO₂, CO, CO₂, or CONR₃;

each R₅ is independently halogen, alkyl, -OR₆, -N(R₆)₂, -SR₆, -SOR₆, -SO₂R₆, -CO₂R₆, a fluorescent moiety, a radioisotope or a therapeutic agent;

each R₆ is independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heterocycloalkyl, a fluorescent moiety, a radioisotope or a therapeutic agent;

each R₇ is independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅, or part of a cyclic structure with a D residue;

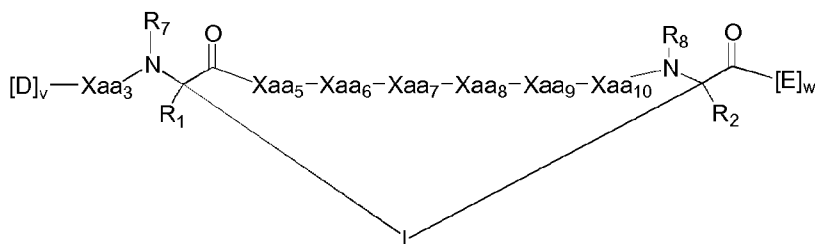
each R₈ is independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅, or part of a cyclic structure with an E residue;

v is an integer from 1-1000, for example 1-500, 1-200, 1-100, 1-50, 1-30, 1-20 or 1-10;

w is an integer from 3-1000, for example 3-500, 3-200, 3-100, 3-50, 3-30, 3-20, or 3-10; and

n is an integer from 1-5.

[00104] In some embodiments, the peptidomimetic macrocycle has the Formula:



wherein:

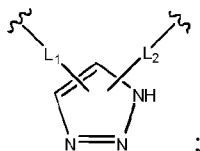
each of Xaa₃, Xaa₅, Xaa₆, Xaa₇, Xaa₈, Xaa₉, and Xaa₁₀ is individually an amino acid, wherein at least three of Xaa₃, Xaa₅, Xaa₆, Xaa₇, Xaa₈, Xaa₉, and Xaa₁₀ are the same amino acid as the amino acid at

the corresponding position of the sequence Phe₃-X₄-Glu₅-Tyr₆-Trp₇-Ala₈-Gln₉-Leu₁₀/Cba₁₀-X₁₁-Ala₁₂, where each X is an amino acid;

each D and E is independently an amino acid;

R₁ and R₂ are independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo-; or at least one of R₁ and R₂ forms a macrocycle-forming linker L' connected to the alpha position of one of said D or E amino acids;

each L and L' is independently a macrocycle-forming linker of the formula



L₁ and L₂ are independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, cycloarylene, heterocycloarylene, or [-R₄-K-R₄]_n, each being optionally substituted with R₅;

R₃ is hydrogen, alkyl, alkenyl, alkynyl, arylalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, cycloalkylalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅;

each R₄ is alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene;

each K is O, S, SO, SO₂, CO, CO₂, or CONR₃;

each R₅ is independently halogen, alkyl, -OR₆, -N(R₆)₂, -SR₆, -SOR₆, -SO₂R₆, -CO₂R₆, a fluorescent moiety, a radioisotope or a therapeutic agent;

each R₆ is independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heterocycloalkyl, a fluorescent moiety, a radioisotope or a therapeutic agent;

R₇ is -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅, or part of a cyclic structure with a D residue;

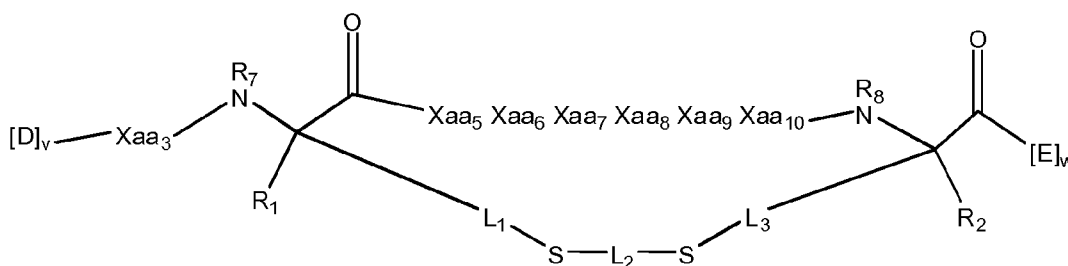
R₈ is -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅, or part of a cyclic structure with an E residue;

v is an integer from 1-1000, for example 1-500, 1-200, 1-100, 1-50, 1-30, 1-20, or 1-10;

w is an integer from 3-1000, for example 3-500, 3-200, 3-100, 3-50, 3-30, 3-20, or 3-10; and

n is an integer from 1-5.

[00105] Peptidomimetic macrocycles are also provided of the formula:



wherein:

each of Xaa₃, Xaa₅, Xaa₆, Xaa₇, Xaa₈, Xaa₉, and Xaa₁₀ is individually an amino acid, wherein at least three of Xaa₃, Xaa₅, Xaa₆, Xaa₇, Xaa₈, Xaa₉, and Xaa₁₀ are the same amino acid as the amino acid at the corresponding position of the sequence Phe₃-X₄-His₅-Tyr₆-Trp₇-Ala₈-Gln₉-Leu₁₀-X₁₁-Ser₁₂, where each X is an amino acid;

each D and E is independently an amino acid;

R₁ and R₂ are independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo-; or at least one of R₁ and R₂ forms a macrocycle-forming linker L' connected to the alpha position of one of said D or E amino acids;

L₁, L₂, L₃ and L₄ are independently alkylene, alkenylene, alkynylene, heteroalkylene,

cycloalkylene, heterocycloalkylene, cycloarylene, heterocycloarylene or [-R₄-K-R₄]-_n, each being unsubstituted or substituted with R₅;

each K is O, S, SO, SO₂, CO, CO₂, or CONR₃;

R₃ is hydrogen, alkyl, alkenyl, alkynyl, arylalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, cycloalkylalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅;

each R₄ is alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene;

each R₅ is independently halogen, alkyl, -OR₆, -N(R₆)₂, -SR₆, -SOR₆, -SO₂R₆, -CO₂R₆, a fluorescent moiety, a radioisotope or a therapeutic agent;

each R₆ is independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heterocycloalkyl, a fluorescent moiety, a radioisotope or a therapeutic agent;

R₇ is -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅, or part of a cyclic structure with a D residue;

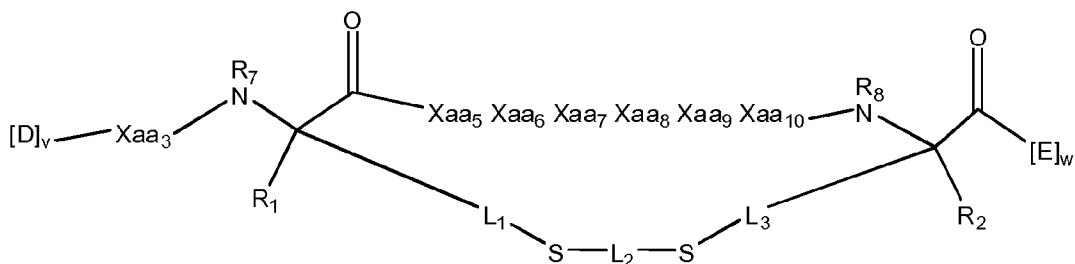
R₈ is -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅, or part of a cyclic structure with an E residue;

v is an integer from 1-1000, for example 1-500, 1-200, 1-100, 1-50, 1-30, 1-20 or 1-10;

w is an integer from 3-1000, for example 3-500, 3-200, 3-100, 3-50, 3-30, 3-20, or 3-10; and

n is an integer from 1-5.

[00106] Peptidomimetic macrocycles are also provided of the formula:



wherein:

each of Xaa₃, Xaa₅, Xaa₆, Xaa₇, Xaa₈, Xaa₉, and Xaa₁₀ is individually an amino acid, wherein at least three of Xaa₃, Xaa₅, Xaa₆, Xaa₇, Xaa₈, Xaa₉, and Xaa₁₀ are the same amino acid as the amino acid at the corresponding position of the sequence Phe₃-X₄-Glu₅-Tyr₆-Trp₇-Ala₈-Gln₉-Leu₁₀/Cba₁₀-X₁₁-Ala₁₂, where each X is an amino acid;

each D and E is independently an amino acid;

R₁ and R₂ are independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo-; or at least one of R₁ and R₂ forms a macrocycle-forming linker L' connected to the alpha position of one of said D or E amino acids;

L₁, L₂, L₃ and L₄ are independently alkylene, alkenylene, alkynylene, heteroalkylene,

cycloalkylene, heterocycloalkylene, cycloarylene, heterocycloarylene or [-R₄-K-R₄]-_n, each being unsubstituted or substituted with R₅;

each K is O, S, SO, SO₂, CO, CO₂, or CONR₃;

R₃ is hydrogen, alkyl, alkenyl, alkynyl, arylalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, cycloalkylalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅;

each R₄ is alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene;

each R₅ is independently halogen, alkyl, -OR₆, -N(R₆)₂, -SR₆, -SOR₆, -SO₂R₆, -CO₂R₆, a fluorescent moiety, a radioisotope or a therapeutic agent;

each R₆ is independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heterocycloalkyl, a fluorescent moiety, a radioisotope or a therapeutic agent;

R₇ is -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅, or part of a cyclic structure with a D residue;

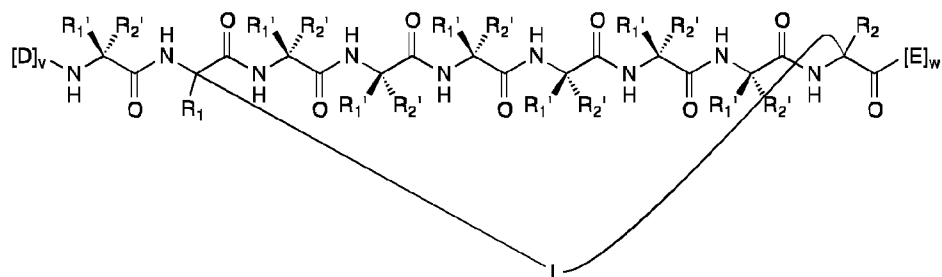
R₈ is -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅, or part of a cyclic structure with an E residue;

v is an integer from 1-1000, for example 1-500, 1-200, 1-100, 1-50, 1-30, 1-20, or 1-10;

w is an integer from 3-1000, for example 3-500, 3-200, 3-100, 3-50, 3-30, 3-20, or 3-10; and

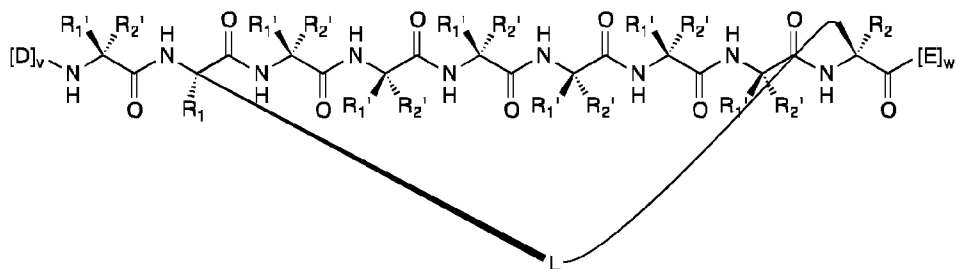
n is an integer from 1-5.

[00107] In one embodiment, the peptidomimetic macrocycle is:



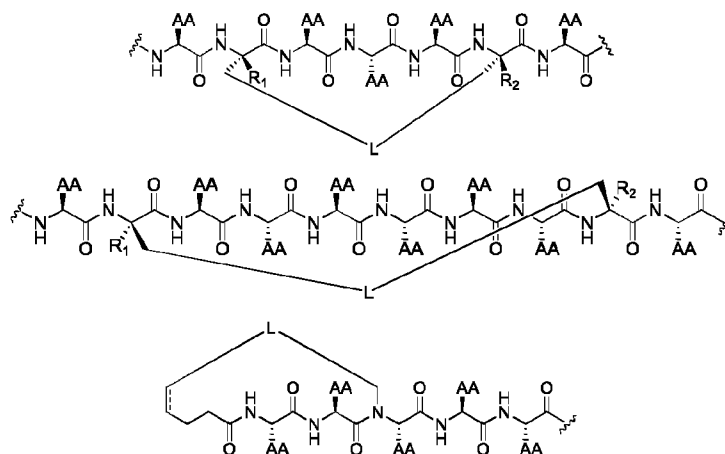
wherein each R₁ and R₂ is independently independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo—.

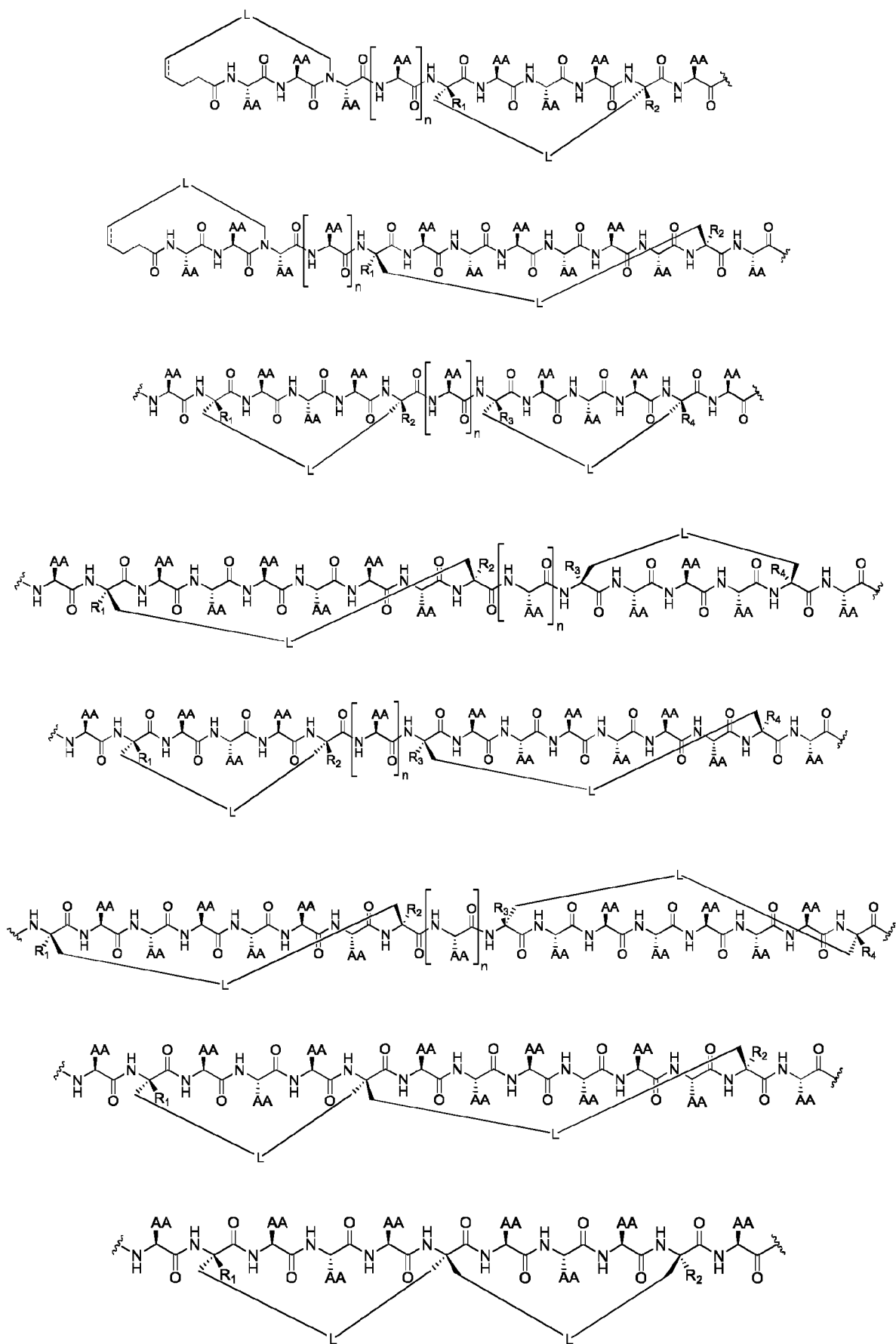
[00108] In related embodiments, the peptidomimetic macrocycle is:

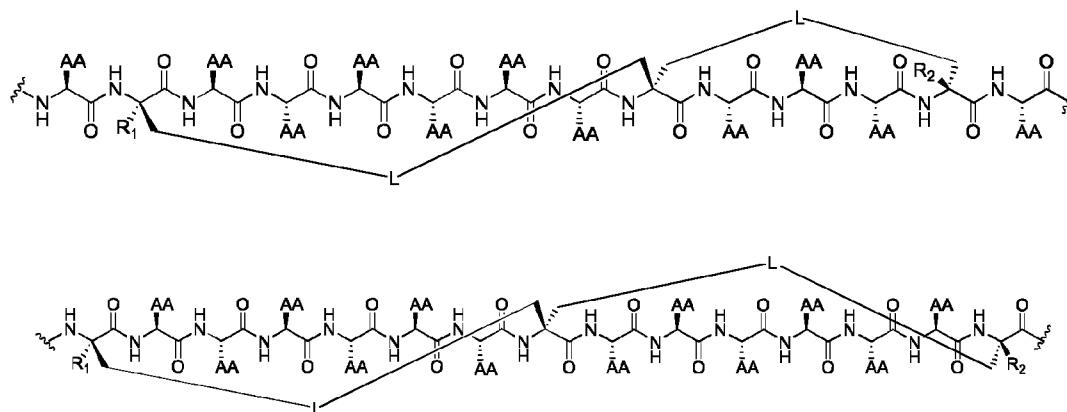


wherein each R₁' and R₂' is independently an amino acid.

[00109] In other embodiments, the peptidomimetic macrocycle is a compound of any of the formulas shown below:

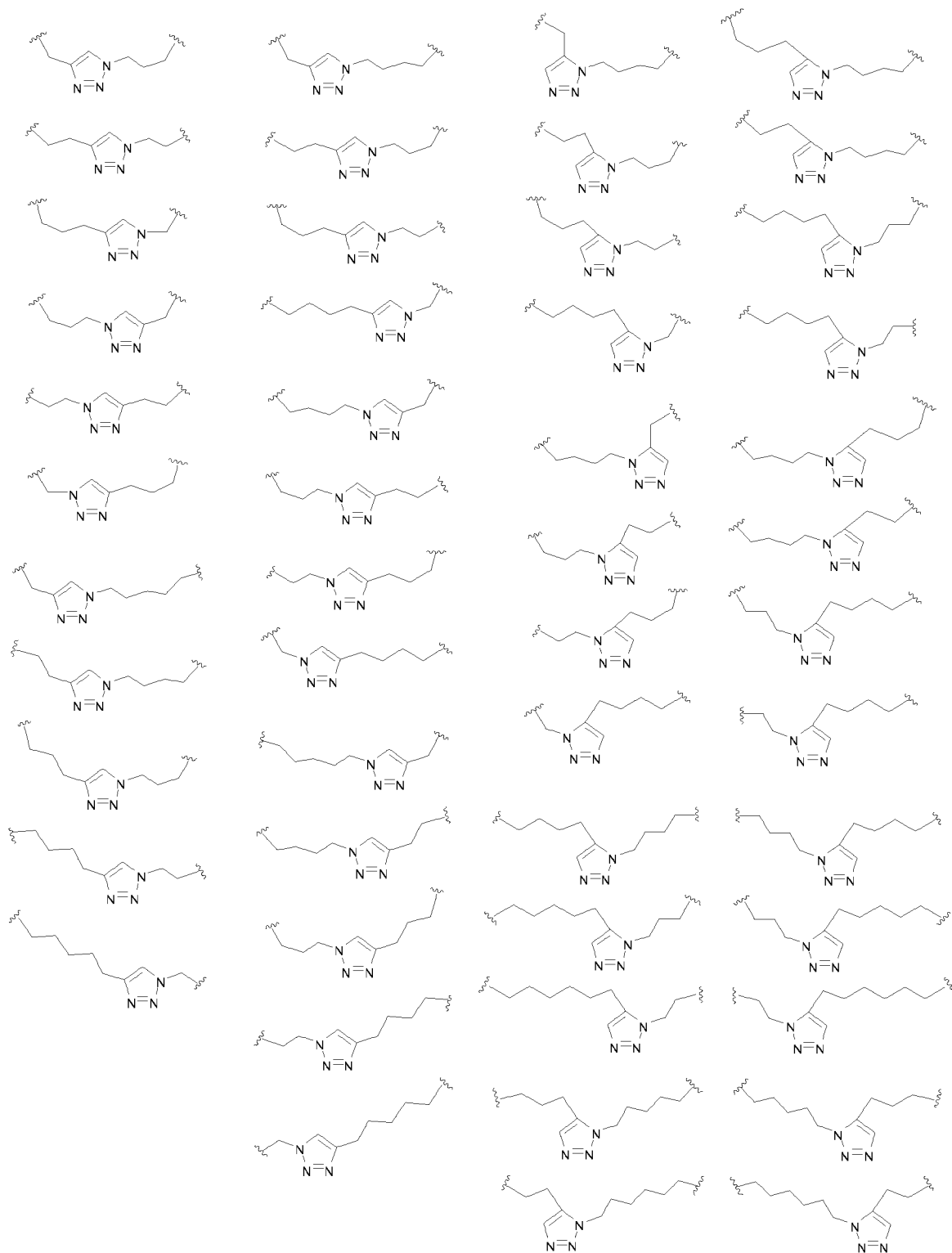


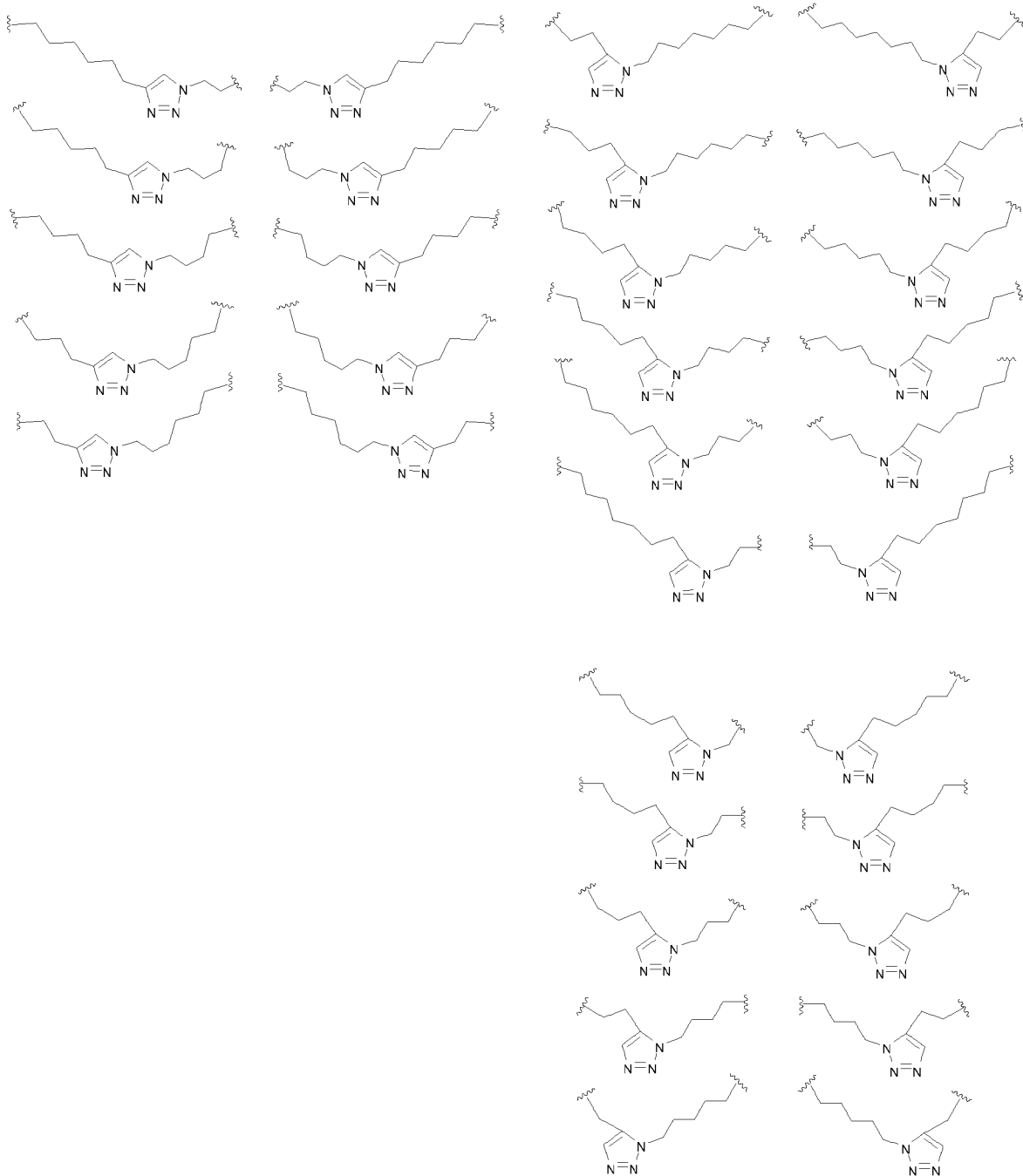


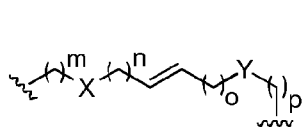


wherein “AA” represents any natural or non-natural amino acid side chain and “ R_1 ” is $[\text{D}]_v$, $[\text{E}]_w$ as defined above, and n is an integer between 0 and 20, 50, 100, 200, 300, 400 or 500. In some embodiments, n is 0. In other embodiments, n is less than 50.

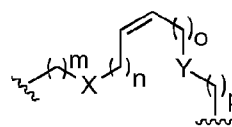
[00110] Exemplary embodiments of the macrocycle-forming linker L for peptidomimetic macrocycles of Formula I are shown below.



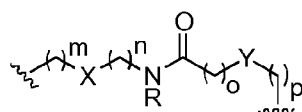




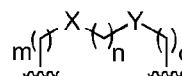
where X, Y = S
m, n, o, p = 0-10



where X, Y = S
m, n, o, p = 0-10



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



where X, Y = S
m, n, o = 0-10

- [00111] In other embodiments, D and/or E in a compound of Formula I or II 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.
- [00112] In other embodiments, at least one of [D] and [E] in a compound of Formula I or II 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. In an embodiment, u is 2.
- [00113] In some embodiments, any of the macrocycle-forming linkers described herein can be used in any combination with any of the sequences shown in Tables 4, 4a, 4b, 6, and 6a and also with any of the R- substituents indicated herein.
- [00114] In some embodiments, the peptidomimetic macrocycle comprises at least one α -helix motif. For example, A, B and/or C in a compound of Formula I or II 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 C α to a second C α 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 Å to 9 Å per turn of the α -helix, or approximately 6 Å to 8 Å 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.

- [00115] Unless otherwise stated, any compounds (including peptidomimetic macrocycles, macrocycle precursors, and other compositions) are also meant to encompass compounds which differ only in the presence of one or more isotopically enriched atoms. For example, compounds having the described structures except for the replacement of a hydrogen by a deuterium or tritium, or the replacement of a carbon by ^{13}C - or ^{14}C -enriched carbon are contemplated herein.
- [00116] In some embodiments, the peptidomimetic macrocycle has improved binding affinity to MDM2 or MDMX relative to a corresponding peptidomimetic macrocycle where w is 0, 1 or 2. In other instances, the peptidomimetic macrocycle has a reduced ratio of binding affinities to MDMX versus MDM2 relative to a corresponding peptidomimetic macrocycle where w is 0, 1 or 2. In still other instances, the peptidomimetic macrocycle has improved *in vitro* anti-tumor efficacy against p53 positive tumor cell lines relative to a corresponding peptidomimetic macrocycle where w is 0, 1 or 2. In some embodiments, the peptidomimetic macrocycle shows improved *in vitro* induction of apoptosis in p53 positive tumor cell lines relative to a corresponding peptidomimetic macrocycle where w is 0, 1 or 2. In other instances, the peptidomimetic macrocycle of claim 1, wherein the peptidomimetic macrocycle has an improved *in vitro* anti-tumor efficacy ratio for p53 positive versus p53 negative or mutant tumor cell lines relative to a corresponding peptidomimetic macrocycle where w is 0, 1 or 2. In still other instances, the peptidomimetic macrocycle has improved *in vivo* anti-tumor efficacy against p53 positive tumors relative to a corresponding peptidomimetic macrocycle where w is 0, 1 or 2. In yet other instances, the peptidomimetic macrocycle has improved *in vivo* induction of apoptosis in p53 positive tumors relative to a corresponding peptidomimetic macrocycle where w is 0, 1 or 2. In some embodiments, the peptidomimetic macrocycle has improved cell permeability relative to a corresponding peptidomimetic macrocycle where w is 0, 1 or 2. In other cases, the peptidomimetic macrocycle has improved solubility relative to a corresponding peptidomimetic macrocycle where w is 0, 1 or 2.
- [00117] In some embodiments, Xaa_5 is Glu or an amino acid analog thereof. In some embodiments, Xaa_5 is Glu or an amino acid analog thereof and wherein the peptidomimetic macrocycle has an improved property, such as improved binding affinity, improved solubility, improved cellular efficacy, improved cell permeability, improved *in vivo* or *in vitro* anti-tumor efficacy, or improved induction of apoptosis relative to a corresponding peptidomimetic macrocycle where Xaa_5 is Ala.
- [00118] In some embodiments, the peptidomimetic macrocycle has improved binding affinity to MDM2 or MDMX relative to a corresponding peptidomimetic macrocycle where Xaa_5 is Ala. In other embodiments, the peptidomimetic macrocycle has a reduced ratio of binding affinities to MDMX vs MDM2 relative to a corresponding peptidomimetic macrocycle where Xaa_5 is Ala. In some

embodiments, the peptidomimetic macrocycle has improved solubility relative to a corresponding peptidomimetic macrocycle where Xaa₅ is Ala, or the peptidomimetic macrocycle has improved cellular efficacy relative to a corresponding peptidomimetic macrocycle where Xaa₅ is Ala.

[00119] In some embodiments, Xaa₅ is Glu or an amino acid analog thereof and wherein the peptidomimetic macrocycle has improved biological activity, such as improved binding affinity, improved solubility, improved cellular efficacy, improved helicity, improved cell permeability, improved *in vivo* or *in vitro* anti-tumor efficacy, or improved induction of apoptosis relative to a corresponding peptidomimetic macrocycle where Xaa₅ is Ala.

[00120] In one embodiment, a compound disclosed herein can contain unnatural proportions of atomic isotopes at one or more of atoms that constitute such compounds. For example, the compounds can be radiolabeled with radioactive isotopes, such as for example tritium (³H), iodine-125 (¹²⁵I) or carbon-14 (¹⁴C). In another embodiment, a compound disclosed herein can have one or more carbon atoms replaced with a silicon atom. All isotopic variations of the compounds disclosed herein, whether radioactive or not, are contemplated herein.

Preparation of Peptidomimetic Macrocycles

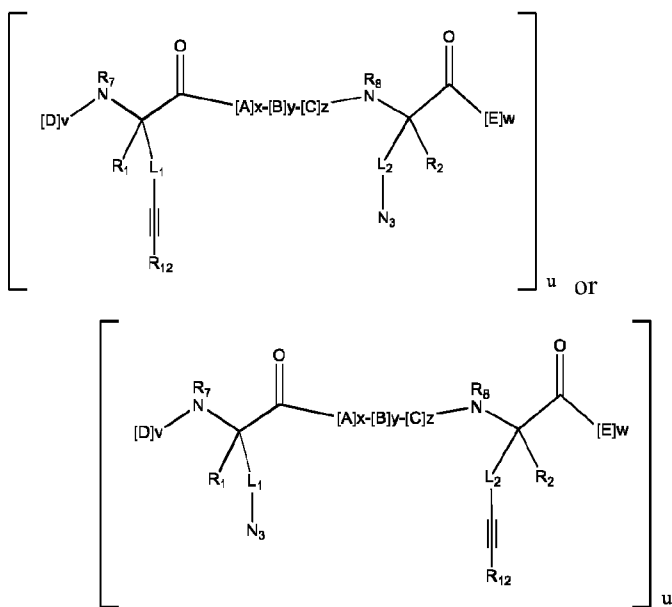
[00121] Peptidomimetic macrocycles of Formulas I and II can be prepared by any of a variety of methods known in the art. For example, macrocycles of Formula I having residues indicated by “\$4rn6” or “\$4a5” in Table 4, Table 4a or Table 4b can be substituted with a residue capable of forming a crosslinker with a second residue in the same molecule or a precursor of such a residue.

[00122] 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. Such a process is described, for example, in US Application 12/037,041, filed on February 25, 2008. 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.

[00123] In some embodiments of macrocycles of Formula I, 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.

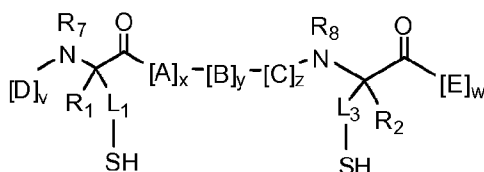
[00124] In some embodiments, provided herein is a method for synthesizing a peptidomimetic macrocycle of Formula I, the method comprising the steps of contacting a peptidomimetic precursor of formulas:



with a macrocyclization reagent;

wherein v, w, x, y, z, A, B, C, D, E, R₁, R₂, R₇, R₈, L₁ and L₂ are as defined above; R₁₂ is -H when the macrocyclization reagent is a Cu reagent and R₁₂ 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 the precursor. For example, R₁₂ may be methyl when the macrocyclization reagent is a Ru reagent.

[00125] In some embodiments, provided herein is a method for synthesizing a peptidomimetic macrocycle of Formula II, the method comprising the steps of contacting a peptidomimetic precursor of formula:



with a compound formula X-L₂-Y,

wherein v, w, x, y, z, A, B, C, D, E, R₁, R₂, R₇, R₈, L₁ and L₂ are as defined for the compound of formula II; and X and Y are each independently a reactive group capable of reacting with a thiol group;

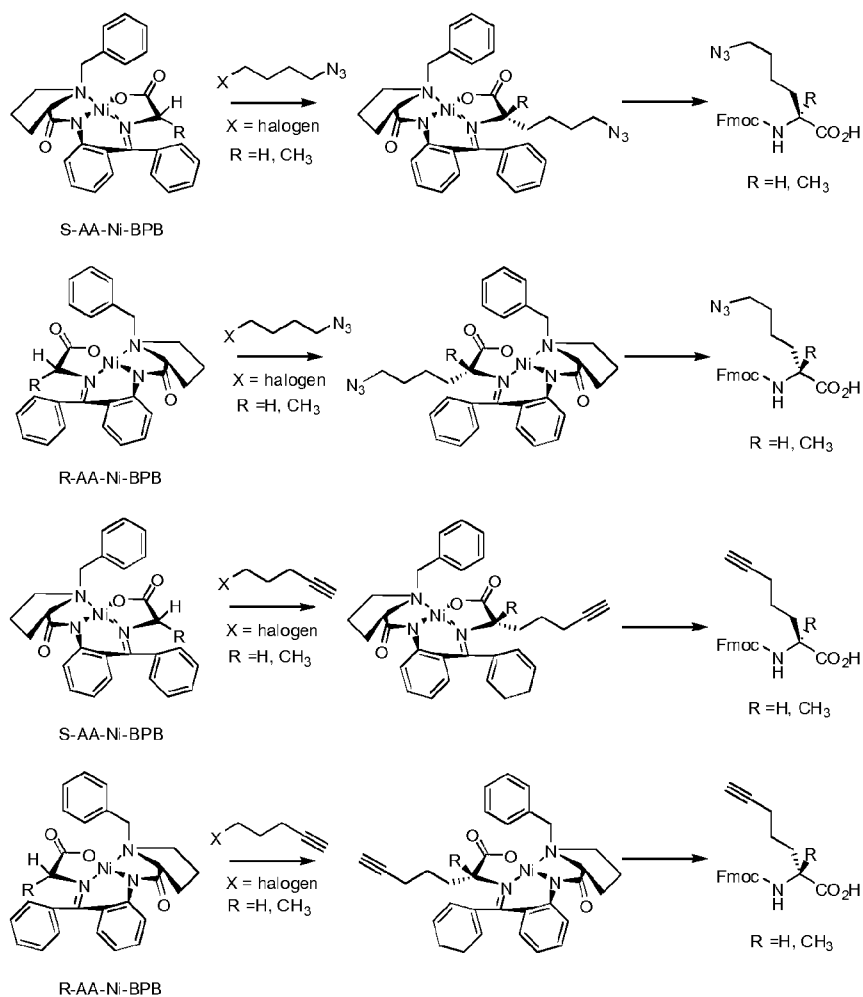
and further wherein said contacting step results in a covalent linkage being formed between the two thiol groups in Formula III.

- [00126] In the peptidomimetic macrocycles disclosed herein, at least one of R_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.
- [00127] For example, at least one of R_1 and R_2 is alkyl, unsubstituted or substituted with halo-. In another example, both R_1 and R_2 are independently alkyl, unsubstituted or substituted with halo-. In some embodiments, at least one of R_1 and R_2 is methyl. In other embodiments, R_1 and R_2 are methyl. The macrocyclization reagent may be a Cu reagent or a Ru reagent.
- [00128] 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.
- [00129] Also envisioned herein is performing the method disclosed herein 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.
- [00130] In some embodiments, an alkyne moiety of the peptidomimetic precursor for making a compound of Formula I 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, an azide moiety of the peptidomimetic precursor for making a compound of Formula I 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.
- [00131] In some embodiments, a thiol group of the peptidomimetic precursor for making a compound of Formula II is a sidechain of an amino acid selected from the group consisting of L-cysteine, D-

- cysteine, L-N-methylcysteine, D-N-methylcysteine, L-homocysteine, D-homocysteine, L-N-methylhomocysteine, D-N-methylhomocysteine, α -methyl-L-cysteine, α -methyl-D-cysteine, α -methyl-L-homocysteine, α -methyl-D-homocysteine, L-penicillamine, D-penicillamine, L-N-methylpenicillamine, D-N-methylpenicillamine and all forms suitably protected for liquid or solid phase peptide synthesis.
- [00132] In some embodiments, $x+y+z$ is 3, and A, B and C are independently natural or non-natural amino acids. In other embodiments, $x+y+z$ is 6, and A, B and C are independently natural or non-natural amino acids.
- [00133] 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 of H_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.
- [00134] 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.
- [00135] The peptidomimetic macrocycles disclosed herein are made, for example, by chemical synthesis methods, such as described in Fields *et al.*, Chapter 3 in Synthetic Peptides: A User's Guide, ed. Grant, W. H. Freeman & Co., New York, N. Y., 1992, p. 77. Hence, 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).
- [00136] 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.

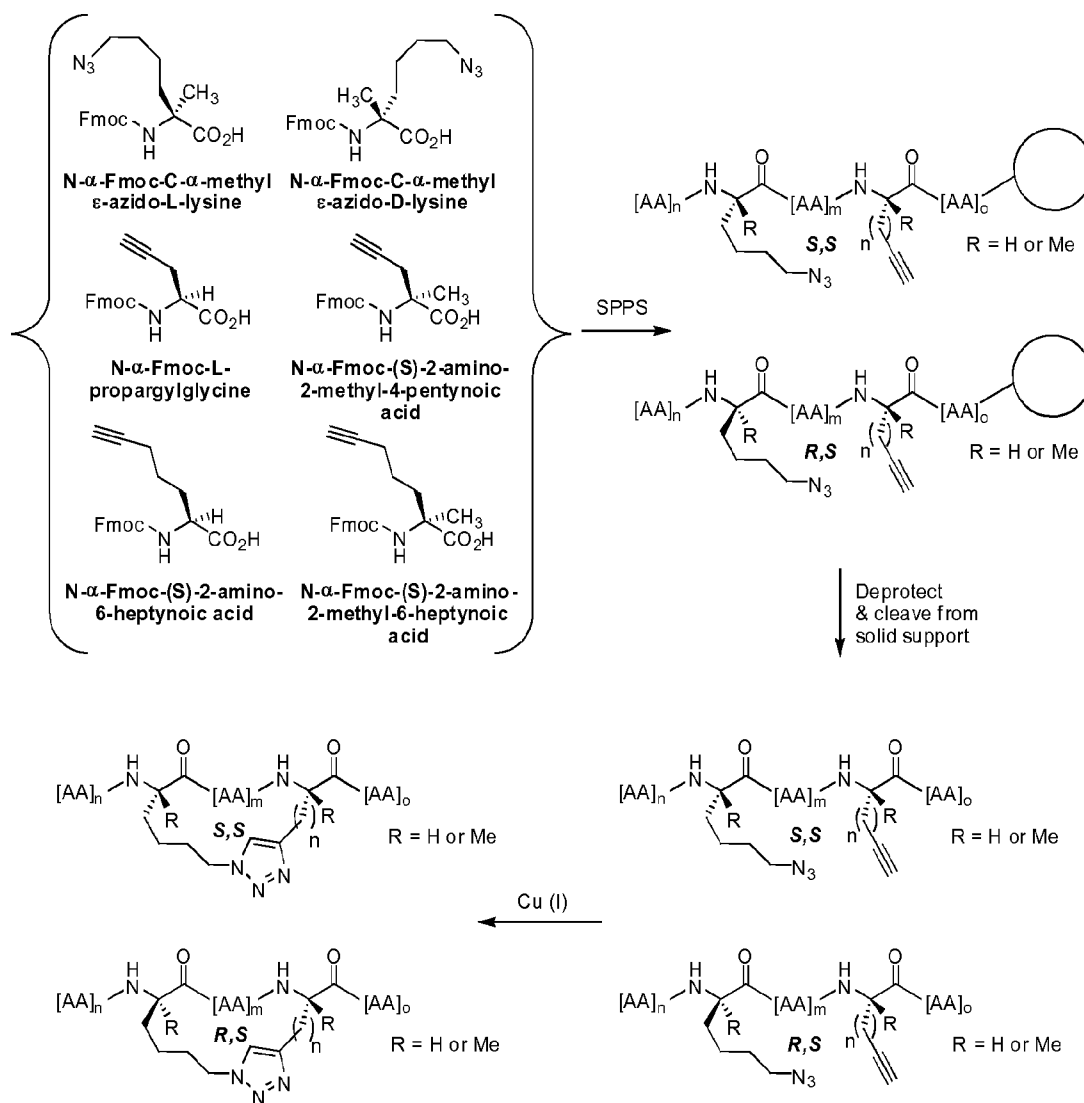
- [00137] Longer peptidomimetic precursors are produced, for example, by conjoining individual synthetic peptides using native chemical ligation. Alternatively, the longer synthetic peptides are biosynthesized by well known recombinant DNA and protein expression techniques. Such techniques are provided in well-known standard manuals with detailed protocols. To construct a gene encoding a peptidomimetic precursor disclosed herein, 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.
- [00138] 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 AAPTEC, Inc., Louisville, KY).
- [00139] 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.
- [00140] Synthetic schemes 1-5 describe the preparation of peptidomimetic macrocycles of Formula I. 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.

[00141] Synthetic Scheme 1:



[00142] Synthetic Scheme 1 describes the preparation of several compounds useful for preparing compounds of Formula I as disclosed herein. Ni(II) complexes of Schiff bases derived from the chiral auxiliary (S)-2-[N-(N'-benzylpropyl)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 disclosed herein. If desired, the resulting compounds can be protected for use in peptide synthesis.

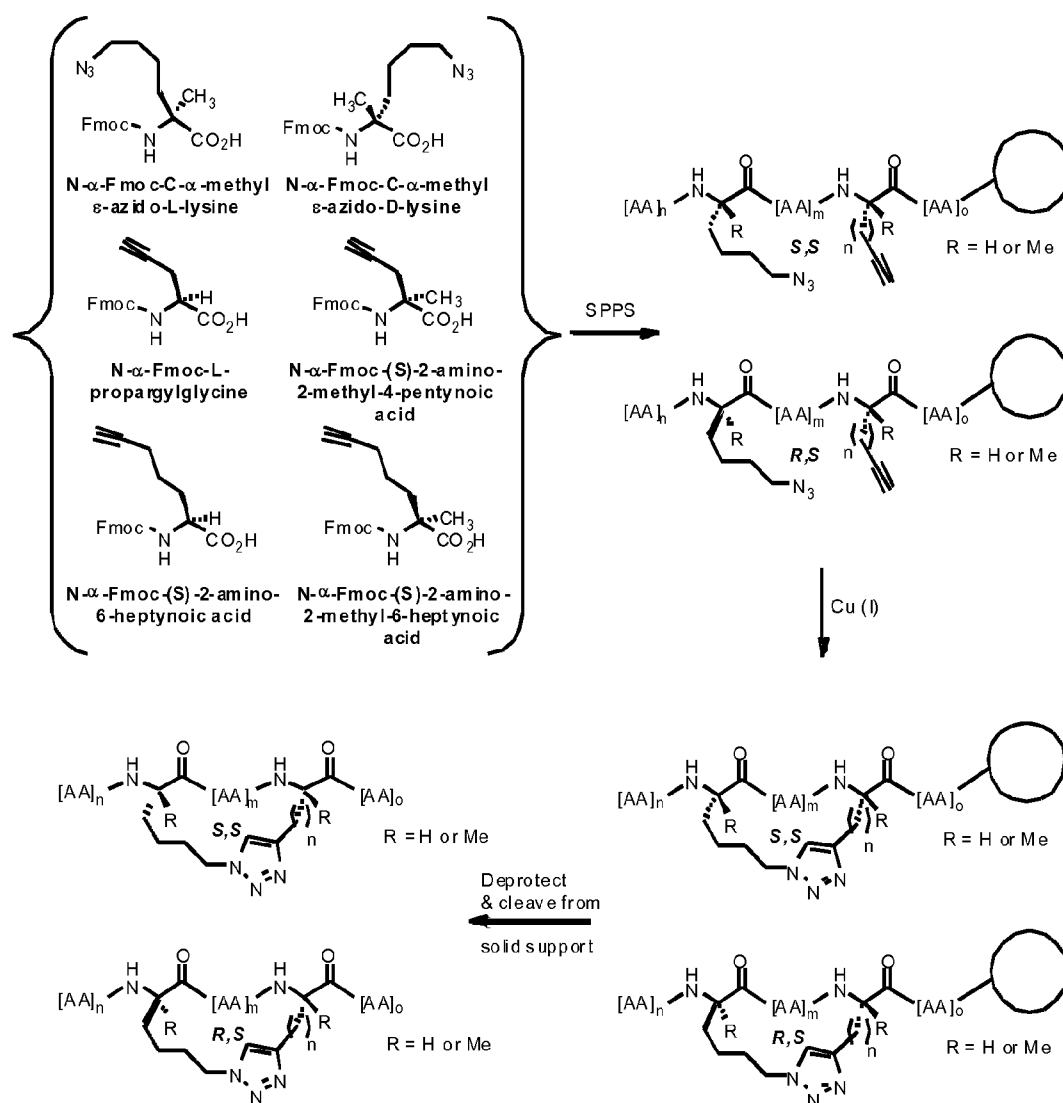
[00143] Synthetic Scheme 2:



[00144] In the general method for the synthesis of peptidomimetic macrocycles of Formula I 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 *et al.* (2002), *Angew. Chem. Int. Ed.* 41:2596-2599; Tornøe *et al.* (2002), *J. Org. Chem.* 67:3057-3064; Deiters *et al.* (2003), *J. Am. Chem. Soc.* 125:11782-11783; Punna *et al.* (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 H₂O, THF, CH₃CN, 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.

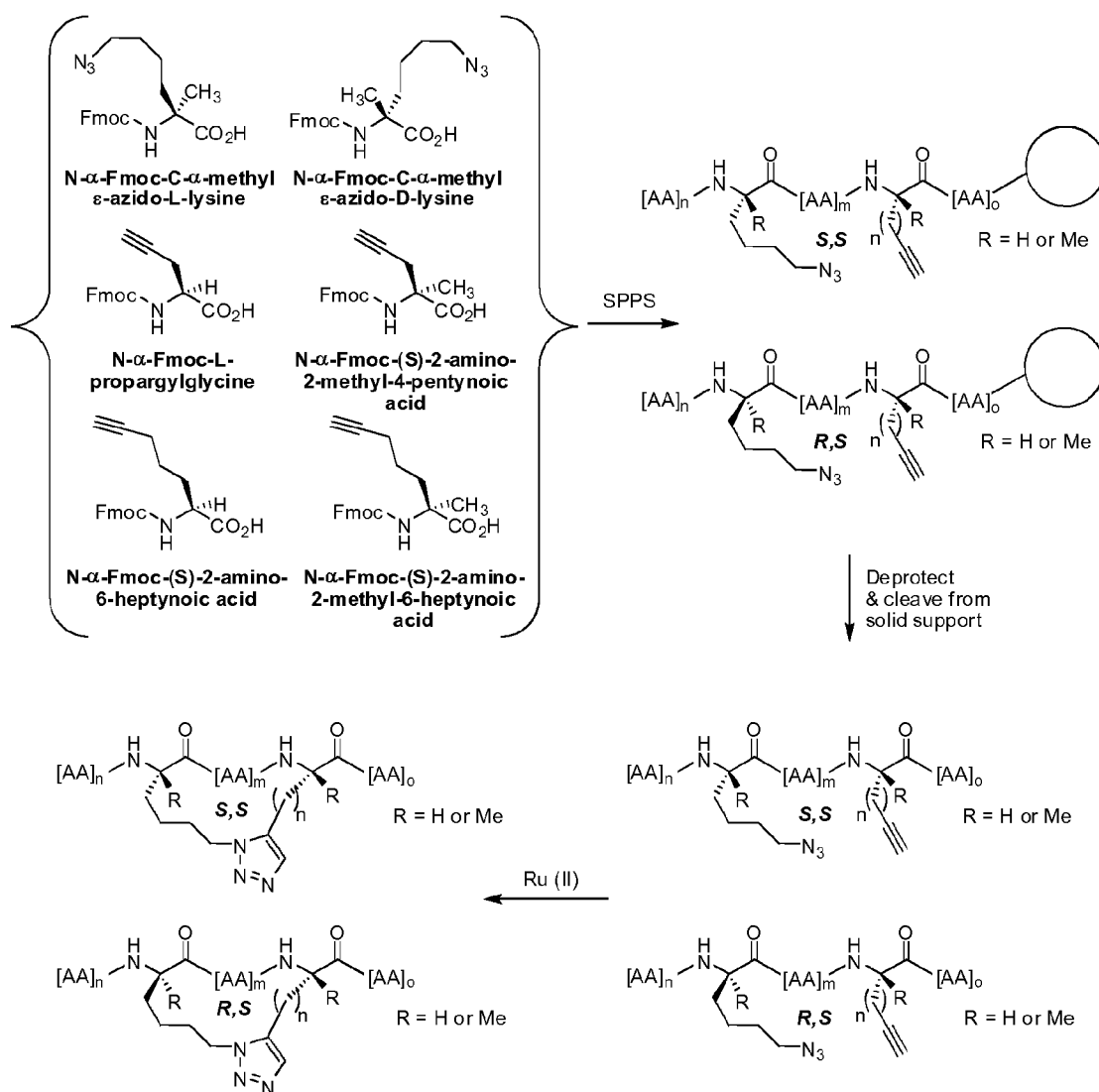
[00145] Synthetic Scheme 3:



[00146] In the general method for the synthesis of peptidomimetic macrocycles of Formula I 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; Tornøe *et al.* (2002), *J. Org. Chem.* 67:3057-3064; Deiters *et al.* (2003), *J. Am. Chem. Soc.* 125:11782-11783; Punna *et 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_2Cl_2 , $\text{ClCH}_2\text{CH}_2\text{Cl}$, 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.

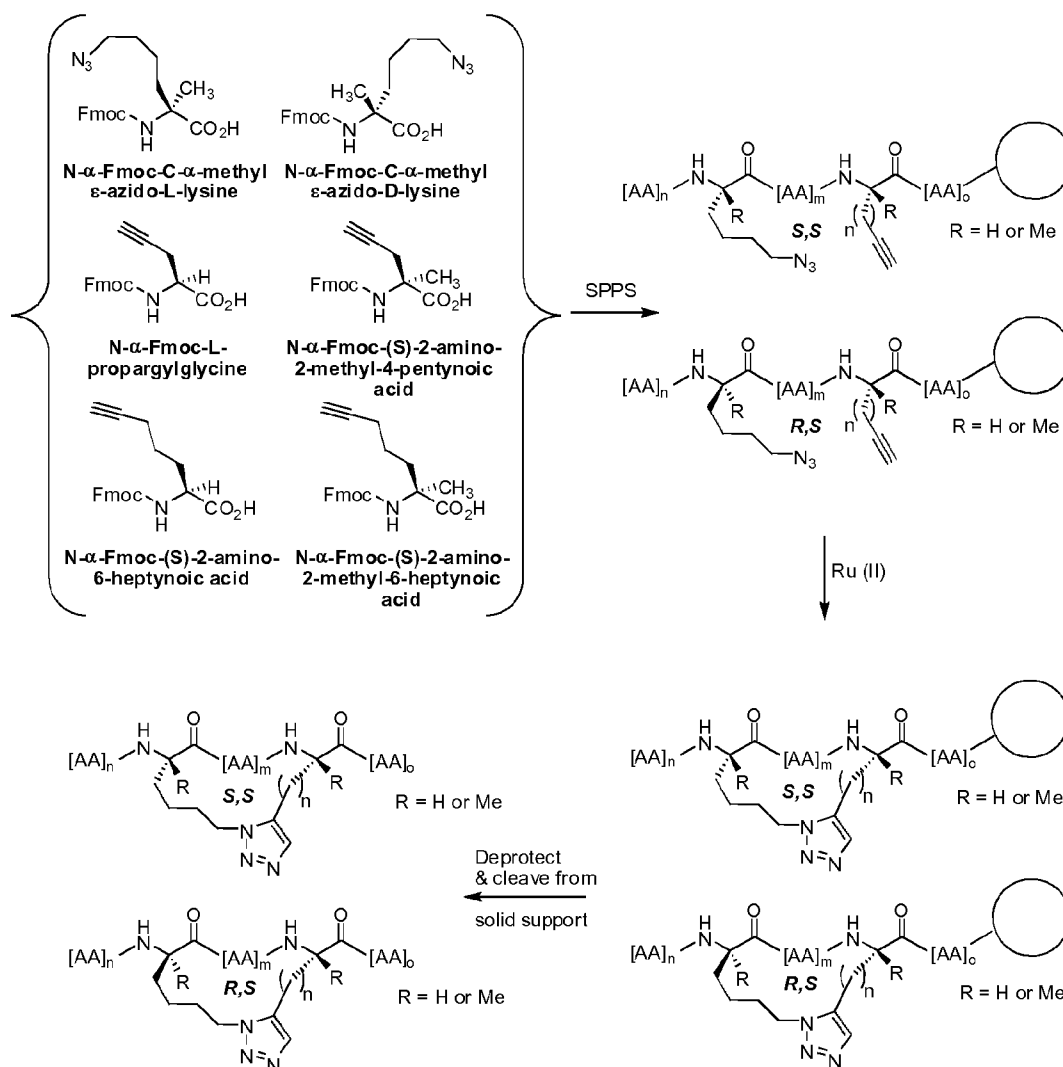
[00147] Synthetic Scheme 4:



[00148] In the general method for the synthesis of peptidomimetic macrocycles of Formula I 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 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 Ru(II) reagents, for example Cp^{*}RuCl(PPh₃)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 DMF, CH₃CN and THF.

[00149] Synthetic Scheme 5:

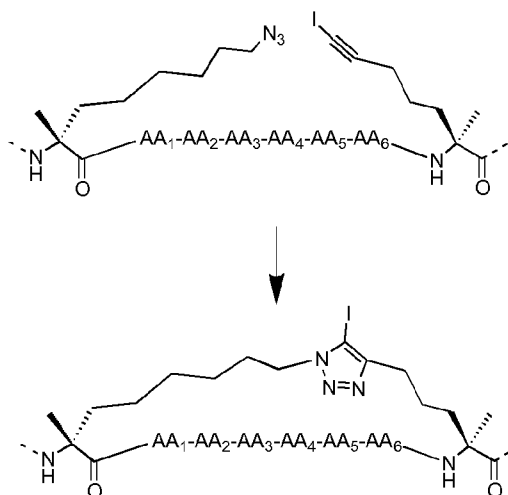


[00150] In the general method for the synthesis of peptidomimetic macrocycles of Formula I shown in Synthetic Scheme 5, the peptidomimetic precursor contains an azide moiety and an alkyne moiety and is synthesized by solid-phase peptide synthesis (SPPS) using the commercially available

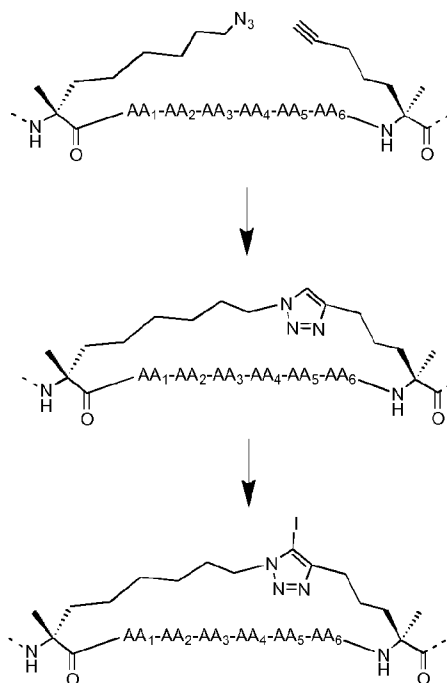
amino acid N- α -Fmoc-L-propargylglycine and the N- α -Fmoc-protected forms of the amino acids (S)-2-amino-2-methyl-4-pentynoic acid, (S)-2-amino-6-heptynoic acid, (S)-2-amino-2-methyl-6-heptynoic acid, N-methyl- ϵ -azido-L-lysine, and N-methyl- ϵ -azido-D-lysine. The peptidomimetic precursor is reacted with a macrocyclization reagent such as a Ru(II) reagent on the resin as a crude mixture. For example, the reagent can be Cp^{*}RuCl(PPh₃)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 CH₂Cl₂, ClCH₂CH₂Cl, CH₃CN, DMF, and THF.

[00151] In some embodiments, a peptidomimetic macrocycle of Formula I comprises a halogen group substitution on a triazole moiety, for example an iodo substitution. Such peptidomimetic macrocycles may be prepared from a precursor having the partial structure and using the cross-linking methods taught herein. Crosslinkers of any length, as described herein, may be prepared comprising such substitutions. In one embodiment, the peptidomimetic macrocycle is prepared according to the scheme shown below. The reaction is performed, for example, in the presence of CuI and an amine ligand such as TEA or TTTA. See, e.g., Hein *et al.* *Angew. Chem., Int. Ed.* 2009, 48, 8018–8021.

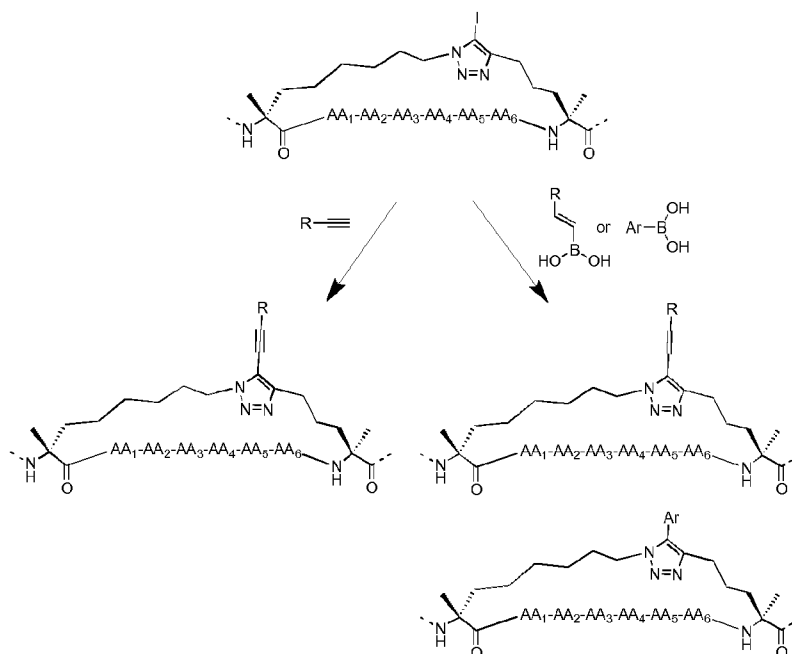
[00152]



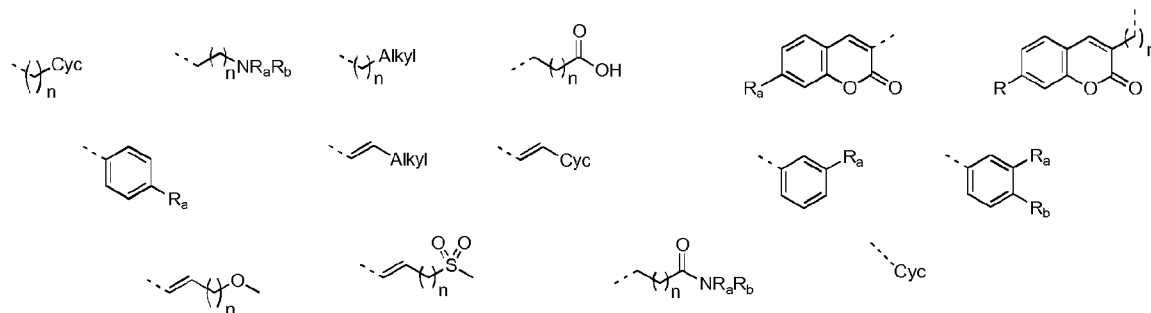
[00153] In other embodiments, an iodo-substituted triazole is generated according to the scheme shown below. For example, the second step in the reaction scheme below is performed using, for example, CuI and N-bromosuccinimide (NBS) in the presence of THF (see, e.g. Zhang *et al.*, *J. Org. Chem.* 2008, 73, 3630–3633). In other embodiments, the second step in the reaction scheme shown below is performed, for example, using CuI and an iodinating agent such as ICl (see, e.g. Wu *et al.*, *Synthesis* 2005, 1314–1318.)



[00154] In some embodiments, an iodo-substituted triazole moiety is used in a cross-coupling reaction, such as a Suzuki or Sonogashira coupling, to afford a peptidomimetic macrocycle comprising a substituted crosslinker. Sonogashira couplings using an alkyne as shown below may be performed, for example, in the presence of a palladium catalyst such as $\text{Pd}(\text{PPh}_3)_2\text{Cl}_2$, CuI , and in the presence of a base such as triethylamine. Suzuki couplings using an arylboronic or substituted alkenyl boronic acid as shown below may be performed, for example, in the presence of a catalyst such as $\text{Pd}(\text{PPh}_3)_4$, and in the presence of a base such as K_2CO_3 .

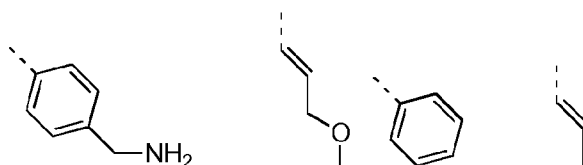


[00155] Any suitable triazole substituent groups which reacts with the iodo-substituted triazole can be used in Suzuki couplings described herein. Example triazole substituents for use in Suzuki couplings are shown below:

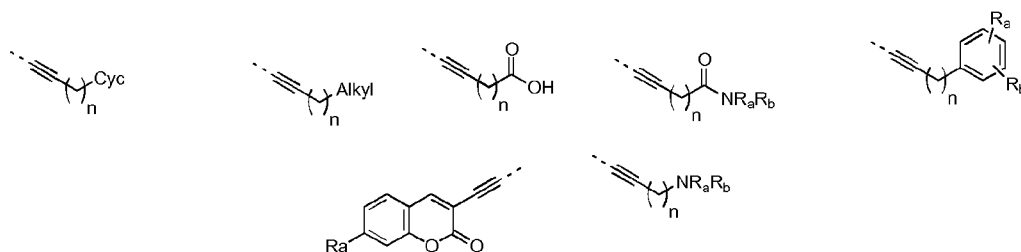


wherein "Cyc" is a suitable aryl, cycloalkyl, cycloalkenyl, heteroaryl, or heterocyclyl group, unsubstituted or optionally substituted with an R_a or R_b group as described below.

[00156] In some embodiments, the substituent is:

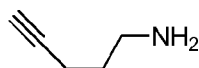


[00157] Any suitable substituent group which reacts with the iodo-substituted triazole can be used in Sonogashira couplings described herein. Example triazole substituents for use in Sonogashira couplings are shown below:

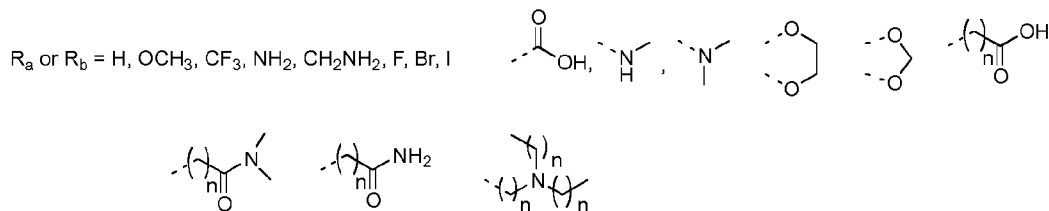


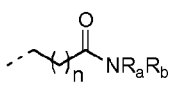
wherein "Cyc" is a suitable aryl, cycloalkyl, cycloalkenyl, heteroaryl, or heterocyclyl group, unsubstituted or optionally substituted with an R_a or R_b group as described below.

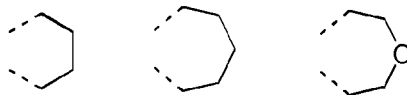
[00158] In some embodiments, the triazole substituent is:



[00159] In some embodiments, the Cyc group shown above is further substituted by at least one R_a or R_b substituent. In some embodiments, at least one of R_a and R_b is independently:



[00160] In other embodiments, the triazole substituent is  and at least one of R_a and R_b is alkyl (including hydrogen, methyl, or ethyl), or:



[00161] The present invention contemplates the use of non-naturally-occurring amino acids and amino acid analogs in the synthesis of the peptidomimetic macrocycles of Formula I 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. The following Table 1 shows some amino acids useful in the preparation of peptidomimetic macrocycles disclosed herein.

TABLE 1

N-α-Fmoc-L-propargyl glycine	N-α-Fmoc-D-propargyl glycine		
N-α-Fmoc-(S)-2-amino-2-methyl-4-pentynoic acid	N-α-Fmoc-(R)-2-amino-2-methyl-4-pentynoic acid		
N-α-Fmoc-(S)-2-amino-2-methyl-5-hexynoic acid	N-α-Fmoc-(R)-2-amino-2-methyl-5-hexynoic acid	N-α-Fmoc-ε-azido-L-lysine	N-α-Fmoc-ε-azido-D-lysine
N-α-Fmoc-(S)-2-amino-2-methyl-6-heptynoic acid	N-α-Fmoc-(R)-2-amino-2-methyl-6-heptynoic acid	N-α-Fmoc-ε-azido-α-methyl-L-lysine	N-α-Fmoc-ε-azido-α-methyl-D-lysine
N-α-Fmoc-(S)-2-amino-2-methyl-7-octynoic acid	N-α-Fmoc-(R)-2-amino-2-methyl-7-octynoic acid	N-α-Fmoc-δ-azido-L-ornithine	N-α-Fmoc-δ-azido-D-ornithine
N-α-Fmoc-(S)-2-amino-2-methyl-8-nonynoic acid	N-α-Fmoc-(R)-2-amino-2-methyl-8-nonynoic acid	N-α-Fmoc-ε-azido-α-methyl-L-ornithine	N-α-Fmoc-ε-azido-α-methyl-D-ornithine

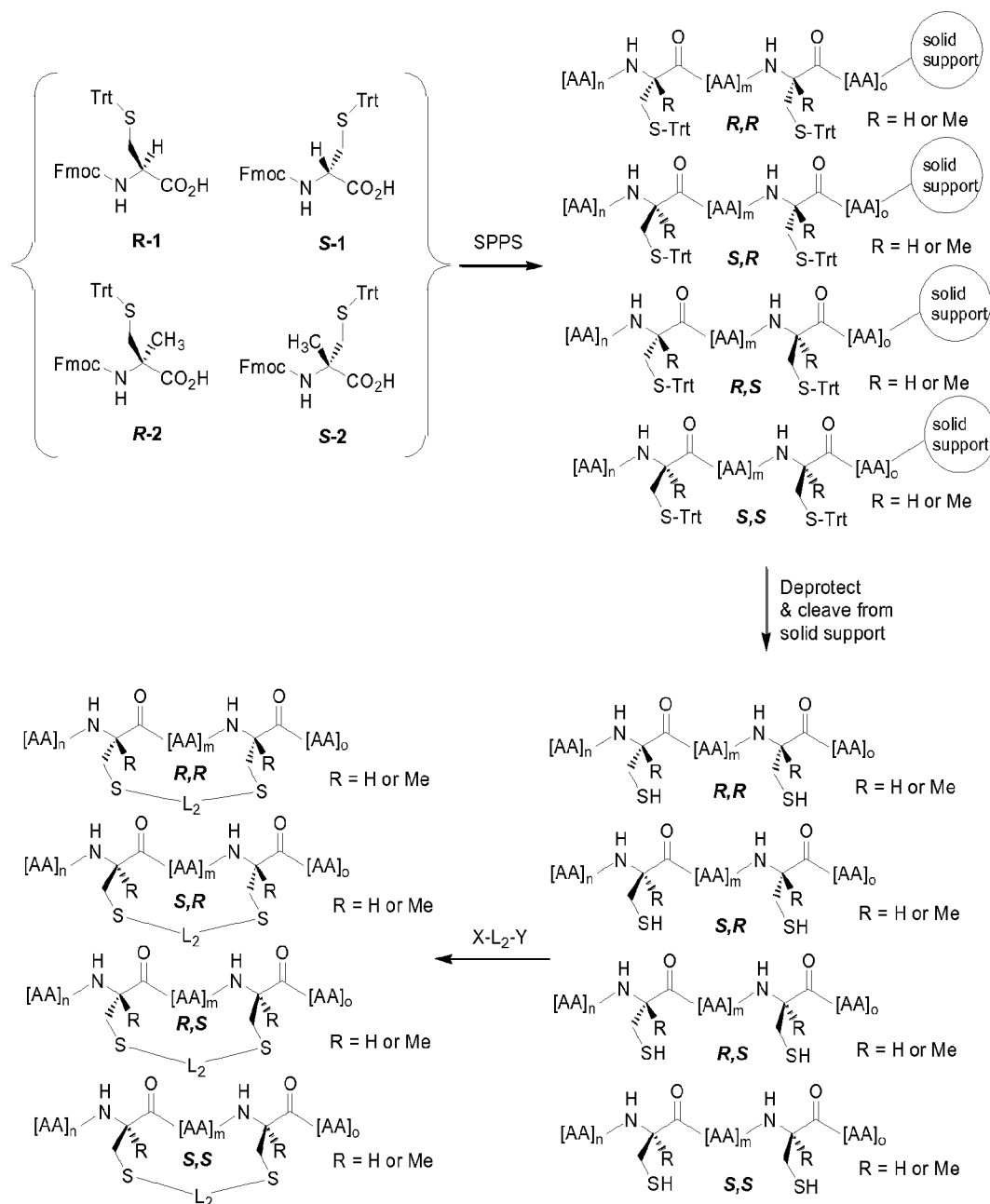
Table 1 shows exemplary amino acids useful in the preparation of peptidomimetic macrocycles disclosed herein.

[00162] 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-propargylglycine, α -methyl-D-propargylglycine, ϵ -azido- α -methyl-L-lysine, and ϵ -azido- α -methyl-D-lysine. In some embodiments the amino acid analogs are N-alkylated, *e.g.*, N-methyl-L-

propargylglycine, N-methyl-D-propargylglycine, N-methyl-ε-azido-L-lysine, and N-methyl-ε-azido-D-lysine.

[00163] The preparation of macrocycles of Formula II is described, for example, in US Application 11/957,325, filed on December 17, 2007 and herein incorporated by reference. Synthetic Schemes 6-9 describe the preparation of such compounds of Formula II. 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]_o” 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.

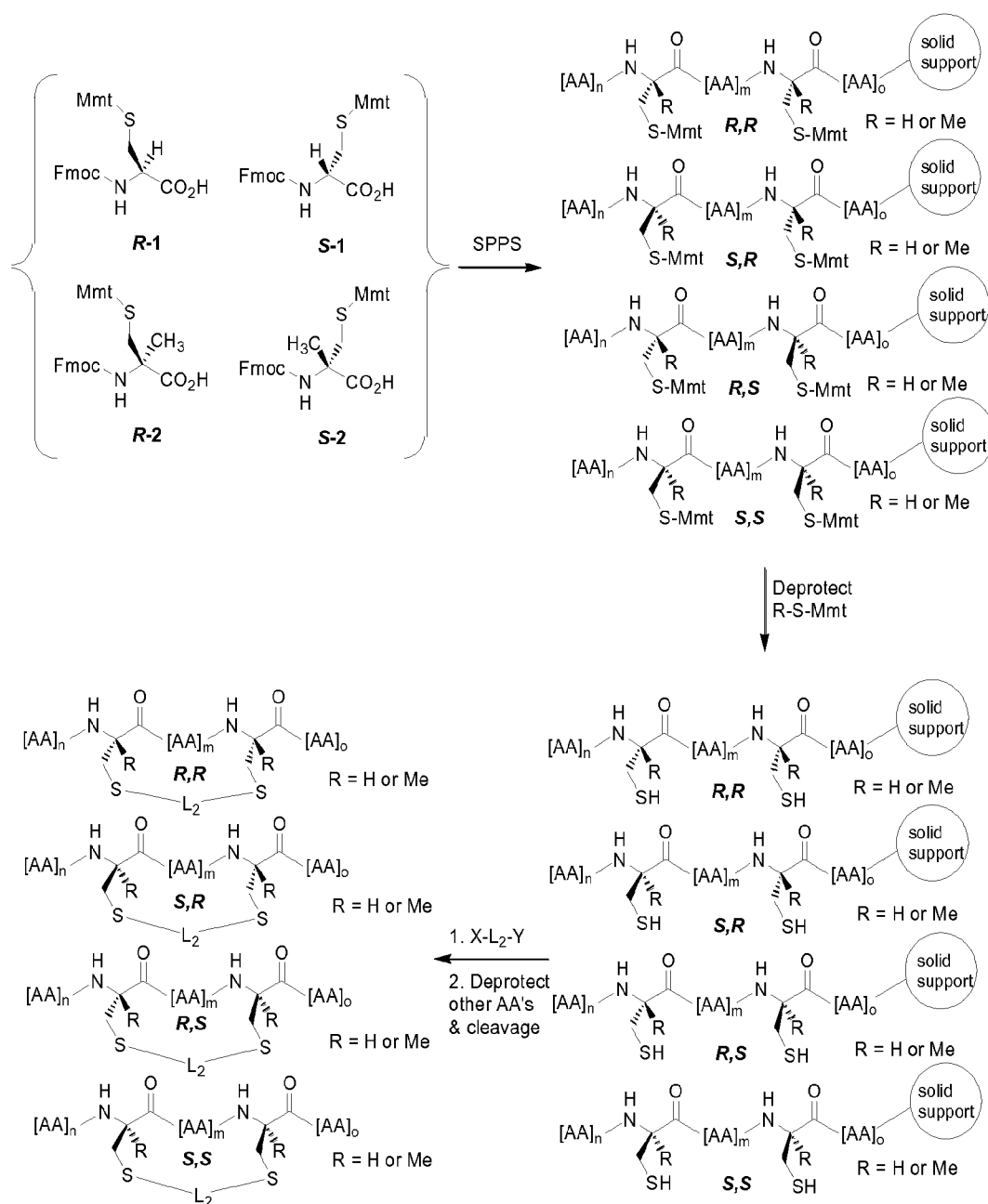
Synthetic Scheme 6:



[00164] 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

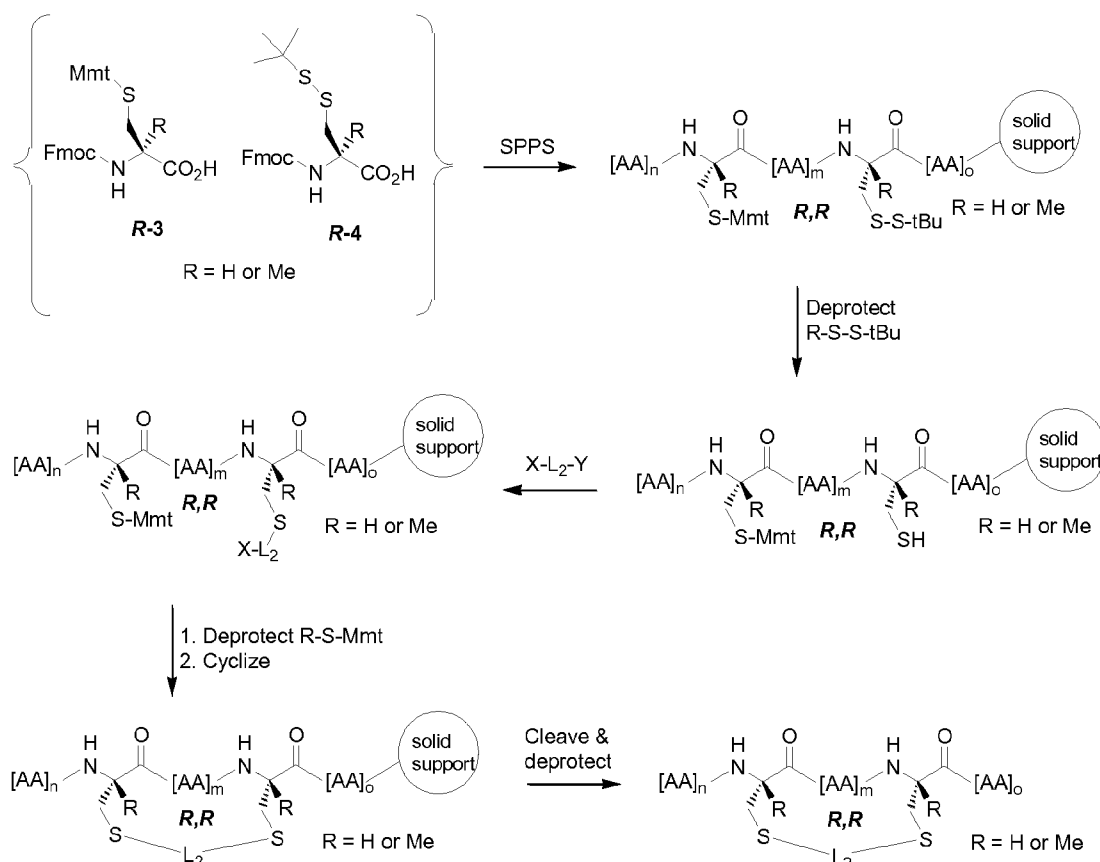
precursor peptidomimetic is reacted as a crude mixture or is purified prior to reaction with X-L₂-Y in organic or aqueous solutions. In some embodiments the alkylation reaction is performed under dilute conditions (i.e. 0.15 mmol/L) to favor macrocyclization and to avoid polymerization. In some embodiments, the alkylation reaction is performed in organic solutions such as liquid NH₃ (Mosberg et al. (1985), J. Am.Chem. Soc. 107:2986-2987; Szewczuk et al. (1992), Int. J. Peptide Protein Res. 40 :233-242), NH₃/MeOH, or NH₃/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 (Brunel 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:



[00165] 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-*p*-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-L₂-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 NH₃ (Mosberg *et al.* (1985), *J. Am. Chem. Soc.* 107:2986-2987; Szewczuk *et al.* (1992), *Int. J. Peptide Protein Res.* 40 :233-242), NH₃/MeOH or NH₃/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).

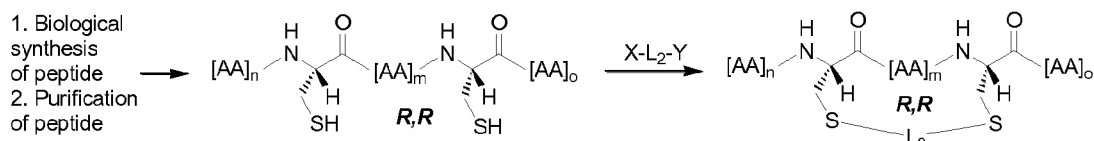
Synthetic Scheme 8:



[00166] 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- p -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- t -Butyl 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 of $X-L_2-Y$ 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_3/MeOH or NH_3/DMF (Or *et al.* (1991), *J. Org. Chem.* 56:3146-3149). 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:



[00167] 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_3/MeOH , 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 (Brunel *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.

[00168] 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 2. For example, X and Y are halides such as $-\text{Cl}$, $-\text{Br}$ or $-\text{I}$. Any of the macrocycle-forming linkers described herein may be used in any combination with any of the sequences shown and also with any of the R- substituents indicated herein.

TABLE 2: Examples of Reactive Groups Capable of Reacting with Thiol Groups and Resulting Linkages

X or Y	Resulting Covalent Linkage
acrylamide	Thioether
halide (<i>e.g.</i> alkyl or aryl halide)	Thioether
sulfonate	Thioether

TABLE 2: Examples of Reactive Groups Capable of Reacting with Thiol Groups and Resulting Linkages

X or Y	Resulting Covalent Linkage
aziridine	Thioether
epoxide	Thioether
haloacetamide	Thioether
maleimide	Thioether
sulfonate ester	Thioether

[00169] The present invention contemplates the use of both naturally-occurring and non-naturally-occurring amino acids and amino acid analogs in the synthesis of the peptidomimetic macrocycles of Formula II. Any amino acid or amino acid analog amenable to the synthetic methods employed for the synthesis of stable bis-sulfhydryl containing peptidomimetic macrocycles can be used in the present invention. For example, cysteine is contemplated as a useful amino acid in the present invention. However, sulfur containing amino acids other than cysteine that contain a different amino 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.

[00170] 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 disclosed herein. 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 macrocycles. 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.

- [00171] 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 of L_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 of L_1 and L_3 .
- [00172] 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.
- [00173] Table 3 shows additional embodiments of X- L_2 -Y groups.

TABLE 3. Exemplary X-L₂-Y groups.

BEB	Bph	Bpy

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

[00174] Additional methods of forming peptidomimetic macrocycles which are envisioned as suitable include those disclosed by Mustapa, M. Firouz Mohd et al., J. Org. Chem (2003), 68, pp. 8193-8198; Yang, Bin 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, amino acid precursors are used containing an additional substituent R- at the alpha position. Such amino acids are incorporated into the macrocycle precursor at the desired positions, which can be at the positions where the crosslinker is substituted or, alternatively, elsewhere in the sequence of the macrocycle precursor.

Cyclization of the precursor is then effected according to the indicated method.

- [00175] 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.

Assays

- [00176] The properties of peptidomimetic macrocycles are assayed, for example, by using the methods described below. In some embodiments, a peptidomimetic macrocycle has improved biological properties relative to a corresponding polypeptide lacking the substituents described herein.

Assay to Determine α -helicity

- [00177] In solution, the secondary structure of polypeptides with α -helical domains will reach a dynamic equilibrium between random coil structures and α -helical structures, often expressed as a “percent helicity”. Thus, for example, alpha-helical domains are predominantly random coils in solution, with α -helical content usually under 25%. Peptidomimetic macrocycles with optimized linkers, on the other hand, possess, for example, an alpha-helicity that is at least two-fold greater than that of a corresponding uncrosslinked polypeptide. In some embodiments, macrocycles will possess an alpha-helicity of greater than 50%. To assay the helicity of peptidomimetic macrocycles, the compounds are dissolved in an aqueous solution (e.g. 50 mM potassium phosphate solution at pH 7, or distilled H₂O, to concentrations of 25-50 μ M). Circular dichroism (CD) spectra are obtained on a spectropolarimeter (e.g., Jasco J-710) using standard measurement parameters (e.g. temperature, 20°C; wavelength, 190-260 nm; step resolution, 0.5 nm; speed, 20 nm/sec; accumulations, 10; response, 1 sec; bandwidth, 1 nm; path length, 0.1 cm). The α -helical content of each peptide is calculated by dividing the mean residue ellipticity (e.g. $[\Phi]_{222\text{obs}}$) by the reported value for a model helical decapeptide (Yang *et al.* (1986), *Methods Enzymol.* 130:208)).

Assay to Determine Melting Temperature (T_m)

- [00178] A peptidomimetic macrocycle comprising a secondary structure such as an α -helix exhibits, for example, a higher melting temperature than a corresponding uncrosslinked polypeptide. Typically peptidomimetic macrocycles exhibit T_m of > 60°C representing a highly stable structure in aqueous solutions. To assay the effect of macrocycle formation on melting temperature, peptidomimetic macrocycles or unmodified peptides are dissolved in distilled H₂O (e.g. at a final concentration of 50 μ M) and the T_m is determined by measuring the change in ellipticity over a

temperature range (e.g. 4 to 95 °C) on a spectropolarimeter (e.g., Jasco J-710) using standard parameters (e.g. wavelength 222nm; step resolution, 0.5 nm; speed, 20 nm/sec; accumulations, 10; response, 1 sec; bandwidth, 1 nm; temperature increase rate: 1°C/min; path length, 0.1 cm).

Protease Resistance Assay.

- [00179] The amide bond of the peptide backbone is susceptible to hydrolysis by proteases, thereby rendering peptidic compounds vulnerable to rapid degradation *in vivo*. Peptide helix formation, however, typically buries the amide backbone and therefore can shield it from proteolytic cleavage. The peptidomimetic macrocycles can be subjected to *in vitro* trypsin proteolysis to assess for any change in degradation rate compared to a corresponding uncrosslinked polypeptide. For example, the peptidomimetic macrocycle and a corresponding uncrosslinked polypeptide are incubated with trypsin agarose and the reactions quenched at various time points by centrifugation and subsequent HPLC injection to quantitate the residual substrate by ultraviolet absorption at 280 nm. Briefly, the peptidomimetic macrocycle and peptidomimetic precursor (5 mcg) are incubated with trypsin agarose (Pierce) (S/E ~125) for 0, 10, 20, 90, and 180 minutes. Reactions are quenched by tabletop centrifugation at high speed; remaining substrate in the isolated supernatant is quantified by HPLC-based peak detection at 280 nm. The proteolytic reaction displays first order kinetics and the rate constant, k , is determined from a plot of $\ln[S]$ versus time ($k = -1 \times \text{slope}$).

Ex Vivo Stability Assay.

- [00180] Peptidomimetic macrocycles with optimized linkers possess, for example, an *ex vivo* half-life that is at least two-fold greater than that of a corresponding uncrosslinked polypeptide, and possess an *ex vivo* half-life of 12 hours or more. For *ex vivo* serum stability studies, a variety of assays can be used. For example, a peptidomimetic macrocycle and a corresponding uncrosslinked polypeptide (2 mcg) are incubated with fresh mouse, rat and/or human serum (2 mL) at 37°C for 0, 1, 2, 4, 8, and 24 hours. To determine the level of intact compound, the following procedure can be used: The samples are extracted by transferring 100 µl of sera to 2 ml centrifuge tubes followed by the addition of 10 µL of 50 % formic acid and 500µL acetonitrile and centrifugation at 14,000 RPM for 10 min at $4 \pm 2^\circ\text{C}$. The supernatants are then transferred to fresh 2 ml tubes and evaporated on Turbovap under $\text{N}_2 < 10$ psi, 37°C. The samples are reconstituted in 100µL of 50:50 acetonitrile:water and submitted to LC-MS/MS analysis.

In vitro Binding Assays.

- [00181] To assess the binding and affinity of peptidomimetic macrocycles and peptidomimetic precursors to acceptor proteins, a fluorescence polarization assay (FPA) is used, for example. The FPA technique measures the molecular orientation and mobility using polarized light and fluorescent tracer. When excited with polarized light, fluorescent tracers (e.g., FITC) attached to molecules with high apparent molecular weights (e.g. FITC-labeled peptides bound to a large protein) emit

higher levels of polarized fluorescence due to their slower rates of rotation as compared to fluorescent tracers attached to smaller molecules (*e.g.* FITC- labeled peptides that are free in solution).

- [00182] For example, fluoresceinated peptidomimetic macrocycles (25 nM) are incubated with the acceptor protein (25- 1000nM) in binding buffer (140mM NaCl, 50 mM Tris-HCL, pH 7.4) for 30 minutes at room temperature. Binding activity is measured, for example, by fluorescence polarization on a luminescence spectrophotometer (*e.g.* Perkin-Elmer LS50B). K_d values can be determined by nonlinear regression analysis using, for example, Graphpad Prism software (GraphPad Software, Inc., San Diego, CA). A peptidomimetic macrocycle shows, in some embodiments, similar or lower K_d than a corresponding uncrosslinked polypeptide.

In vitro Displacement Assays To Characterize Antagonists of Peptide-Protein Interactions.

- [00183] To assess the binding and affinity of compounds that antagonize the interaction between a peptide and an acceptor protein, a fluorescence polarization assay (FPA) utilizing a fluoresceinated peptidomimetic macrocycle derived from a peptidomimetic precursor sequence is used, for example. The FPA technique measures the molecular orientation and mobility using polarized light and fluorescent tracer. When excited with polarized light, fluorescent tracers (*e.g.*, FITC) attached to molecules with high apparent molecular weights (*e.g.* FITC-labeled peptides bound to a large protein) emit higher levels of polarized fluorescence due to their slower rates of rotation as compared to fluorescent tracers attached to smaller molecules (*e.g.* FITC-labeled peptides that are free in solution). A compound that antagonizes the interaction between the fluoresceinated peptidomimetic macrocycle and an acceptor protein will be detected in a competitive binding FPA experiment.
- [00184] For example, putative antagonist compounds (1 nM to 1 mM) and a fluoresceinated peptidomimetic macrocycle (25 nM) are incubated with the acceptor protein (50 nM) in binding buffer (140mM NaCl, 50 mM Tris-HCL, pH 7.4) for 30 minutes at room temperature. Antagonist binding activity is measured, for example, by fluorescence polarization on a luminescence spectrophotometer (*e.g.* Perkin-Elmer LS50B). K_d values can be determined by nonlinear regression analysis using, for example, Graphpad Prism software (GraphPad Software, Inc., San Diego, CA).
- [00185] Any class of molecule, such as small organic molecules, peptides, oligonucleotides or proteins can be examined as putative antagonists in this assay.

Assay for Protein-ligand binding by Affinity Selection-Mass Spectrometry

- [00186] To assess the binding and affinity of test compounds for proteins, an affinity-selection mass spectrometry assay is used, for example. Protein-ligand binding experiments are conducted according to the following representative procedure outlined for a system-wide control experiment using 1 μ M peptidomimetic macrocycle plus 5 μ M hMDM2. A 1 μ L DMSO aliquot

of a 40 μM stock solution of peptidomimetic macrocycle is dissolved in 19 μL of PBS (Phosphate-buffered saline: 50 mM, pH 7.5 Phosphate buffer containing 150 mM NaCl). The resulting solution is mixed by repeated pipetting and clarified by centrifugation at 10 000g for 10 min. To a 4 μL aliquot of the resulting supernatant is added 4 μL of 10 μM hMDM2 in PBS. Each 8.0 μL experimental sample thus contains 40 pmol (1.5 μg) of protein at 5.0 μM concentration in PBS plus 1 μM peptidomimetic macrocycle and 2.5% DMSO. Duplicate samples thus prepared for each concentration point are incubated for 60 min at room temperature, and then chilled to 4 $^{\circ}\text{C}$ prior to size-exclusion chromatography-LC-MS analysis of 5.0 μL injections. Samples containing a target protein, protein–ligand complexes, and unbound compounds are injected onto an SEC column, where the complexes are separated from non-binding component by a rapid SEC step. The SEC column eluate is monitored using UV detectors to confirm that the early-eluting protein fraction, which elutes in the void volume of the SEC column, is well resolved from unbound components that are retained on the column. After the peak containing the protein and protein–ligand complexes elutes from the primary UV detector, it enters a sample loop where it is excised from the flow stream of the SEC stage and transferred directly to the LC-MS via a valving mechanism. The $(\text{M} + 3\text{H})^{3+}$ ion of the peptidomimetic macrocycle is observed by ESI-MS at the expected m/z , confirming the detection of the protein–ligand complex.

Assay for Protein-ligand K_d Titration Experiments.

- [00187] To assess the binding and affinity of test compounds for proteins, a protein-ligand K_d titration experiment is performed, for example. Protein-ligand K_d titrations experiments are conducted as follows: 2 μL DMSO aliquots of a serially diluted stock solution of titrant peptidomimetic macrocycle (5, 2.5, ..., 0.098 mM) are prepared then dissolved in 38 μL of PBS. The resulting solutions are mixed by repeated pipetting and clarified by centrifugation at 10 000g for 10 min. To 4.0 μL aliquots of the resulting supernatants is added 4.0 μL of 10 μM hMDM2 in PBS. Each 8.0 μL experimental sample thus contains 40 pmol (1.5 μg) of protein at 5.0 μM concentration in PBS, varying concentrations (125, 62.5, ..., 0.24 μM) of the titrant peptide, and 2.5% DMSO. Duplicate samples thus prepared for each concentration point are incubated at room temperature for 30 min, then chilled to 4 $^{\circ}\text{C}$ prior to SEC-LC-MS analysis of 2.0 μL injections. The $(\text{M} + \text{H})^{1+}$, $(\text{M} + 2\text{H})^{2+}$, $(\text{M} + 3\text{H})^{3+}$, and/or $(\text{M} + \text{Na})^{1+}$ ion is observed by ESI-MS; extracted ion chromatograms are quantified, then fit to equations to derive the binding affinity K_d as described in “*A General Technique to Rank Protein-Ligand Binding Affinities and Determine Allosteric vs. Direct Binding Site Competition in Compound Mixtures.*” Annis, D. A.; Nazef, N.; Chuang, C. C.; Scott, M. P.; Nash, H. M. *J. Am. Chem. Soc.* **2004**, 126, 15495-15503; also in “*ALIS: An Affinity Selection-Mass Spectrometry System for the Discovery and Characterization of Protein-Ligand Interactions*” D. A. Annis, C.-C. Chuang, and N. Nazef. In *Mass Spectrometry in*

Medicinal Chemistry. Edited by Wanner K, Höfner G: Wiley-VCH; **2007**:121-184. Mannhold R, Kubinyi H, Folkers G (Series Editors): Methods and Principles in Medicinal Chemistry.

Assay for Competitive Binding Experiments by Affinity Selection-Mass Spectrometry

[00188] To determine the ability of test compounds to bind competitively to proteins, an affinity selection mass spectrometry assay is performed, for example. A mixture of ligands at 40 μM per component is prepared by combining 2 μL aliquots of 400 μM stocks of each of the three compounds with 14 μL of DMSO. Then, 1 μL aliquots of this 40 μM per component mixture are combined with 1 μL DMSO aliquots of a serially diluted stock solution of titrant peptidomimetic macrocycle (10, 5, 2.5, ..., 0.078 mM). These 2 μL samples are dissolved in 38 μL of PBS. The resulting solutions were mixed by repeated pipetting and clarified by centrifugation at 10 000g for 10 min. To 4.0 μL aliquots of the resulting supernatants is added 4.0 μL of 10 μM hMDM2 protein in PBS. Each 8.0 μL experimental sample thus contains 40 pmol (1.5 μg) of protein at 5.0 μM concentration in PBS plus 0.5 μM ligand, 2.5% DMSO, and varying concentrations (125, 62.5, ..., 0.98 μM) of the titrant peptidomimetic macrocycle. Duplicate samples thus prepared for each concentration point are incubated at room temperature for 60 min, then chilled to 4 $^{\circ}\text{C}$ prior to SEC-LC-MS analysis of 2.0 μL injections. Additional details on these and other methods are provided in “*A General Technique to Rank Protein-Ligand Binding Affinities and Determine Allosteric vs. Direct Binding Site Competition in Compound Mixtures.*” Annis, D. A.; Nazef, N.; Chuang, C. C.; Scott, M. P.; Nash, H. M. *J. Am. Chem. Soc.* **2004**, 126, 15495-15503; also in “*ALIS: An Affinity Selection-Mass Spectrometry System for the Discovery and Characterization of Protein-Ligand Interactions*” D. A. Annis, C.-C. Chuang, and N. Nazef. In Mass Spectrometry in Medicinal Chemistry. Edited by Wanner K, Höfner G: Wiley-VCH; **2007**:121-184. Mannhold R, Kubinyi H, Folkers G (Series Editors): Methods and Principles in Medicinal Chemistry.

Binding Assays in Intact Cells.

[00189] It is possible to measure binding of peptides or peptidomimetic macrocycles to their natural acceptors in intact cells by immunoprecipitation experiments. For example, intact cells are incubated with fluoresceinated (FITC-labeled) compounds for 4 hrs in the absence of serum, followed by serum replacement and further incubation that ranges from 4-18 hrs. Cells are then pelleted and incubated in lysis buffer (50mM Tris [pH 7.6], 150 mM NaCl, 1% CHAPS and protease inhibitor cocktail) for 10 minutes at 4 $^{\circ}\text{C}$. Extracts are centrifuged at 14,000 rpm for 15 minutes and supernatants collected and incubated with 10 μL goat anti-FITC antibody for 2 hrs, rotating at 4 $^{\circ}\text{C}$ followed by further 2 hrs incubation at 4 $^{\circ}\text{C}$ with protein A/G Sepharose (50 μL of 50% bead slurry). After quick centrifugation, the pellets are washed in lysis buffer containing increasing salt concentration (e.g., 150, 300, 500 mM). The beads are then re-equilibrated at 150 mM NaCl before addition of SDS-containing sample buffer and boiling. After centrifugation, the supernatants are optionally electrophoresed using 4%-12% gradient Bis-Tris gels followed by

transfer into Immobilon-P membranes. After blocking, blots are optionally incubated with an antibody that detects FITC and also with one or more antibodies that detect proteins that bind to the peptidomimetic macrocycle.

Cellular Penetrability Assays.

- [00190] A peptidomimetic macrocycle is, for example, more cell penetrable compared to a corresponding uncrosslinked macrocycle. Peptidomimetic macrocycles with optimized linkers possess, for example, cell penetrability that is at least two-fold greater than a corresponding uncrosslinked macrocycle, and often 20% or more of the applied peptidomimetic macrocycle will be observed to have penetrated the cell after 4 hours. To measure the cell penetrability of peptidomimetic macrocycles and corresponding uncrosslinked macrocycle, intact cells are incubated with fluorescently-labeled (e.g. fluoresceinated) peptidomimetic macrocycles or corresponding uncrosslinked macrocycle (10 μ M) for 4 hrs in serum free media at 37°C, washed twice with media and incubated with trypsin (0.25%) for 10 min at 37°C. The cells are washed again and resuspended in PBS. Cellular fluorescence is analyzed, for example, by using either a FACSCalibur flow cytometer or Cellomics' KineticScan ® HCS Reader.

Cellular Efficacy Assays.

- [00191] The efficacy of certain peptidomimetic macrocycles is determined, for example, in cell-based killing assays using a variety of tumorigenic and non-tumorigenic cell lines and primary cells derived from human or mouse cell populations. Cell viability is monitored, for example, over 24-96 hrs of incubation with peptidomimetic macrocycles (0.5 to 50 μ M) to identify those that kill at $EC_{50} < 10 \mu$ M. Several standard assays that measure cell viability are commercially available and are optionally used to assess the efficacy of the peptidomimetic macrocycles. In addition, assays that measure Annexin V and caspase activation are optionally used to assess whether the peptidomimetic macrocycles kill cells by activating the apoptotic machinery. For example, the Cell Titer-glo assay is used which determines cell viability as a function of intracellular ATP concentration.

In Vivo Stability Assay.

- [00192] To investigate the *in vivo* stability of the peptidomimetic macrocycles, the compounds are, for example, administered to mice and/or rats by IV, IP, PO or inhalation routes at concentrations ranging from 0.1 to 50 mg/kg and blood specimens withdrawn at 0', 5', 15', 30', 1 hr, 4 hrs, 8 hrs and 24 hours post-injection. Levels of intact compound in 25 μ L of fresh serum are then measured by LC-MS/MS as above.

In vivo Efficacy in Animal Models.

- [00193] To determine the anti-oncogenic activity of peptidomimetic macrocycles *in vivo*, the compounds are, for example, given alone (IP, IV, PO, by inhalation or nasal routes) or in combination with

sub-optimal doses of relevant chemotherapy (*e.g.*, cyclophosphamide, doxorubicin, etoposide). In one example, 5×10^6 RS4;11 cells (established from the bone marrow of a patient with acute lymphoblastic leukemia) that stably express luciferase are injected by tail vein in NOD-SCID mice 3 hrs after they have been subjected to total body irradiation. If left untreated, this form of leukemia is fatal in 3 weeks in this model. The leukemia is readily monitored, for example, by injecting the mice with D-luciferin (60 mg/kg) and imaging the anesthetized animals (*e.g.*, Xenogen In Vivo Imaging System, Caliper Life Sciences, Hopkinton, MA). Total body bioluminescence is quantified by integration of photonic flux (photons/sec) by Living Image Software (Caliper Life Sciences, Hopkinton, MA). Peptidomimetic macrocycles alone or in combination with sub-optimal doses of relevant chemotherapeutic agents are, for example, administered to leukemic mice (10 days after injection/day 1 of experiment, in bioluminescence range of 14-16) by tail vein or IP routes at doses ranging from 0.1mg/kg to 50 mg/kg for 7 to 21 days. Optionally, the mice are imaged throughout the experiment every other day and survival monitored daily for the duration of the experiment. Expired mice are optionally subjected to necropsy at the end of the experiment. Another animal model is implantation into NOD-SCID mice of DoHH2, a cell line derived from human follicular lymphoma, that stably expresses luciferase. These *in vivo* tests optionally generate preliminary pharmacokinetic, pharmacodynamic and toxicology data.

Clinical Trials.

- [00194] To determine the suitability of the peptidomimetic macrocycles for treatment of humans, clinical trials are performed. For example, patients diagnosed with cancer and in need of treatment can be selected and separated in treatment and one or more control groups, wherein the treatment group is administered a peptidomimetic macrocycle, while the control groups receive a placebo or a known anti-cancer drug. The treatment safety and efficacy of the peptidomimetic macrocycles can thus be evaluated by performing comparisons of the patient groups with respect to factors such as survival and quality-of-life. In this example, the patient group treated with a peptidomimetic macrocycle can show improved long-term survival compared to a patient control group treated with a placebo.

Pharmaceutical Compositions and Routes of Administration

- [00195] Pharmaceutical compositions disclosed herein include peptidomimetic macrocycles and 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 disclosed herein which, upon administration to a recipient, is capable of providing (directly or indirectly) a compound disclosed herein. Particularly favored pharmaceutically acceptable derivatives are those that increase the bioavailability of the compounds 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.
- [00196] In some embodiments, peptidomimetic macrocycles 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.
- [00197] Pharmaceutically acceptable salts of the compounds disclosed herein 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.
- [00198] For preparing pharmaceutical compositions from the compounds provided herein, 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.
- [00199] 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.
- [00200] 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.

- [00201] Liquid form preparations include, without limitation, 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.
- [00202] The pharmaceutical preparation can be 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.
- [00203] When one or more compositions disclosed herein 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 one or more compounds disclosed herein. Alternatively, those agents are part of a single dosage form, mixed together with one or more compounds disclosed herein in a single composition.

Methods of Use

- [00204] In one aspect, provided herein are novel peptidomimetic macrocycles that are useful in competitive binding assays to identify agents which bind to the natural ligand(s) of the proteins or peptides upon which the peptidomimetic macrocycles are modeled. For example, in the p53/MDMX system, labeled peptidomimetic macrocycles based on p53 can be used in a MDMX binding assay along with small molecules that competitively bind to MDMX. Competitive binding studies allow for rapid *in vitro* evaluation and determination of drug candidates specific for the p53/MDMX system. Such binding studies can be performed with any of the peptidomimetic macrocycles disclosed herein and their binding partners.
- [00205] Provided herein is the generation of antibodies against the peptidomimetic macrocycles. In some embodiments, these antibodies specifically bind both the peptidomimetic macrocycle and the precursor peptides, such as p53, to which the peptidomimetic macrocycles are related. Such antibodies, for example, disrupt the native protein-protein interaction, for example, binding between p53 and MDMX.
- [00206] In other aspects, provided herein are both prophylactic and therapeutic methods of treating a subject at risk of (or susceptible to) a disorder or having a disorder associated with aberrant (*e.g.*, insufficient or excessive) expression or activity of the molecules including p53, MDM2 or MDMX.
- [00207] In another embodiment, a disorder is caused, at least in part, by an abnormal level of p53 or MDM2 or MDMX, (*e.g.*, over or under expression), or by the presence of p53 or MDM2 or

MDMX exhibiting abnormal activity. As such, the reduction in the level and/or activity of p53 or MDM2 or MDMX, or the enhancement of the level and/or activity of p53 or MDM2 or MDMX, by peptidomimetic macrocycles derived from p53, is used, for example, to ameliorate or reduce the adverse symptoms of the disorder.

- [00208] In another aspect, provided herein are methods for treating or preventing a disease including hyperproliferative disease and inflammatory disorder by interfering with the interaction or binding between binding partners, for example, between p53 and MDM2 or p53 and MDMX. These methods comprise administering an effective amount of a compound to a warm blooded animal, including a human. In some embodiments, the administration of one or more compounds disclosed herein induces cell growth arrest or apoptosis.
- [00209] As used herein, the term “treatment” is defined as the application or administration of a therapeutic agent to a patient, or application or administration of a therapeutic agent to an isolated tissue or cell line from a patient, who has a disease, a symptom of disease or a predisposition toward a disease, with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve or affect the disease, the symptoms of disease or the predisposition toward disease.
- [00210] In some embodiments, the peptidomimetics macrocycles can 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 can be categorized as pathologic, *i.e.*, characterizing or constituting a disease state, or can 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 peptidomimetics macrocycles are novel therapeutic agents for controlling breast cancer, ovarian cancer, colon cancer, lung cancer, metastasis of such cancers and the like.
- [00211] 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.

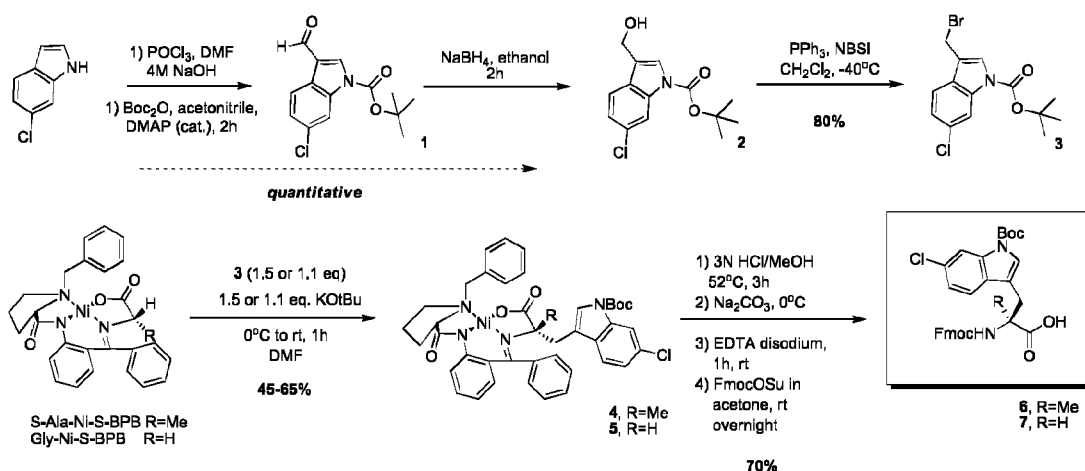
- [00212] Examples of proliferative disorders include hematopoietic neoplastic disorders. As used herein, the term "hematopoietic neoplastic disorders" includes diseases involving hyperplastic/neoplastic cells of hematopoietic origin, *e.g.*, arising from myeloid, lymphoid or erythroid lineages, or precursor cells thereof. The diseases can 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./Hematol.* 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 (LGL), Hodgkin's disease and Reed-Stenberg disease.
- [00213] 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.
- [00214] Examples of cellular proliferative and/or differentiative disorders of the skin include, but are not limited to proliferative skin disease such as melanomas, including mucosal melanoma, superficial spreading melanoma, nodular melanoma, lentigo (*e.g.* lentigo maligna, lentigo maligna melanoma, or acral lentiginous melanoma), amelanotic melanoma, desmoplastic melanoma, melanoma with features of a Spitz nevus, melanoma with small nevus-like cells, polypoid

melanoma, and soft-tissue melanoma; basal cell carcinomas including micronodular basal cell carcinoma, superficial basal cell carcinoma, nodular basal cell carcinoma (rodent ulcer), cystic basal cell carcinoma, cicatricial basal cell carcinoma, pigmented basal cell carcinoma, aberrant basal cell carcinoma, infiltrative basal cell carcinoma, nevoid basal cell carcinoma syndrome, polypoid basal cell carcinoma, pore-like basal cell carcinoma, and fibroepithelioma of Pinkus; squamous cell carcinomas including acanthoma (large cell acanthoma), adenoid squamous cell carcinoma, basaloid squamous cell carcinoma, clear cell squamous cell carcinoma, signet-ring cell squamous cell carcinoma, spindle cell squamous cell carcinoma, Marjolin's ulcer, erythroplasia of Queyrat, and Bowen's disease; or other skin or subcutaneous tumors.

- [00215] 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.
- [00216] 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.
- [00217] 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.
- [00218] 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-stromal tumors such as, granulosa-theca cell tumors, thecomafibromas, androblastomas, hil cell tumors, and gonadoblastoma; and metastatic tumors such as Krukenberg tumors.
- [00219] 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 described herein can be employed in practicing the invention. It is intended that the following claims define the scope and that methods and structures within the scope of these claims and their equivalents be covered thereby.

Examples

Example 1: Synthesis of 6-chlorotryptophan Fmoc amino acids



[00220] Tert-butyl 6-chloro-3-formyl-1H-indole-1-carboxylate, **1**. To a stirred solution of dry DMF (12 mL) was added dropwise POCl₃ (3.92 mL, 43 mmol, 1.3 equiv) at 0 °C under Argon. The solution was stirred at the same temperature for 20 min before a solution of 6-chloroindole (5.0 g, 33 mmol, 1 eq.) in dry DMF (30 mL) was added dropwise. The resulting mixture was allowed to warm to room temperature and stirred for an additional 2.5h. Water (50 mL) was added and the solution was neutralized with 4M aqueous NaOH (pH ~ 8). The resulting solid was filtered off, washed with water and dried under vacuum. This material was directly used in the next step without additional purification. To a stirred solution of the crude formyl indole (33 mmol, 1 eq.) in THF (150 mL) was added successively Boc₂O (7.91 g, 36.3 mmol, 1.1 equiv) and DMAP (0.4 g, 3.3 mmol, 0.1 equiv) at room temperature under N₂. The resulting mixture was stirred at room temperature for 1.5h and the solvent was evaporated under reduced pressure. The residue was taken up in EtOAc and washed with 1N HCl, dried and concentrated to give the formyl indole **1** (9 g, 98 % over 2 steps) as a white solid. ¹H NMR (CDCl₃) δ: 1.70 (s, Boc, 9H); 7.35 (dd, 1H); 8.21 (m, 3H); 10.07 (s, 1H).

[00221] Tert-butyl 6-chloro-3-(hydroxymethyl)-1H-indole-1-carboxylate, **2**. To a solution of compound **1** (8.86g, 32 mmol, 1 eq.) in ethanol (150 mL) was added NaBH₄ (2.4g, 63 mmol, 2 eq.). The reaction was stirred for 3 h at room temperature. The reaction mixture was concentrated and the residue was poured into diethyl ether and water. The organic layer was separated, dried over magnesium sulfate and concentrated to give a white solid (8.7g, 98%). This material was directly used in the next step without additional purification. ¹H NMR (CDCl₃) δ: 1.65 (s, Boc, 9H); 4.80 (s, 2H, CH₂); 7.21 (dd, 1H); 7.53 (m, 2H); 8.16 (bs, 1H).

[00222] Tert-butyl 3-(bromomethyl)-6-chloro-1H-indole-1-carboxylate, **3**. To a solution of compound **2** (4.1g, 14.6 mmol, 1 eq.) in dichloromethane (50 mL) under argon was added a solution of

triphenylphosphine (4.59g, 17.5 mmol, 1.2 eq.) in dichloromethane (50 mL) at -40°C. The reaction solution was stirred an additional 30 min at 40°C. Then NBS (3.38g, 19 mmol, 1.3 eq.) was added. The resulting mixture was allowed to warm to room temperature and stirred overnight. Dichloromethane was evaporated, Carbon Tetrachloride (100 mL) was added and the mixture was stirred for 1h and filtrated. The filtrate was concentrated, loaded in a silica plug and quickly eluted with 25% EtOAc in Hexanes. The solution was concentrated to give a white foam (3.84g, 77%). ¹H NMR (CDCl₃) δ: 1.66 (s, Boc, 9H); 4.63 (s, 2H, CH₂); 7.28 (dd, 1H); 7.57 (d, 1H); 7.64 (bs, 1H); 8.18 (bs, 1H).

[00223] αMe-6Cl-Trp(Boc)-Ni-S-BPB, **4**. To **S-Ala-Ni-S-BPB** (2.66g, 5.2 mmol, 1 eq.) and KO-*t*Bu (0.87g, 7.8 mmol, 1.5 eq.) was added 50 mL of DMF under argon. The bromide derivative compound **3** (2.68g, 7.8 mmol, 1.5 eq.) in solution of DMF (5.0 mL) was added via syringe. The reaction mixture was stirred at ambient temperature for 1h. The solution was then quenched with 5 % aqueous acetic acid and diluted with water. The desired product was extracted in dichloromethane, dried and concentrated. The oily product **4** was purified by flash chromatography (solid loading) on normal phase using EtOAc and Hexanes as eluents to give a red solid (1.78g, 45% yield). αMe-6Cl-Trp(Boc)-Ni-S-BPB, **4**: M+H calc. 775.21, M+H obs. 775.26; ¹H NMR (CDCl₃) δ: 1.23 (s, 3H, αMe); 1.56 (m, 11H, Boc + CH₂); 1.82-2.20 (m, 4H, 2CH₂); 3.03 (m, 1H, CH_α); 3.24 (m, 2H, CH₂); 3.57 and 4.29 (AB system, 2H, CH₂ (benzyl), J= 12.8Hz); 6.62 (d, 2H); 6.98 (d, 1H); 7.14 (m, 2H); 7.23 (m, 1H); 7.32-7.36 (m, 5H); 7.50 (m, 2H); 7.67 (bs, 1H); 7.98 (d, 2H); 8.27 (m, 2H).

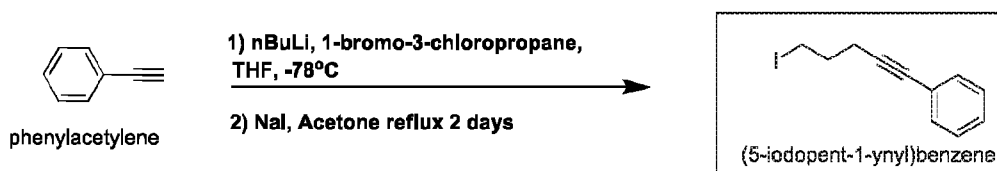
[00224] Fmoc-αMe-6Cl-Trp(Boc)-OH, **6**. To a solution of 3N HCl/MeOH (1/3, 15 mL) at 50°C was added a solution of compound **4** (1.75g, 2.3 mmol, 1 eq.) in MeOH (5 ml) dropwise. The starting material disappeared within 3-4 h. The acidic solution was then cooled to 0°C with an ice bath and quenched with an aqueous solution of Na₂CO₃ (1.21g, 11.5 mmol, 5 eq.). Methanol was removed and 8 more equivalents of Na₂CO₃ (1.95g, 18.4 mmol) were added to the suspension. The Nickel scavenging EDTA disodium salt dihydrate (1.68g, 4.5 mmol, 2 eq.) was then added and the suspension was stirred for 2h. A solution of Fmoc-OSu (0.84g, 2.5 mmol, 1.1 eq.) in acetone (50 mL) was added and the reaction was stirred overnight. Afterwards, the reaction was diluted with diethyl ether and 1N HCl. The organic layer was then dried over magnesium sulfate and concentrated in vacuo. The desired product **6** was purified on normal phase using acetone and dichloromethane as eluents to give a white foam (0.9g, 70% yield). Fmoc-αMe-6Cl-Trp(Boc)-OH, **6**: M+H calc. 575.19, M+H obs. 575.37; ¹H NMR (CDCl₃) 1.59 (s, 9H, Boc); 1.68 (s, 3H, Me); 3.48 (bs, 2H, CH₂); 4.22 (m, 1H, CH); 4.39 (bs, 2H, CH₂); 5.47 (s, 1H, NH); 7.10 (m, 1H); 7.18 (m, 2H); 7.27 (m, 2H); 7.39 (m, 2H); 7.50 (m, 2H); 7.75 (d, 2H); 8.12 (bs, 1H).

[00225] 6Cl-Trp(Boc)-Ni-S-BPB, **5**. To **Gly-Ni-S-BPB** (4.6g, 9.2 mmol, 1 eq.) and KO-*t*Bu (1.14g, 10.1 mmol, 1.1 eq.) was added 95 mL of DMF under argon. The bromide derivative compound **3** (3.5g, 4.6 mmol, 1.1 eq.) in solution of DMF (10 mL) was added via syringe. The reaction

mixture was stirred at ambient temperature for 1h. The solution was then quenched with 5 % aqueous acetic acid and diluted with water. The desired product was extracted in dichloromethane, dried and concentrated. The oily product **5** was purified by flash chromatography (solid loading) on normal phase using EtOAc and Hexanes as eluents to give a red solid (5g, 71% yield). 6Cl-Trp(Boc)-Ni-S-BPB, **5**: M+H calc. 761.20, M+H obs. 761.34; ^1H NMR (CDCl_3) δ : 1.58 (m, 11H, Boc + CH_2); 1.84 (m, 1H); 1.96 (m, 1H); 2.24 (m, 2H, CH_2); 3.00 (m, 1H, CH_α); 3.22 (m, 2H, CH_2); 3.45 and 4.25 (AB system, 2H, CH_2 (benzyl), $J=12.8\text{Hz}$); 4.27 (m, 1H, CH_α); 6.65 (d, 2H); 6.88 (d, 1H); 7.07 (m, 2H); 7.14 (m, 2H); 7.28 (m, 3H); 7.35-7.39 (m, 2H); 7.52 (m, 2H); 7.96 (d, 2H); 8.28 (m, 2H).

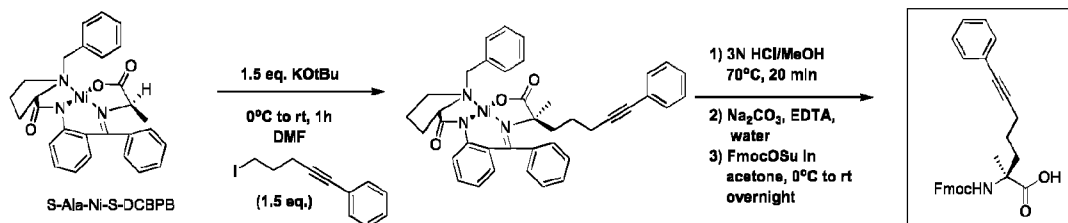
[00226] Fmoc-6Cl-Trp(Boc)-OH, **7**. To a solution of 3N HCl/MeOH (1/3, 44 mL) at 50°C was added a solution of compound **5** (5g, 6.6 mmol, 1 eq.) in MeOH (10 ml) dropwise. The starting material disappeared within 3-4 h. The acidic solution was then cooled to 0°C with an ice bath and quenched with an aqueous solution of Na_2CO_3 (3.48g, 33 mmol, 5 eq.). Methanol was removed and 8 more equivalents of Na_2CO_3 (5.57g, 52 mmol) were added to the suspension. The Nickel scavenging EDTA disodium salt dihydrate (4.89g, 13.1 mmol, 2 eq.) and the suspension was stirred for 2h. A solution of Fmoc-OSu (2.21g, 6.55 mmol, 1.1 eq.) in acetone (100 mL) was added and the reaction was stirred overnight. Afterwards, the reaction was diluted with diethyl ether and 1N HCl. The organic layer was then dried over magnesium sulfate and concentrated in vacuo. The desired product **7** was purified on normal phase using acetone and dichloromethane as eluents to give a white foam (2.6g, 69% yield). Fmoc-6Cl-Trp(Boc)-OH, **7**: M+H calc. 561.17, M+H obs. 561.37; ^1H NMR (CDCl_3) 1.63 (s, 9H, Boc); 3.26 (m, 2H, CH_2); 4.19 (m, 1H, CH); 4.39 (m, 2H, CH_2); 4.76 (m, 1H); 5.35 (d, 1H, NH); 7.18 (m, 2H); 7.28 (m, 2H); 7.39 (m, 3H); 7.50 (m, 2H); 7.75 (d, 2H); 8.14 (bs, 1H).

Example 1a: Synthesis of alkyne compounds for use in synthesis of compounds of Formula I.



[00227] **Synthesis of (5-iodopent-1-ynyl)benzene.** To a solution of THF (200mL) into reaction flask was added (5-chloropent-1-ynyl)benzene Phenylacetylene (10g, 97.91mmol). Then the reaction mixture was cooled to -78°C in a dry ice bath. nBuLi (95.95mmol, 38.39mL) was added dropwise and allowed to react for 0.5h at -78°C. At -78°C, 1-bromo-3-chloropropane was added. Stirred for 5 hours during which the reaction was allowed to warm up to room temperature. Then reaction was refluxed at 90°C for 3 hours. The solvent was distilled off, then water (150mL) and ether (150mL) was added. The crude product was extracted, ether was distilled off and the resulting crude mixture was dissolved in acetone. Sodium iodide (22.92mmol, 3.44g) was added into the solution. The reaction mixture, with reflux condenser attached, was heated to

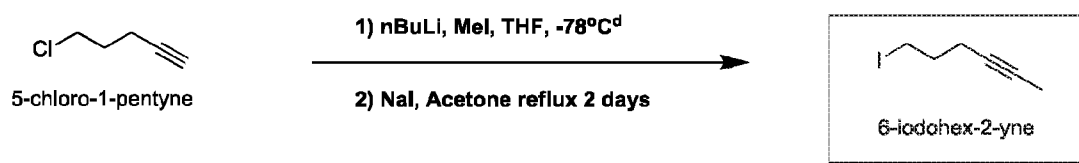
70°C for two days. Acetone was distilled off using short path distillation apparatus. Ether (150mL) and water (150mL) was added and carried out extraction. Ether was then dried over magnesium sulfate and distilled off resulting in 5.00g of product (yield 98%). No further purification was carried out. ¹H NMR (500 MHz, CDCl₃) δ 2.072 (m, 2H, CH₂); 2.605 (t, 2H, CH₂); 3.697 (m, 2H, CH₂); 7.276 (m, 2H, Phenyl); 7.389 (m, 2H, phenyl); 7.484 (m, 1H, phenyl).



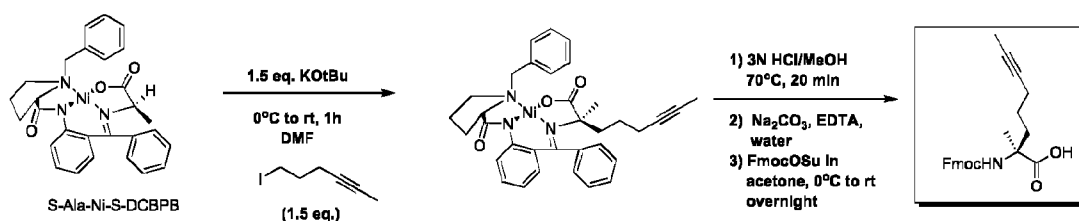
[00228] Synthesis of MeS5-PhenylAlkyne-Ni-S-BPB. To S-Ala-Ni-S-BPB (18.17 mmol, 9.30g) and KO-^tBu (27.26mmol, 3.05g) was added 200 mL of DMF under argon. (5-iodopent-1-ynyl)benzene (27.26mmol, 7.36g) in solution of DMF (50 mL) was added via syringe. The reaction mixture was stirred at ambient temperature for 1h. The solution was then quenched with acetic acid (27.26mmol, 1.58mL) and diluted with water (100mL). The product was extracted with dichloromethane (100mL), separated and dried over magnesium sulfate. The crude product was purified by flash chromatography on normal phase using acetone and dichloromethane as eluents to afford the desired product as a red solid (9.48g, 79.8 %). M+H calc. 654.22, M+H obs. 654.4; ¹H NMR (500 MHz, CDCl₃) δ 1.17 (s, 3H, Me ($\perp$ Me-Phe)); 1.57 (m, 1H, CH₂); 1.67 (m, 1H, CH₂); 1.89 (m, 1H, CH₂); 2.06 (m, 1H, CH₂); 2.24 (m, 2H, CH₂); 3.05 (m, 1H); 3.18 (s, 2H); 3.26 (m, 1H); 3.56 and 4.31 (AB system, 2H, CH₂ (benzyl), J= 12.8Hz); 6.64 (m, 2H); 6.94 (d, 1H); 7.12 (m, 1H); 7.20 (m, 1H); 7.20-7.40 (m, 10H); 7.43 (m, 2H); 8.01 (d, 2H); 8.13 (m, 1H).

[00229] Synthesis of (S)-2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-2-methyl-7-phenylhept-6-ynoic acid.

To a solution of 3N HCl/MeOH (1/1, 23 mL) at 70°C was added a solution of MeS5-PhenylAlkyne-Ni-S-BPB (14.5 mmol, 9.48g) in MeOH (70 ml) dropwise. The starting material disappeared within 10-20 min. The green reaction mixture was then concentrated in vacuo. Water was added (100mL) and the resulting precipitate (S-BPB HCl salt) was filtered off. Sodium carbonate (116 mmol, 12.29g) and EDTA (29mmol, 10.79g) were added to the mother liquor. The mixture was stirred at room temperature for 3 hours to scavenge the free nickel. After addition of 50 mL of acetone, the reaction was cooled to 0°C with an ice bath. Fmoc-OSu (16.68mmol, 5.62g) dissolved in acetone (50ml) was added and the reaction was allowed to warm up to ambient temperature with stirring overnight. Afterwards, the reaction was diluted with ethyl acetate (300mL). Then the organic layer was separated. The aqueous layer was acidified with conc. HCl. The desired product was extracted with dichloromethane (400mL), dried over magnesium sulfate and concentrated in vacuo. The crude product was purified by flash chromatography on normal phase using 10%MBTE/DCM as eluents to afford the desired product as a white solid (6.05g, 51 %). M+H calc. 454.53, M+H obs. 454.2; ¹H NMR (CDCl₃) δ 1.50 (bs, 2H, CH₂); 1.60 (bs, 3H, CH₃); 2.05 (bs, 1H, CH₂); 2.30 (bs, 1H, CH₂); 2.42 (bs, 2H, CH₂); 4.20 (m, 1H, CH); 4.40 (bs, 2H, CH₂); 5.58 (s, 1H, NH); 7.26 (m, 3H); 7.32 (m, 2H); 7.37 (m, 4H); 7.58 (d, 2H); 7.76 (d, 2H).



[00230] Synthesis of 6-iodohex-2-yne. To a solution of THF (250mL) into reaction flask was added 5-chloro-1-pentyne (48.7mmol, 5.0g). Then the reaction mixture was cooled to -78°C in a dry ice bath. $n\text{BuLi}$ (51.1mmol, 20.44mL) was added dropwise and allowed to react for 0.5h at -78°C and allowed to warm to room temperature. Then methyl iodide (54.5mmol, 3.39mL) was added to the reaction mixture. The reaction was stirred for 5 hours. Water was added (1.5mL) and the THF was distilled off. The crude product was extracted with pentane (100mL) and water (100mL). Pentane was distilled off and the resulting crude mixture was dissolved in acetone (300mL). Sodium iodide (172.9mmol, 25.92g) was added into the solution. The reaction mixture, with reflux condenser attached, was heated to 70°C for two days. Acetone was distilled off using short path distillation apparatus. Ether (100mL) and water (100mL) was added and carried out extraction. Ether was then dried over magnesium sulfate and distilled off resulting in 8.35g of product (yield 83%). No further purification was carried out. ^1H NMR (500 MHz, CDCl_3) δ 1.762 (t, 3H, CH_3); 1.941 (m, 2H, CH_2); 2.245 (m, 2H, CH_2); 3.286 (m, 2H, CH_2).



[00231] Synthesis of MeS5-MethylAlkyne-Ni-S-BPB. To S-Ala-Ni-S-BPB (19.53 mmol, 10g) and $\text{KO}-t\text{Bu}$ (29.29mmol, 3.28g) was added 200 mL of DMF under argon. 6-iodohex-2-yne (29.29mmol, 6.09g) in solution of DMF (50 mL) was added via syringe. The reaction mixture was stirred at ambient temperature for 1h. The solution was then quenched with acetic acid (29.29mmol, 1.69mL) and diluted with water (100mL). The product was extracted with dichloromethane (300mL), separated and dried over magnesium sulfate. The crude product was purified by flash chromatography on normal phase using acetone and dichloromethane as eluents to afford the desired product as a red solid (8.10g, 70 %). $\text{M}+\text{H}$ calc. 592.2, $\text{M}+\text{H}$ obs. 592.4; ^1H NMR (500 Mz, CDCl_3) δ 1.17 (s, 3H, CH_3 ($\alpha\text{Me-Phe}$)); 1.57 (m, 1H, CH_2); 1.67 (m, 1H, CH_2); 1.89 (m, 1H, CH_2); 2.06 (m, 1H, CH_2); 2.24 (m, 2H, CH_2); 3.05 (m, 1H); 3.18 (s, 2H); 3.26 (m, 1H); 3.56 and 4.31 (AB system, 2H, CH_2 (benzyl), $J=12.8\text{Hz}$); 6.64 (m, 2H); 6.94 (d, 1H); 7.12 (m, 1H); 7.20 (m, 1H); 7.20-7.40 (m, 10H); 7.43 (m, 2H); 8.01 (d, 2H); 8.13 (m, 1H).

[00232] Synthesis of (S)-2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-2-methyloct-6-ynoic acid. To a solution of 3N HCl/MeOH (1/1, 23 mL) at 70°C was added a solution of MeS5-MethylAlkyne-Ni-S-BPB (13.70 mmol, 8.10g) in methanol (70 mL) dropwise. The starting material disappeared within 10-20 min. The green reaction mixture was then concentrated in vacuo. Water (150mL) was added and the resulting precipitate (S-BPB HCl salt) was filtered off. Sodium carbonate (116 mmol, 12.29g) EDTA (29mmol,

10.79g) were added to the mother liquor. The mixture was stirred at room temperature for 3 hours to scavenge the free nickel. After addition of 75 mL of acetone, the reaction was cooled to 0°C with an ice bath. Fmoc-OSu (15.76mmol, 5.31g) dissolved in acetone (75 ml) was added and the reaction was allowed to warm up to ambient temperature with stirring overnight. Afterwards, the reaction was diluted with ethyl acetate (200mL). Then the organic layer was separated. The aqueous layer was acidified with conc. HCl. The desired product was extracted with dichloromethane (200mL), dried over magnesium sulfate and concentrated in vacuo. The crude product was purified by flash chromatography on normal phase using 10%MBTE/DCM as eluents to afford the desired product as a white solid (2.40g, 45 %). M+H calc. 392.18, M+H obs. 392.3; ¹H NMR (500 Mz, CDCl₃) δ 1.38 (bs, 1H, CH₂); 1.50 (bs, 1H, CH₂); 1.60 (bs, 2H, CH₂); 1.75 (s, 3H, CH₃); 1.95 (bs, 2H, CH₂); 2.10 (bs, 3H, CH₃); 4.20 (m, 1H, CH); 4.40 (bs, 2H, CH₂); 5.58 (s, 1H, NH); 7.32 (m, 2H); 7.42 (m, 2H); 7.59 (d, 2H); 7.78 (d, 2H).

Example 2: Peptidomimetic macrocycles of Formula I.

- [00233] Peptidomimetic macrocycles are prepared as described herein and as in pending U.S. Patent Application No. 12/037,041, filed February 25, 2008, which is hereby incorporated by reference in its entirety. Peptidomimetic macrocycles are designed by replacing two or more naturally occurring amino acids with the corresponding synthetic amino acids. Substitutions are made at i and i+4, and i and i+7 positions. Peptide synthesis is performed either manually or on an automated peptide synthesizer (Applied Biosystems, model 433A), using solid phase conditions, rink amide AM resin (Novabiochem), and Fmoc main-chain protecting group chemistry. For the coupling of natural Fmoc-protected amino acids (Novabiochem), 10 equivalents of amino acid and a 1:1:2 molar ratio of coupling reagents HBTU/HOBt (Novabiochem)/DIEA are employed. Non-natural amino acids (4 equiv) are coupled with a 1:1:2 molar ratio of HATU (Applied Biosystems)/HOBt/DIEA. The N-termini of the synthetic peptides are acetylated, while the C-termini are amidated.
- [00234] Generally, fully protected resin-bound peptides were synthesized on a PEG-PS resin (loading 0.45 mmol/g) on a 0.5 mmol scale. Deprotection of the temporary Fmoc group was achieved by 3 × 10 min treatments of the resin bound peptide with 20% (v/v) piperidine in DMF. After washing with NMP (3x), dichloromethane (3x) and NMP (3x), coupling of each successive amino acid was achieved with 1 × 60 min incubation with the appropriate preactivated Fmoc-amino acid derivative. All protected amino acids (1.0 mmol) were dissolved in NMP and activated with HCTU (1.0 mmol), Cl-HOBt (1.0 mmol) and DIEA (2.0 mmol) prior to transfer of the coupling solution to the deprotected resin-bound peptide. After coupling was completed, the resin was washed in preparation for the next deprotection/coupling cycle. Acetylation of the amino terminus was carried out in the presence of acetic anhydride/DIEA in NMP. The LC-MS analysis of a cleaved and deprotected sample obtained from an aliquot of the fully assembled resin-bound peptide was accomplished in order to verifying the completion of each coupling.

- [00235] In a typical example for the preparation of a peptidomimetic macrocycle comprising a 1,4-triazole group (e.g. SP153), 20% (v/v) 2,6-lutidine in DMF was added to the peptide resin (0.5 mmol) in a 40ml glass vial and shaken for 10 minutes. Sodium ascorbate (0.25g, 1.25 mmol) and diisopropylethylamine (0.22ml, 1.25 mmol) were then added, followed by copper(I) iodide (0.24g, 1.25 mmol) and the resulting reaction mixture was mechanically shaken 16 hours at ambient temperature.
- [00236] In a typical example for the preparation of a peptidomimetic macrocycle comprising a 1,5-triazole group (SP932, SP933), a peptide resin (0.25 mmol) was washed with anhydrous DCM. Resin was loaded into a microwave vial. Vessel was evacuated and purged with nitrogen. Chloro(pentamethylcyclopentadienyl) bis(triphenylphosphine)ruthenium(II), 10% loading, (Strem 44-0117) was added. Anhydrous toluene was added to the reaction vessel. The reaction was then loaded into the microwave and held at 90°C for 10 minutes. Reaction may need to be pushed a subsequent time for completion. In other cases, Chloro(1,5-cyclooctadiene)(pentamethylcyclopentadienyl)ruthenium ("Cp*RuCl(cod)") may be used, for example at room temperature in a solvent comprising toluene.
- [00237] In a typical example for the preparation of a peptidomimetic macrocycle comprising an iodo-substituted triazole group (e.g. SP457), THF (2 ml) was added to the peptide resin (0.05 mmol) in a 40ml glass vial and shaken for 10 minutes. N-bromosuccinimide (0.04g, 0.25 mmol), copper(I) iodide (0.05g, 0.25 mmol) and diisopropylethylamine (0.04 ml, 0.25 mmol) were then added and the resulting reaction mixture was mechanically shaken 16 hours at ambient temperature. Iodo-triazole crosslinkers may be further substituted by a coupling reaction, for example with boronic acids, to result in a peptidomimetic macrocycle such as SP465. In a typical example, DMF (3 ml) was added to the iodo-triazole peptide resin (0.1 mmol) in a 40ml glass vial and shaken for 10 minutes. Phenyl boronic acid (0.04g, 0.3 mmol), tetrakis(triphenylphosphine)palladium(0) (0.006g, 0.005 mmol) and potassium carbonate (0.083g, 0.6 mmol) were then added and the resulting reaction mixture was mechanically shaken 16 hours at 70° C. Iodo-triazole crosslinkers may also be further substituted by a coupling reaction, for example with a terminal alkyne (e.g. Sonogashira coupling), to result in a peptidomimetic macrocycle such as SP468. In a typical example, 2:1 THF:triethylamine (3 ml) was added to the iodo-triazole peptide resin (0.1 mmol) in a 40ml glass vial and shaken for 10 minutes. N-BOC-4-pentyne-1-amine (0.04g, 0.2 mmol) and bis(triphenylphosphine)palladiumchloride (0.014g, 0.02 mmol) were added and shaken for 5 minutes. Copper(I) iodide (0.004g, 0.02 mmol) was then added and the resulting reaction mixture was mechanically shaken 16 hours at 70° C.
- [00238] The triazole-cyclized resin-bound peptides were deprotected and cleaved from the solid support by treatment with TFA/H₂O/TIS (95/5/5 v/v) for 2.5 h at room temperature. After filtration of the resin the TFA solution was precipitated in cold diethyl ether and centrifuged to yield the desired product as a solid. The crude product was purified by preparative HPLC. For example,

purification of cross-linked compounds is achieved by high performance liquid chromatography (HPLC) (Varian ProStar) on a reverse phase C18 column (Varian) to yield the pure compounds. Chemical composition of the pure products is confirmed by LC/MS mass spectrometry (Micromass LCT interfaced with Agilent 1100 HPLC system) and amino acid analysis (Applied Biosystems, model 420A).

[00239] Table 4 shows a list of peptidomimetic macrocycles of Formula I.

Table 4

SP-	Sequence
1	Ac-F\$4rn6AYWEAc3cL\$4a5AAA-NH2
2	Ac-F\$4rn6AYWEAc3cL\$4a5AAibA-NH2
3	Ac-LTF\$4rn6AYWAQL\$4a5SANle-NH2
4	Ac-LTF\$4rn6AYWAQL\$4a5SAL-NH2
5	Ac-LTF\$4rn6AYWAQL\$4a5SAM-NH2
6	Ac-LTF\$4rn6AYWAQL\$4a5SAhL-NH2
7	Ac-LTF\$4rn6AYWAQL\$4a5SAF-NH2
8	Ac-LTF\$4rn6AYWAQL\$4a5SAI-NH2
9	Ac-LTF\$4rn6AYWAQL\$4a5SACHg-NH2
10	Ac-LTF\$4rn6AYWAQL\$4a5SAAib-NH2
11	Ac-LTF\$4rn6AYWAQL\$4a5SAA-NH2
12	Ac-LTF\$4rn6AYWA\$4a5L\$S\$Nle-NH2
13	Ac-LTF\$4rn6AYWA\$4a5L\$S\$A-NH2
14	Ac-F\$4rn6AYWEAc3cL\$4a5AANle-NH2
15	Ac-F\$4rn6AYWEAc3cL\$4a5AAL-NH2
16	Ac-F\$4rn6AYWEAc3cL\$4a5AAM-NH2
17	Ac-F\$4rn6AYWEAc3cL\$4a5AAhL-NH2
18	Ac-F\$4rn6AYWEAc3cL\$4a5AAF-NH2
19	Ac-F\$4rn6AYWEAc3cL\$4a5AAI-NH2
20	Ac-F\$4rn6AYWEAc3cL\$4a5AACHg-NH2
21	Ac-F\$4rn6AYWEAc3cL\$4a5AACHa-NH2
22	Ac-F\$4rn6AYWEAc3cL\$4a5AAAib-NH2
23	Ac-LTF\$4rn6AYWAQL\$4a5AAAibV-NH2
24	Ac-LTF\$4rn6AYWAQL\$4a5AAAibV-NH2
25	Ac-LTF\$4rn6AYWAQL\$4a5SAibAA-NH2
26	Ac-LTF\$4rn6AYWAQL\$4a5SAibAA-NH2
27	Ac-HLTF\$4rn6HHWHQL\$4a5AANleNle-NH2
28	Ac-DLTF\$4rn6HHWHQL\$4a5RRLV-NH2
29	Ac-HHTF\$4rn6HHWHQL\$4a5AAML-NH2
30	Ac-F\$4rn6HHWHQL\$4a5RRDCha-NH2
31	Ac-F\$4rn6HHWHQL\$4a5HRFV-NH2
32	Ac-HLTF\$4rn6HHWHQL\$4a5AAhLA-NH2
33	Ac-DLTF\$4rn6HHWHQL\$4a5RRChg1-NH2
34	Ac-DLTF\$4rn6HHWHQL\$4a5RRChg1-NH2
35	Ac-HHTF\$4rn6HHWHQL\$4a5AACHav-NH2
36	Ac-F\$4rn6HHWHQL\$4a5RRDa-NH2
37	Ac-F\$4rn6HHWHQL\$4a5HRAibG-NH2
38	Ac-F\$4rn6AYWAQL\$4a5HHNleL-NH2
39	Ac-F\$4rn6AYWSAL\$4a5HQNle-NH2
40	Ac-F\$4rn6AYWVQL\$4a5QHChg1-NH2
41	Ac-F\$4rn6AYWTAL\$4a5QQNlev-NH2
42	Ac-F\$4rn6AYWYQL\$4a5HAibAa-NH2
43	Ac-LTF\$4rn6AYWAQL\$4a5HHLA-NH2
44	Ac-LTF\$4rn6AYWAQL\$4a5HHLA-NH2
45	Ac-LTF\$4rn6AYWAQL\$4a5HQNlev-NH2

SP-	Sequence
46	Ac-LTF\$4rn6AYWAQL\$4a5HQNlev-NH2
47	Ac-LTF\$4rn6AYWAQL\$4a5QQMl-NH2
48	Ac-LTF\$4rn6AYWAQL\$4a5QQMl-NH2
49	Ac-LTF\$4rn6AYWAQL\$4a5HAibhLV-NH2
50	Ac-LTF\$4rn6AYWAQL\$4a5AHFA-NH2
51	Ac-HLTF\$4rn6HHWHQL\$4a5AANlel-NH2
52	Ac-DLTF\$4rn6HHWHQL\$4a5RRLa-NH2
53	Ac-HHTF\$4rn6HHWHQL\$4a5AAMv-NH2
54	Ac-F\$4rn6HHWHQL\$4a5RRDA-NH2
55	Ac-F\$4rn6HHWHQL\$4a5HRFCha-NH2
56	Ac-F\$4rn6AYWEAL\$4a5AA-NHAm
57	Ac-F\$4rn6AYWEAL\$4a5AA-NHiAm
58	Ac-F\$4rn6AYWEAL\$4a5AA-NHnPr3Ph
59	Ac-F\$4rn6AYWEAL\$4a5AA-NHnBu33Me
60	Ac-F\$4rn6AYWEAL\$4a5AA-NHnPr
61	Ac-F\$4rn6AYWEAL\$4a5AA-NHnEt2Ch
62	Ac-F\$4rn6AYWEAL\$4a5AA-NHnEt2Cp
63	Ac-F\$4rn6AYWEAL\$4a5AA-NHHex
64	Ac-LTF\$4rn6AYWAQL\$4a5AAIA-NH2
65	Ac-LTF\$4rn6AYWAQL\$4a5AAIA-NH2
66	Ac-LTF\$4rn6AYWAAL\$4a5AAMA-NH2
67	Ac-LTF\$4rn6AYWAAL\$4a5AAMA-NH2
68	Ac-LTF\$4rn6AYWAQL\$4a5AANleA-NH2
69	Ac-LTF\$4rn6AYWAQL\$4a5AANleA-NH2
70	Ac-LTF\$4rn6AYWAQL\$4a5AAIa-NH2
71	Ac-LTF\$4rn6AYWAQL\$4a5AAIa-NH2
72	Ac-LTF\$4rn6AYWAAL\$4a5AAMa-NH2
73	Ac-LTF\$4rn6AYWAAL\$4a5AAMa-NH2
74	Ac-LTF\$4rn6AYWAQL\$4a5AANlea-NH2
75	Ac-LTF\$4rn6AYWAQL\$4a5AANlea-NH2
76	Ac-LTF\$4rn6AYWAAL\$4a5AAIv-NH2
77	Ac-LTF\$4rn6AYWAAL\$4a5AAIv-NH2
78	Ac-LTF\$4rn6AYWAQL\$4a5AAMv-NH2
79	Ac-LTF\$4rn6AYWAAL\$4a5AANlev-NH2
80	Ac-LTF\$4rn6AYWAAL\$4a5AANlev-NH2
81	Ac-LTF\$4rn6AYWAQL\$4a5AAIl-NH2
82	Ac-LTF\$4rn6AYWAQL\$4a5AAIl-NH2
83	Ac-LTF\$4rn6AYWAAL\$4a5AAMl-NH2
84	Ac-LTF\$4rn6AYWAQL\$4a5AANlel-NH2
85	Ac-LTF\$4rn6AYWAQL\$4a5AANlel-NH2
86	Ac-F\$4rn6AYWEAL\$4a5AAMA-NH2
87	Ac-F\$4rn6AYWEAL\$4a5AANleA-NH2
88	Ac-F\$4rn6AYWEAL\$4a5AAIa-NH2
89	Ac-F\$4rn6AYWEAL\$4a5AAMa-NH2
90	Ac-F\$4rn6AYWEAL\$4a5AANlea-NH2
91	Ac-F\$4rn6AYWEAL\$4a5AAIv-NH2
92	Ac-F\$4rn6AYWEAL\$4a5AAMv-NH2
93	Ac-F\$4rn6AYWEAL\$4a5AANlev-NH2
94	Ac-F\$4rn6AYWEAL\$4a5AAIl-NH2
95	Ac-F\$4rn6AYWEAL\$4a5AAMl-NH2
96	Ac-F\$4rn6AYWEAL\$4a5AANlel-NH2
97	Ac-F\$4rn6AYWEAL\$4a5AANlel-NH2
98	Ac-LTF\$4rn6AY6clWAQL\$4a5SAA-NH2
99	Ac-LTF\$4rn6AY6clWAQL\$4a5SAA-NH2
100	Ac-WTF\$4rn6FYWSQL\$4a5AVAA-NH2
101	Ac-WTF\$4rn6FYWSQL\$4a5AVAA-NH2
102	Ac-WTF\$4rn6VYWSQL\$4a5AVA-NH2
103	Ac-WTF\$4rn6VYWSQL\$4a5AVA-NH2

SP-	Sequence
104	Ac-WTF\$4rn6FYWSQL\$4a5SAAa-NH2
105	Ac-WTF\$4rn6FYWSQL\$4a5SAAa-NH2
106	Ac-WTF\$4rn6VYWSQL\$4a5AVAAA-NH2
107	Ac-WTF\$4rn6VYWSQL\$4a5AVAAA-NH2
108	Ac-LTF\$4rn6AYWAQL\$4a5AVG-NH2
109	Ac-LTF\$4rn6AYWAQL\$4a5AVG-NH2
110	Ac-LTF\$4rn6AYWAQL\$4a5AVQ-NH2
111	Ac-LTF\$4rn6AYWAQL\$4a5AVQ-NH2
112	Ac-LTF\$4rn6AYWAQL\$4a5SAa-NH2
113	Ac-LTF\$4rn6AYWAQL\$4a5SAa-NH2
114	Ac-LTF\$4rn6AYWAQhL\$4a5SAA-NH2
115	Ac-LTF\$4rn6AYWAQhL\$4a5SAA-NH2
116	Ac-LTF\$4rn6AYWEQLStSA\$4a5-NH2
117	Ac-LTF\$4rn6AYWAQL\$4a5SLA-NH2
118	Ac-LTF\$4rn6AYWAQL\$4a5SLA-NH2
119	Ac-LTF\$4rn6AYWAQL\$4a5SWA-NH2
120	Ac-LTF\$4rn6AYWAQL\$4a5SWA-NH2
121	Ac-LTF\$4rn6AYWAQL\$4a5SVS-NH2
122	Ac-LTF\$4rn6AYWAQL\$4a5SAS-NH2
123	Ac-LTF\$4rn6AYWAQL\$4a5SVG-NH2
124	Ac-ETF\$4rn6VYWAQL\$4a5SAa-NH2
125	Ac-ETF\$4rn6VYWAQL\$4a5SAA-NH2
126	Ac-ETF\$4rn6VYWAQL\$4a5SVA-NH2
127	Ac-ETF\$4rn6VYWAQL\$4a5SLA-NH2
128	Ac-ETF\$4rn6VYWAQL\$4a5SWA-NH2
129	Ac-ETF\$4rn6KYWAQL\$4a5SWA-NH2
130	Ac-ETF\$4rn6VYWAQL\$4a5SVS-NH2
131	Ac-ETF\$4rn6VYWAQL\$4a5SAS-NH2
132	Ac-ETF\$4rn6VYWAQL\$4a5SVG-NH2
133	Ac-LTF\$4rn6VYWAQL\$4a5SSa-NH2
134	Ac-ETF\$4rn6VYWAQL\$4a5SSa-NH2
135	Ac-LTF\$4rn6VYWAQL\$4a5SNa-NH2
136	Ac-ETF\$4rn6VYWAQL\$4a5SNa-NH2
137	Ac-LTF\$4rn6VYWAQL\$4a5SAa-NH2
138	Ac-LTF\$4rn6VYWAQL\$4a5SVA-NH2
139	Ac-LTF\$4rn6VYWAQL\$4a5SVA-NH2
140	Ac-LTF\$4rn6VYWAQL\$4a5SWA-NH2
141	Ac-LTF\$4rn6VYWAQL\$4a5SVS-NH2
142	Ac-LTF\$4rn6VYWAQL\$4a5SVS-NH2
143	Ac-LTF\$4rn6VYWAQL\$4a5SAS-NH2
144	Ac-LTF\$4rn6VYWAQL\$4a5SAS-NH2
145	Ac-LTF\$4rn6VYWAQL\$4a5SVG-NH2
146	Ac-LTF\$4rn6VYWAQL\$4a5SVG-NH2
147	Ac-LTF\$4rn6EYWAQCha\$4a5SAA-NH2
148	Ac-LTF\$4rn6EYWAQCha\$4a5SAA-NH2
149	Ac-LTF\$4rn6EYWAQCpg\$4a5SAA-NH2
150	Ac-LTF\$4rn6EYWAQCpg\$4a5SAA-NH2
151	Ac-LTF\$4rn6EYWAQF\$4a5SAA-NH2
152	Ac-LTF\$4rn6EYWAQF\$4a5SAA-NH2
153	Ac-LTF\$4rn6EYWAQCb\$4a5SAA-NH2
154	Ac-LTF\$4rn6EYWAQCb\$4a5SAA-NH2
155	Ac-LTF3Cl\$4rn6EYWAQL\$4a5SAA-NH2
156	Ac-LTF3Cl\$4rn6EYWAQL\$4a5SAA-NH2
157	Ac-LTF34F2\$4rn6EYWAQL\$4a5SAA-NH2
158	Ac-LTF34F2\$4rn6EYWAQL\$4a5SAA-NH2
159	Ac-LTF34F2\$4rn6EYWAQhL\$4a5SAA-NH2
160	Ac-LTF34F2\$4rn6EYWAQhL\$4a5SAA-NH2
161	Ac-ETF\$4rn6EYWAQL\$4a5SAA-NH2

SP-	Sequence
162	Ac-LTF\$4rn6AYWVQL\$4a5SAA-NH2
163	Ac-LTF\$4rn6AHWAQL\$4a5SAA-NH2
164	Ac-LTF\$4rn6AEWAQL\$4a5SAA-NH2
165	Ac-LTF\$4rn6ASWAQL\$4a5SAA-NH2
166	Ac-LTF\$4rn6AEWAQL\$4a5SAA-NH2
167	Ac-LTF\$4rn6ASWAQL\$4a5SAA-NH2
168	Ac-LTF\$4rn6AF4coohWAQL\$4a5SAA-NH2
169	Ac-LTF\$4rn6AF4coohWAQL\$4a5SAA-NH2
170	Ac-LTF\$4rn6AHWAQL\$4a5AAIa-NH2
171	Ac-LTF\$4rn6FYWAQL\$4a5AAIa-NH2
172	Ac-ITF\$4rn6EHWAQL\$4a5AAIa-NH2
173	Ac-ITF\$4rn6EHWAQL\$4a5AAIa-NH2
174	Ac-ETF\$4rn6EHWAQL\$4a5AAIa-NH2
175	Ac-ETF\$4rn6EHWAQL\$4a5AAIa-NH2
176	Ac-LTF\$4rn6AHWVQL\$4a5AAIa-NH2
177	Ac-ITF\$4rn6FYWVQL\$4a5AAIa-NH2
178	Ac-ITF\$4rn6EYWVQL\$4a5AAIa-NH2
179	Ac-ITF\$4rn6EHWVQL\$4a5AAIa-NH2
180	Ac-LTF\$4rn6AEWAQL\$4a5AAIa-NH2
181	Ac-LTF\$4rn6AF4coohWAQL\$4a5AAIa-NH2
182	Ac-LTF\$4rn6AF4coohWAQL\$4a5AAIa-NH2
183	Ac-LTF\$4rn6AHWAQL\$4a5AHFA-NH2
184	Ac-ITF\$4rn6FYWAQL\$4a5AHFA-NH2
185	Ac-ITF\$4rn6FYWAQL\$4a5AHFA-NH2
186	Ac-ITF\$4rn6FHWAQL\$4a5AEFA-NH2
187	Ac-ITF\$4rn6FHWAQL\$4a5AEFA-NH2
188	Ac-ITF\$4rn6EHWAQL\$4a5AHFA-NH2
189	Ac-ITF\$4rn6EHWAQL\$4a5AHFA-NH2
190	Ac-LTF\$4rn6AHWVQL\$4a5AHFA-NH2
191	Ac-ITF\$4rn6FYWVQL\$4a5AHFA-NH2
192	Ac-ITF\$4rn6EYWVQL\$4a5AHFA-NH2
193	Ac-ITF\$4rn6EHWVQL\$4a5AHFA-NH2
194	Ac-ITF\$4rn6EHWVQL\$4a5AHFA-NH2
195	Ac-ETF\$4rn6EYWAAL\$4a5SAA-NH2
196	Ac-LTF\$4rn6AYWVAL\$4a5SAA-NH2
197	Ac-LTF\$4rn6AHWAAL\$4a5SAA-NH2
198	Ac-LTF\$4rn6AEWAAL\$4a5SAA-NH2
199	Ac-LTF\$4rn6AEWAAL\$4a5SAA-NH2
200	Ac-LTF\$4rn6ASWAAL\$4a5SAA-NH2
201	Ac-LTF\$4rn6ASWAAL\$4a5SAA-NH2
202	Ac-LTF\$4rn6AYWAAL\$4a5AAIa-NH2
203	Ac-LTF\$4rn6AYWAAL\$4a5AAIa-NH2
204	Ac-LTF\$4rn6AYWAAL\$4a5AHFA-NH2
205	Ac-LTF\$4rn6EHWAQL\$4a5AHIa-NH2
206	Ac-LTF\$4rn6EHWAQL\$4a5AHIa-NH2
207	Ac-LTF\$4rn6AHWAQL\$4a5AHIa-NH2
208	Ac-LTF\$4rn6EYWAQL\$4a5AHIa-NH2
209	Ac-LTF\$4rn6AYWAQL\$4a5AAFa-NH2
210	Ac-LTF\$4rn6AYWAQL\$4a5AAFa-NH2
211	Ac-LTF\$4rn6AYWAQL\$4a5AAWa-NH2
212	Ac-LTF\$4rn6AYWAQL\$4a5AAVa-NH2
213	Ac-LTF\$4rn6AYWAQL\$4a5AAVa-NH2
214	Ac-LTF\$4rn6AYWAQL\$4a5AALa-NH2
215	Ac-LTF\$4rn6AYWAQL\$4a5AALa-NH2
216	Ac-LTF\$4rn6EYWAQL\$4a5AAIa-NH2
217	Ac-LTF\$4rn6EYWAQL\$4a5AAIa-NH2
218	Ac-LTF\$4rn6EYWAQL\$4a5AAFa-NH2
219	Ac-LTF\$4rn6EYWAQL\$4a5AAFa-NH2

SP-	Sequence
220	Ac-LTF\$4rn6EYWAQL\$4a5AAVa-NH2
221	Ac-LTF\$4rn6EYWAQL\$4a5AAVa-NH2
222	Ac-LTF\$4rn6EHWAQL\$4a5AAIa-NH2
223	Ac-LTF\$4rn6EHWAQL\$4a5AAIa-NH2
224	Ac-LTF\$4rn6EHWAQL\$4a5AAWa-NH2
225	Ac-LTF\$4rn6EHWAQL\$4a5AAWa-NH2
226	Ac-LTF\$4rn6EHWAQL\$4a5AALa-NH2
227	Ac-LTF\$4rn6EHWAQL\$4a5AALa-NH2
228	Ac-ETF\$4rn6EHWVQL\$4a5AALa-NH2
229	Ac-LTF\$4rn6AYWAQL\$4a5AAAa-NH2
230	Ac-LTF\$4rn6AYWAQL\$4a5AAAa-NH2
231	Ac-LTF\$4rn6AYWAQL\$4a5AAAibA-NH2
232	Ac-LTF\$4rn6AYWAQL\$4a5AAAibA-NH2
233	Ac-LTF\$4rn6AYWAQL\$4a5AAAAa-NH2
234	Ac-LTF\$r5AYWAQL\$4a5s8AAIa-NH2
235	Ac-LTF\$r5AYWAQL\$4a5s8SAA-NH2
236	Ac-LTF\$4rn6AYWAQCba\$4a5AANleA-NH2
237	Ac-ETF\$4rn6AYWAQCba\$4a5AANleA-NH2
238	Ac-LTF\$4rn6EYWAQCba\$4a5AANleA-NH2
239	Ac-LTF\$4rn6AYWAQCba\$4a5AWNleA-NH2
240	Ac-ETF\$4rn6AYWAQCba\$4a5AWNleA-NH2
241	Ac-LTF\$4rn6EYWAQCba\$4a5AWNleA-NH2
242	Ac-LTF\$4rn6EYWAQCba\$4a5SAFA-NH2
243	Ac-LTF34F2\$4rn6EYWAQCba\$4a5SANleA-NH2
244	Ac-LTF\$4rn6EF4coohWAQCba\$4a5SANleA-NH2
245	Ac-LTF\$4rn6EYWSQCba\$4a5SANleA-NH2
246	Ac-LTF\$4rn6EYWWQCba\$4a5SANleA-NH2
247	Ac-LTF\$4rn6EYWAQCba\$4a5AAIa-NH2
248	Ac-LTF34F2\$4rn6EYWAQCba\$4a5AAIa-NH2
249	Ac-LTF\$4rn6EF4coohWAQCba\$4a5AAIa-NH2
250	Pam-ETF\$4rn6EYWAQCba\$4a5SAA-NH2
251	Ac-LThF\$4rn6EFWAQCba\$4a5SAA-NH2
252	Ac-LTA\$4rn6EYWAQCba\$4a5SAA-NH2
253	Ac-LTF\$4rn6EYAAQCba\$4a5SAA-NH2
254	Ac-LTF\$4rn6EY2NalaQCba\$4a5SAA-NH2
255	Ac-LTF\$4rn6AYWAQCba\$4a5SAA-NH2
256	Ac-LTF\$4rn6EYWAQCba\$4a5SAF-NH2
257	Ac-LTF\$4rn6EYWAQCba\$4a5SAFa-NH2
258	Ac-LTF\$4rn6AYWAQCba\$4a5SAF-NH2
259	Ac-LTF34F2\$4rn6AYWAQCba\$4a5SAF-NH2
260	Ac-LTF\$4rn6AF4coohWAQCba\$4a5SAF-NH2
261	Ac-LTF\$4rn6EY6clWAQCba\$4a5SAF-NH2
262	Ac-LTF\$4rn6AYWSQCba\$4a5SAF-NH2
263	Ac-LTF\$4rn6AYWWQCba\$4a5SAF-NH2
264	Ac-LTF\$4rn6AYWAQCba\$4a5AAIa-NH2
265	Ac-LTF34F2\$4rn6AYWAQCba\$4a5AAIa-NH2
266	Ac-LTF\$4rn6AY6clWAQCba\$4a5AAIa-NH2
267	Ac-LTF\$4rn6AF4coohWAQCba\$4a5AAIa-NH2
268	Ac-LTF\$4rn6EYWAQCba\$4a5AAFa-NH2
269	Ac-LTF\$4rn6EYWAQCba\$4a5AAFa-NH2
270	Ac-ETF\$4rn6AYWAQCba\$4a5AWNlea-NH2
271	Ac-LTF\$4rn6EYWAQCba\$4a5AWNlea-NH2
272	Ac-ETF\$4rn6EYWAQCba\$4a5AWNlea-NH2
273	Ac-ETF\$4rn6EYWAQCba\$4a5AWNlea-NH2
274	Ac-LTF\$4rn6AYWAQCba\$4a5SAFa-NH2
275	Ac-LTF\$4rn6AYWAQCba\$4a5SAFa-NH2
276	Ac-ETF\$4rn6AYWAQL\$4a5AWNlea-NH2
277	Ac-LTF\$4rn6EYWAQL\$4a5AWNlea-NH2

SP-	Sequence
278	Ac-ETF\$4rn6EYWAQL\$4a5AWNlea-NH2
279	Dmaac-LTF\$4rn6EYWAQhL\$4a5SAA-NH2
280	Hexac-LTF\$4rn6EYWAQhL\$4a5SAA-NH2
281	Napac-LTF\$4rn6EYWAQhL\$4a5SAA-NH2
282	Decac-LTF\$4rn6EYWAQhL\$4a5SAA-NH2
283	Admac-LTF\$4rn6EYWAQhL\$4a5SAA-NH2
284	Tmac-LTF\$4rn6EYWAQhL\$4a5SAA-NH2
285	Pam-LTF\$4rn6EYWAQhL\$4a5SAA-NH2
286	Ac-LTF\$4rn6AYWAQCba\$4a5AANlea-NH2
287	Ac-LTF34F2\$4rn6EYWAQCba\$4a5AAIa-NH2
288	Ac-LTF34F2\$4rn6EYWAQCba\$4a5SAA-NH2
289	Ac-LTF34F2\$4rn6EYWAQCba\$4a5SAA-NH2
290	Ac-LTF\$4rn6EF4coohWAQCba\$4a5SAA-NH2
291	Ac-LTF\$4rn6EF4coohWAQCba\$4a5SAA-NH2
292	Ac-LTF\$4rn6EYWSQCba\$4a5SAA-NH2
293	Ac-LTF\$4rn6EYWSQCba\$4a5SAA-NH2
294	Ac-LTF\$4rn6EYWAQhL\$4a5SAA-NH2
295	Ac-LTF\$4rn6AYWAQhL\$4a5SAF-NH2
296	Ac-LTF\$4rn6AYWAQhL\$4a5SAF-NH2
297	Ac-LTF34F2\$4rn6AYWAQhL\$4a5SAA-NH2
298	Ac-LTF34F2\$4rn6AYWAQhL\$4a5SAA-NH2
299	Ac-LTF\$4rn6AF4coohWAQhL\$4a5SAA-NH2
300	Ac-LTF\$4rn6AF4coohWAQhL\$4a5SAA-NH2
301	Ac-LTF\$4rn6AYWSQhL\$4a5SAA-NH2
302	Ac-LTF\$4rn6AYWSQhL\$4a5SAA-NH2
303	Ac-LTF\$4rn6EYWAQL\$4a5AANlea-NH2
304	Ac-LTF34F2\$4rn6AYWAQL\$4a5AANlea-NH2
305	Ac-LTF\$4rn6AF4coohWAQL\$4a5AANlea-NH2
306	Ac-LTF\$4rn6AYWSQL\$4a5AANlea-NH2
307	Ac-LTF34F2\$4rn6AYWAQhL\$4a5AANlea-NH2
308	Ac-LTF34F2\$4rn6AYWAQhL\$4a5AANlea-NH2
309	Ac-LTF\$4rn6AF4coohWAQhL\$4a5AANlea-NH2
310	Ac-LTF\$4rn6AF4coohWAQhL\$4a5AANlea-NH2
311	Ac-LTF\$4rn6AYWSQhL\$4a5AANlea-NH2
312	Ac-LTF\$4rn6AYWSQhL\$4a5AANlea-NH2
313	Ac-LTF\$4rn6AYWAQhL\$4a5AAAAa-NH2
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315	Ac-LTF\$4rn6AYWAQL\$4a5AAAAa-NH2
316	Ac-LTF\$4rn6AYWAQL\$4a5AAAAa-NH2
317	Ac-LTF\$4rn6AYWAQL\$4a5AAAAa-NH2
318	Ac-LTF\$4rn6EYWAQhL\$4a5AANlea-NH2
319	Ac-AATF\$4rn6AYWAQL\$4a5AANlea-NH2
320	Ac-LTF\$4rn6AYWAQL\$4a5AANleAA-NH2
321	Ac-ALTF\$4rn6AYWAQL\$4a5AANleAA-NH2
322	Ac-LTF\$4rn6AYWAQCba\$4a5AANleAA-NH2
323	Ac-LTF\$4rn6AYWAQhL\$4a5AANleAA-NH2
324	Ac-LTF\$4rn6EYWAQCba\$4a5SAAA-NH2
325	Ac-LTF\$4rn6EYWAQCba\$4a5SAAA-NH2
326	Ac-LTF\$4rn6EYWAQCba\$4a5SAAA-NH2
327	Ac-LTF\$4rn6EYWAQCba\$4a5SAAA-NH2
328	Ac-ALTF\$4rn6EYWAQCba\$4a5SAA-NH2
329	Ac-ALTF\$4rn6EYWAQCba\$4a5SAAA-NH2
330	Ac-ALTF\$4rn6EYWAQCba\$4a5SAA-NH2
331	Ac-LTF\$4rn6EYWAQL\$4a5AAAAa-NH2
332	Ac-LTF\$4rn6EY6clWAQCba\$4a5SAA-NH2
333	Ac-LTF\$4rn6EF4cooh6clWAQCba\$4a5SANlea-NH2
334	Ac-LTF\$4rn6EF4cooh6clWAQCba\$4a5SANlea-

SP-	Sequence
	NH2
335	Ac-LTF\$4rn6EF4cooh6clWAQCba\$4a5AAIa-NH2
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337	Ac-LTF\$4rn6AY6clWAQL\$4a5AAAAAa-NH2
338	Ac-LTF\$4rn6AY6clWAQL\$4a5AAAAAa-NH2
339	Ac-F\$4rn6AY6clWEAL\$4a5AAAAAAa-NH2
340	Ac-ETF\$4rn6EYWAQL\$4a5AAAAAAa-NH2
341	Ac-ETF\$4rn6EYWAQL\$4a5AAAAAAa-NH2
342	Ac-LTF\$4rn6EYWAQL\$4a5AAAAAAa-NH2
343	Ac-LTF\$4rn6EYWAQL\$4a5AAAAAAa-NH2
344	Ac-LTF\$4rn6AYWAQL\$4a5AANleAAa-NH2
345	Ac-LTF\$4rn6AYWAQL\$4a5AANleAAa-NH2
346	Ac-LTF\$4rn6EYWAQCba\$4a5AAAAAAa-NH2
347	Ac-LTF\$4rn6EYWAQCba\$4a5AAAAAAa-NH2
348	Ac-LTF\$4rn6EF4coohWAQCba\$4a5AAAAAAa-NH2
349	Ac-LTF\$4rn6EF4coohWAQCba\$4a5AAAAAAa-NH2
350	Ac-LTF\$4rn6EYWSQCba\$4a5AAAAAAa-NH2
351	Ac-LTF\$4rn6EYWSQCba\$4a5AAAAAAa-NH2
352	Ac-LTF\$4rn6EYWAQCba\$4a5SAAa-NH2
353	Ac-LTF\$4rn6EYWAQCba\$4a5SAAa-NH2
354	Ac-ALTF\$4rn6EYWAQCba\$4a5SAAa-NH2
355	Ac-ALTF\$4rn6EYWAQCba\$4a5SAAa-NH2
356	Ac-ALTF\$4rn6EYWAQCba\$4a5SAAa-NH2
357	Ac-ALTF\$4rn6EYWAQCba\$4a5SAAa-NH2
358	Ac-AALTF\$4rn6EYWAQCba\$4a5SAAa-NH2
359	Ac-AALTF\$4rn6EYWAQCba\$4a5SAAa-NH2
360	Ac-RTF\$4rn6EYWAQCba\$4a5SAA-NH2
361	Ac-LRF\$4rn6EYWAQCba\$4a5SAA-NH2
362	Ac-LTF\$4rn6EYWRQCba\$4a5SAA-NH2
363	Ac-LTF\$4rn6EYWARCba\$4a5SAA-NH2
364	Ac-LTF\$4rn6EYWAQCba\$4a5RAA-NH2
365	Ac-LTF\$4rn6EYWAQCba\$4a5SRA-NH2
366	Ac-LTF\$4rn6EYWAQCba\$4a5SAR-NH2
367	5-FAM-BaLTF\$4rn6EYWAQCba\$4a5SAA-NH2
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371	Ac-AAF\$4rn6EYWAQL\$4a5AANleA-NH2
372	Ac-AAAF\$4rn6EYWAQL\$4a5AANleA-NH2
373	Ac-AAAAF\$4rn6EYWAQL\$4a5AANleA-NH2
374	Ac-AATF\$4rn6EYWAQL\$4a5AANleA-NH2
375	Ac-AALTF\$4rn6EYWAQL\$4a5AANleA-NH2
376	Ac-AAALTF\$4rn6EYWAQL\$4a5AANleA-NH2
377	Ac-LTF\$4rn6EYWAQL\$4a5AANleAA-NH2
378	Ac-ALTF\$4rn6EYWAQL\$4a5AANleAA-NH2
379	Ac-AALTF\$4rn6EYWAQL\$4a5AANleAA-NH2
380	Ac-LTF\$4rn6EYWAQCba\$4a5AANleAA-NH2
381	Ac-LTF\$4rn6EYWAQhL\$4a5AANleAA-NH2
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383	Ac-LTF\$4rn6ANmYWAQL\$4a5AANleA-NH2
384	Ac-LTF\$4rn6ANmYWAQL\$4a5AANleA-NH2
385	Ac-LTF\$4rn6AYNmWAQL\$4a5AANleA-NH2
386	Ac-LTF\$4rn6AYNmWAQL\$4a5AANleA-NH2
387	Ac-LTF\$4rn6AYAmwAQL\$4a5AANleA-NH2
388	Ac-LTF\$4rn6AYAmwAQL\$4a5AANleA-NH2
389	Ac-LTF\$4rn6AYWAibQL\$4a5AANleA-NH2

SP-	Sequence
390	Ac-LTF\$4rn6AYWAibQL\$4a5AANleA-NH2
391	Ac-LTF\$4rn6AYWAQL\$4a5AAibNleA-NH2
392	Ac-LTF\$4rn6AYWAQL\$4a5AAibNleA-NH2
393	Ac-LTF\$4rn6AYWAQL\$4a5AaNleA-NH2
394	Ac-LTF\$4rn6AYWAQL\$4a5AaNleA-NH2
395	Ac-LTF\$4rn6AYWAQL\$4a5ASarNleA-NH2
396	Ac-LTF\$4rn6AYWAQL\$4a5ASarNleA-NH2
397	Ac-LTF\$4rn6AYWAQL\$4a5AANleAib-NH2
398	Ac-LTF\$4rn6AYWAQL\$4a5AANleAib-NH2
399	Ac-LTF\$4rn6AYWAQL\$4a5AANleNmA-NH2
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401	Ac-LTF\$4rn6AYWAQL\$4a5AANleSar-NH2
402	Ac-LTF\$4rn6AYWAQL\$4a5AANleSar-NH2
403	Ac-LTF\$4rn6AYWAQL\$4a5AANleAAib-NH2
404	Ac-LTF\$4rn6AYWAQL\$4a5AANleAAib-NH2
405	Ac-LTF\$4rn6AYWAQL\$4a5AANleANmA-NH2
406	Ac-LTF\$4rn6AYWAQL\$4a5AANleANmA-NH2
407	Ac-LTF\$4rn6AYWAQL\$4a5AANleAa-NH2
408	Ac-LTF\$4rn6AYWAQL\$4a5AANleAa-NH2
409	Ac-LTF\$4rn6AYWAQL\$4a5AANleASar-NH2
410	Ac-LTF\$4rn6AYWAQL\$4a5AANleASar-NH2
413	Ac-LTF\$4rn6Cou4YWAQL\$4a5AANleA-NH2
414	Ac-LTF\$4rn6Cou4YWAQL\$4a5AANleA-NH2
415	Ac-LTF\$4rn6AYWCou4QL\$4a5AANleA-NH2
416	Ac-LTF\$4rn6AYWAQL\$4a5Cou4ANleA-NH2
417	Ac-LTF\$4rn6AYWAQL\$4a5Cou4ANleA-NH2
418	Ac-LTF\$4rn6AYWAQL\$4a5ACou4NleA-NH2
419	Ac-LTF\$4rn6AYWAQL\$4a5ACou4NleA-NH2
420	Ac-LTF\$4rn6AYWAQL\$4a5AANleA-OH
421	Ac-LTF\$4rn6AYWAQL\$4a5AANleA-OH
422	Ac-LTF\$4rn6AYWAQL\$4a5AANleA-NHnPr
423	Ac-LTF\$4rn6AYWAQL\$4a5AANleA-NHnPr
424	Ac-LTF\$4rn6AYWAQL\$4a5AANleA-NHnBu33Me
425	Ac-LTF\$4rn6AYWAQL\$4a5AANleA-NHnBu33Me
426	Ac-LTF\$4rn6AYWAQL\$4a5AANleA-NHHex
427	Ac-LTF\$4rn6AYWAQL\$4a5AANleA-NHHex
428	Ac-LTA\$4rn6AYWAQL\$4a5AANleA-NH2
429	Ac-LThL\$4rn6AYWAQL\$4a5AANleA-NH2
430	Ac-LTF\$4rn6AYAAQL\$4a5AANleA-NH2
431	Ac-LTF\$4rn6AY2Na1AQL\$4a5AANleA-NH2
432	Ac-LTF\$4rn6EYWCou4QCba\$4a5SAA-NH2
433	Ac-LTF\$4rn6EYWCou7QCba\$4a5SAA-NH2
435	Dmaac-LTF\$4rn6EYWAQCba\$4a5SAA-NH2
436	Dmaac-LTF\$4rn6AYWAQL\$4a5AAAAAa-NH2
437	Dmaac-LTF\$4rn6AYWAQL\$4a5AAAAAa-NH2
438	Dmaac-LTF\$4rn6EYWAQL\$4a5AAAAAa-NH2
439	Dmaac-LTF\$4rn6EYWAQL\$4a5AAAAAa-NH2
440	Dmaac-LTF\$4rn6EF4coohWAQCba\$4a5AAIa-NH2
441	Dmaac-LTF\$4rn6EF4coohWAQCba\$4a5AAIa-NH2
442	Dmaac-LTF\$4rn6AYWAQL\$4a5AANleA-NH2
443	Dmaac-LTF\$4rn6AYWAQL\$4a5AANleA-NH2
444	Ac-LTF\$4rn6AYWAQL\$4a5AANleA-NH2
445	Ac-LTF\$4rn6EYWAQL\$4a5AAAAAa-NH2
446	Cou6BaLTF\$4rn6EYWAQhL\$4a5SAA-NH2
447	Cou8BaLTF\$4rn6EYWAQhL\$4a5SAA-NH2
448	Ac-LTF4I\$4rn6EYWAQL\$4a5AAAAAa-NH2

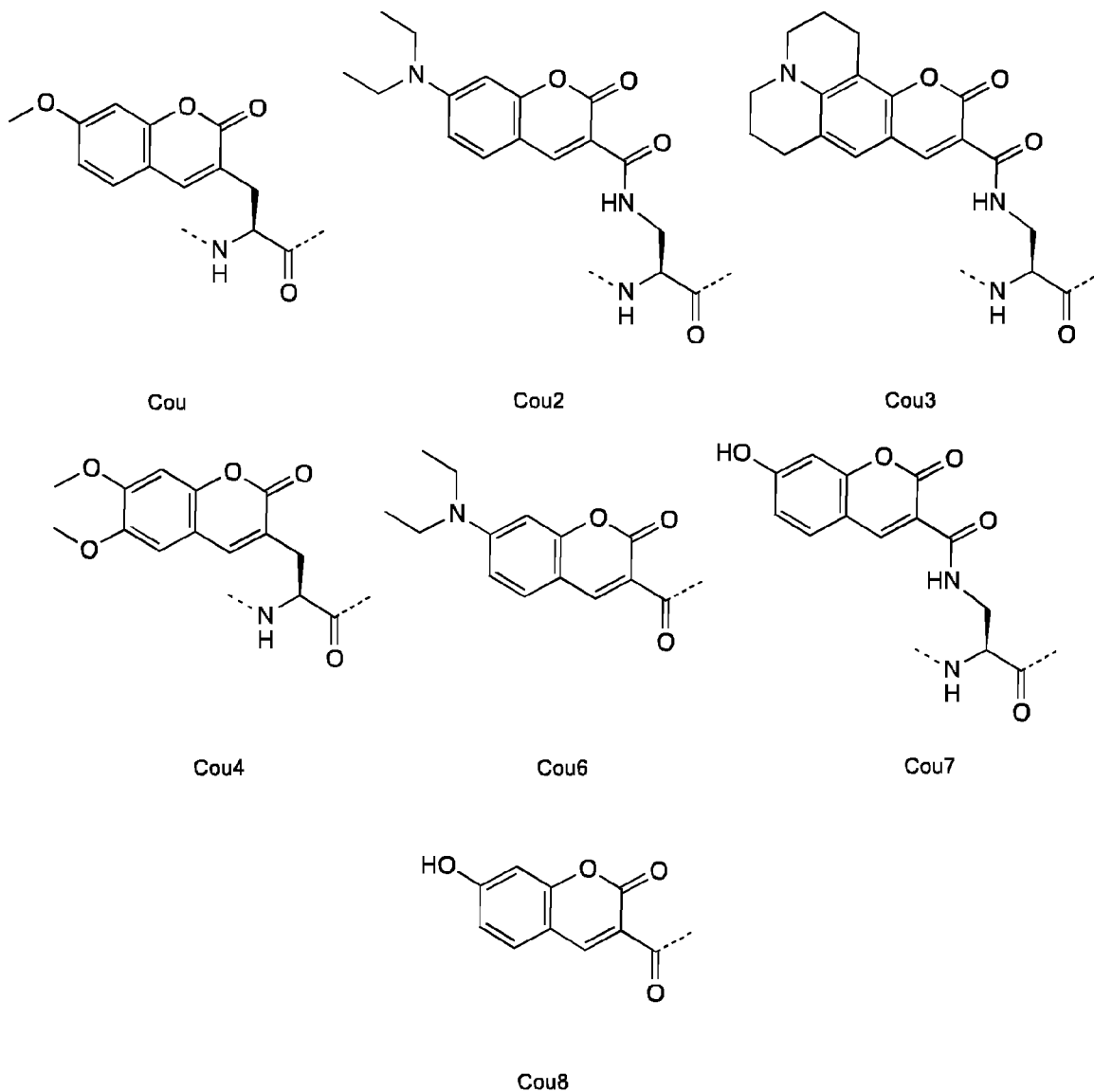
Table 4a

SP	Sequence	Exact Mass	Found Mass	Calc (M+1)/1	Calc (M+2)/2	Calc (M+3)/3
449	Ac-LTF\$4rn6AYWAQL\$4a5AANleA-NH2	1812.01	907.89	1813.02	907.01	605.01
450	Ac-LTF\$4rn6AYWAQL\$4a5AAAAAa-NH2	1912.04	957.75	1913.05	957.03	638.35
451	Ac-LTF\$4rn6EYWAQL\$4a5AAAAAa-NH2	1970.04	986.43	1971.05	986.03	657.69
452	Ac-LTF\$5rn6AYWAQL\$5a5AAAAAa-NH2	1912.04	957.38	1913.05	957.03	638.35
153	Ac-LTF\$4rn6EYWAQCba\$4a5SAA-NH2	1784.93	894.38	1785.94	893.47	595.98
454	Ac-LTF\$4rn4EYWAQCba\$4a5SAA-NH2	1756.89	880.05	1757.9	879.45	586.64
455	Ac-LTF\$4rn5EYWAQCba\$4a5SAA-NH2	1770.91	887.08	1771.92	886.46	591.31
456	Ac-LTF\$5rn6EYWAQCba\$5a5SAA-NH2	1784.92	894.11	1785.93	893.47	595.98
457	Ac-LTF\$4rn6EYWAQCba5I-\$4a5SAA-NH2	1910.82	957.01	1911.83	956.42	637.95
459	Ac-LTA\$5rn6EYWAQCba\$5a5SAA-NH2	1708.89	856	1709.9	855.45	570.64
460	Ac-LTA\$4rn6EYWAQCba\$4a5SAA-NH2	1708.89	856	1709.9	855.45	570.64
461	5-FAM-BaLTF\$4rn6EYWAQCba\$4a5SAA-NH2	2172	1087.81	2173.01	1087.01	725.01
462	5-FAM-BaLTA\$4rn6EYWAQCba\$4a5SAA-NH2	2095.97	1049.79	2096.98	1048.99	699.66
463	5-FAM-BaLTF\$5rn6EYWAQCba\$5a5SAA-NH2	2172	1087.53	2173.01	1087.01	725.01
464	5-FAM-BaLTA\$5rn6EYWAQCba\$5a5SAA-NH2	2095.97	1049.98	2096.98	1048.99	699.66
465	Ac-LTF\$4rn6EYWAQCba5Ph-\$4a5SAA-NH2	1675.87	932.31	1676.88	931.48	559.63
466	Ac-LTF\$4rn6EYWAQCba5Prp-\$4a5SAA-NH2	1675.87	914.46	1676.88	913.48	559.63
467	Ac-LTF\$4rn6AYWAAL\$4a5AAAAAa-NH2	1855.01		1856.02	928.51	619.34
468	Ac-LTF\$4rn6EYWAQCba5penNH2-\$4a5SAA-NH2	1675.87		1676.88	838.94	559.63
469	Ac-LTF\$4rn6EYWAQCba5BnzNH2-\$4a5SAA-NH2	1675.87		1676.88	838.94	559.63
470	Ac-LTF\$4rn6EYWAQCba5prpOMe-\$4a5SAA-NH2		929.17		928.48	
932	Ac-LTF\$5rn6EYWAQL4Me\$5a5AAAAAa-NH2	1926.05		1927.06	964.03	643.02
933	Ac-LTF\$5rn6EYWAQL4Ph\$5a5AAAAAa-NH2	1988.07		1989.07	995.04	663.70
934	Ac-LTF\$5rn6EYWAQCba4Me\$5a5SAANH2	1740.93		1741.94	871.48	581.32
935	Ac-LTF\$5rn6EYWAQCba4Ph\$5a5SAANH2	1802.95		1803.96	902.48	601.99

[00240] In the sequences shown above and elsewhere, the following abbreviations are used: “Nle” represents norleucine, “Aib” represents 2-aminoisobutyric acid, “Ac” represents acetyl, and “Pr” represents propionyl. Amino acids represented as “\$” are alpha-Me S5-pentenyl-alanine olefin amino acids connected by an all-carbon crosslinker comprising one double bond. Amino acids represented as “\$r5” are alpha-Me R5-pentenyl-alanine olefin amino acids connected by an all-carbon comprising one double bond. Amino acids represented as “\$s8” are alpha-Me S8-octenyl-alanine olefin amino acids connected by an all-carbon crosslinker comprising one double bond. Amino acids represented as “\$r8” are alpha-Me R8-octenyl-alanine olefin amino acids connected by an all-carbon crosslinker comprising one double bond. “Ahx” represents an aminocyclohexyl linker. The crosslinkers are linear all-carbon crosslinker comprising eight or eleven carbon atoms between the alpha carbons of each amino acid. Amino acids represented as “\$/” are alpha-Me S5-pentenyl-alanine olefin amino acids that are not connected by any crosslinker. Amino acids represented as “\$/r5” are alpha-Me R5-pentenyl-alanine olefin amino acids that are not connected by any crosslinker. Amino acids represented as “\$/s8” are alpha-Me S8-octenyl-alanine olefin amino acids that are not connected by any crosslinker. Amino acids represented as “\$/r8” are

alpha-Me R8-octenyl-alanine olefin amino acids that are not connected by any crosslinker. Amino acids represented as “Amw” are alpha-Me tryptophan amino acids. Amino acids represented as “Aml” are alpha-Me leucine amino acids. Amino acids represented as “Amf” are alpha-Me phenylalanine amino acids. Amino acids represented as “2ff” are 2-fluoro-phenylalanine amino acids. Amino acids represented as “3ff” are 3-fluoro-phenylalanine amino acids. Amino acids represented as “St” are amino acids comprising two pentenyl-alanine olefin side chains, each of which is crosslinked to another amino acid as indicated. Amino acids represented as “St//” are amino acids comprising two pentenyl-alanine olefin side chains that are not crosslinked. Amino acids represented as “%St” are amino acids comprising two pentenyl-alanine olefin side chains, each of which is crosslinked to another amino acid as indicated via fully saturated hydrocarbon crosslinks. Amino acids represented as “Ba” are beta-alanine. The lower-case character “e” or “z” within the designation of a crosslinked amino acid (e.g. “\$er8” or “\$zr8”) represents the configuration of the double bond (*E* or *Z*, respectively). In other contexts, lower-case letters such as “a” or “f” represent D amino acids (e.g. D-alanine, or D-phenylalanine, respectively). Amino acids designated as “NmW” represent N-methyltryptophan. Amino acids designated as “NmY” represent N-methyltyrosine. Amino acids designated as “NmA” represent N-methylalanine. Amino acids designated as “Sar” represent sarcosine. Amino acids designated as “Cha” represent cyclohexyl alanine. Amino acids designated as “Cpg” represent cyclopentyl glycine. Amino acids designated as “Chg” represent cyclohexyl glycine. Amino acids designated as “Cba” represent cyclobutyl alanine. Amino acids designated as “F4I” represent 4-iodo phenylalanine. Amino acids designated as “F3Cl” represent 3-chloro phenylalanine. Amino acids designated as “F4cooh” represent 4-carboxy phenylalanine. Amino acids designated as “F34F2” represent 3,4-difluoro phenylalanine. Amino acids designated as “6clW” represent 6-chloro tryptophan. The designation “iso1” or “iso2” indicates that the peptidomimetic macrocycle is a single isomer. “Ac3c” represents a aminocyclopropane carboxylic acid residue.

[00241] Amino acids designated as “Cou4”, “Cou6”, “Cou7” and “Cou8”, respectively, represent the following structures:



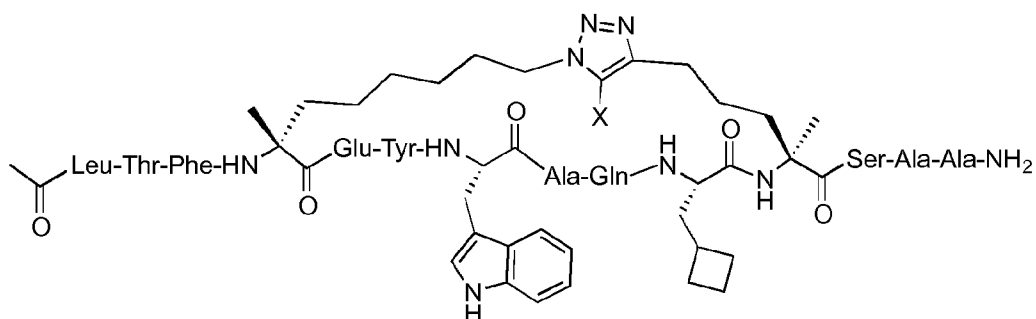
[00242] In some embodiments, a peptidomimetic macrocycle is obtained in more than one isomer, for example due to the configuration of a double bond within the structure of the crosslinker (*E* vs *Z*). Such isomers can or can not be separable by conventional chromatographic methods. In some embodiments, one isomer has improved biological properties relative to the other isomer. In one embodiment, an *E* crosslinker olefin isomer of a peptidomimetic macrocycle has better solubility, better target affinity, better in vivo or in vitro efficacy, higher helicity, or improved cell permeability relative to its *Z* counterpart. In another embodiment, a *Z* crosslinker olefin isomer of a peptidomimetic macrocycle has better solubility, better target affinity, better in vivo or in vitro efficacy, higher helicity, or improved cell permeability relative to its *E* counterpart.

[00243] Amino acids forming crosslinkers are represented according to the legend indicated below.

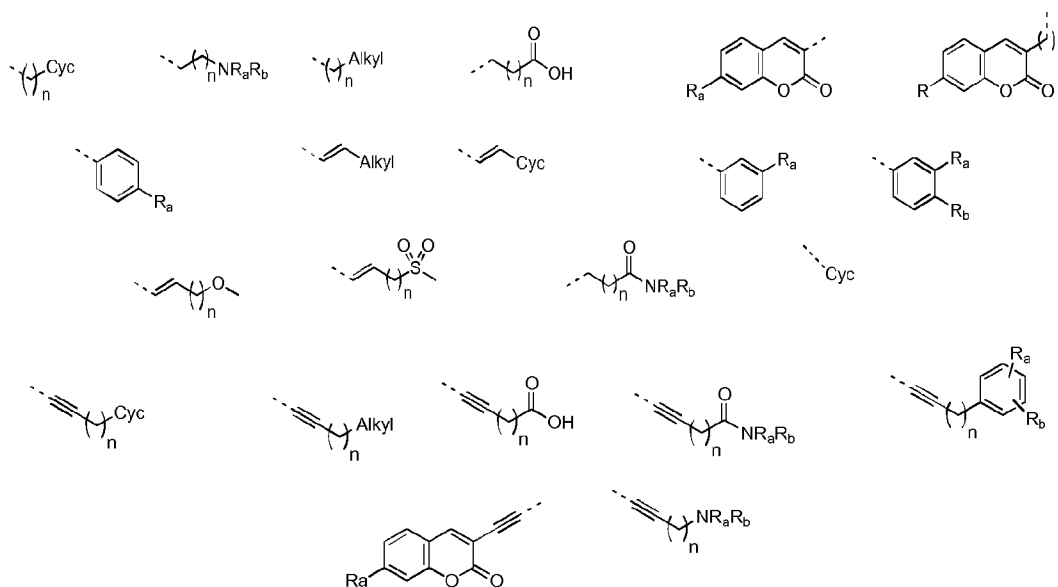
[00244] Stereochemistry at the alpha position of each amino acid is S unless otherwise indicated. Amino acids labeled “4Me” were prepared using an amino acid comprising an alkyne which was methyl-substituted (internal alkyne), resulting in triazole groups comprising a methyl group at the 4-position. Amino acids labeled “4Ph” were prepared using an amino acid comprising an alkyne which was phenyl-substituted (internal alkyne), resulting in triazole groups comprising a phenyl group at the 4-position. For azide amino acids, the number of carbon atoms indicated refers to the number of methylene units between the alpha carbon and the terminal azide. For alkyne amino acids, the number of carbon atoms indicated is the number of methylene units between the alpha position and the triazole moiety plus the two carbon atoms within the triazole group derived from the alkyne.

\$5n3	Alpha-Me azide 1,5 triazole (3 carbon)
#5n3	Alpha-H azide 1,5 triazole (3 carbon)
\$4a5	Alpha-Me alkyne 1,4 triazole (5 carbon)
\$4a6	Alpha-Me alkyne 1,4 triazole (6 carbon)
\$5a5	Alpha-Me alkyne 1,5 triazole (5 carbon)
\$5a6	Alpha-Me alkyne 1,5 triazole (6 carbon)
#4a5	Alpha-H alkyne 1,4 triazole (5 carbon)
#5a5	Alpha-H alkyne 1,5 triazole (5 carbon)
\$5n5	Alpha-Me azide 1,5 triazole (5 carbon)
\$5n6	Alpha-Me azide 1,5 triazole (6 carbon)
\$4n5	Alpha-Me azide 1,4 triazole (5 carbon)
\$4n6	Alpha-Me azide 1,4 triazole (6 carbon)
\$4ra5	Alpha-Me R-alkyne 1,4 triazole (5 carbon)
\$4ra6	Alpha-Me R-alkyne 1,4 triazole (6 carbon)
\$4rn4	Alpha-Me R-azide 1,4 triazole (4 carbon)
\$4rn5	Alpha-Me R-azide 1,4 triazole (5 carbon)
\$4rn6	Alpha-Me R-azide 1,4 triazole (6 carbon)
\$5rn5	Alpha-Me R-azide 1,5 triazole (5 carbon)
\$5ra5	Alpha-Me R-alkyne 1,5 triazole (5 carbon)
\$5ra6	Alpha-Me R-alkyne 1,5 triazole (6 carbon)
\$5rn6	Alpha-Me R-azide 1,5 triazole (6 carbon)
#5rn6	Alpha-H R-azide 1,5 triazole (6 carbon)
\$4rn5	Alpha-Me R-azide 1,4 triazole (5 carbon)
#4rn5	Alpha-H R-azide 1,4 triazole (5 carbon)
4Me\$5rn6	Alpha-Me R-azide 1,5 triazole (6 carbon); 4-Me substituted triazole
4Me\$5a5	Alpha-Me alkyne 1,5 triazole (5 carbon); 4-Me substituted triazole
4Ph\$5a5	Alpha-Me alkyne 1,5 triazole (5 carbon); 4-phenyl substituted triazole

[00245] Amino acids designated as “5I”, “5penNH₂”, “5BnzNH₂”, “5prpOMe”, “5Ph”, and “5prp”, refer to crosslinked amino acids of the type shown in the following exemplary peptidomimetic macrocycle of Formula I:

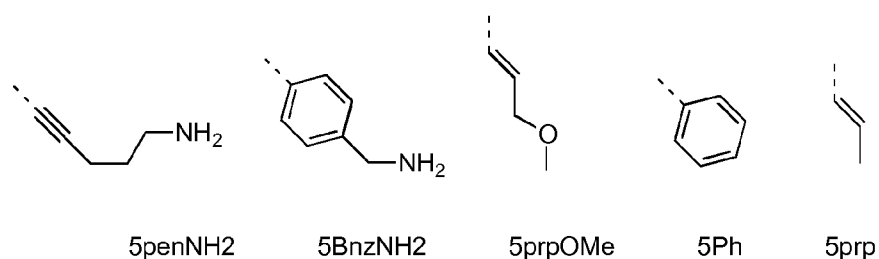


[00246] In the above structure, X is, for example, one of the following substituents:



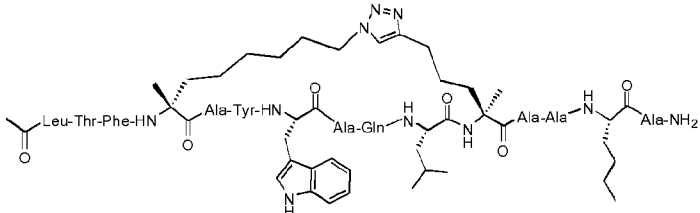
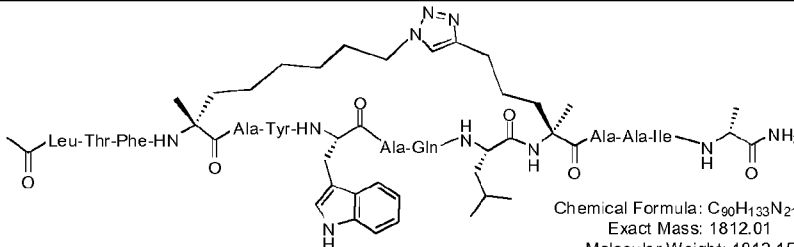
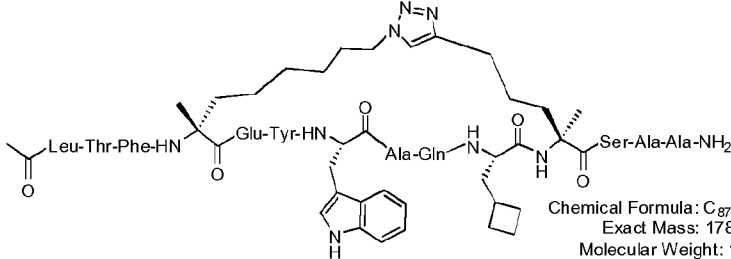
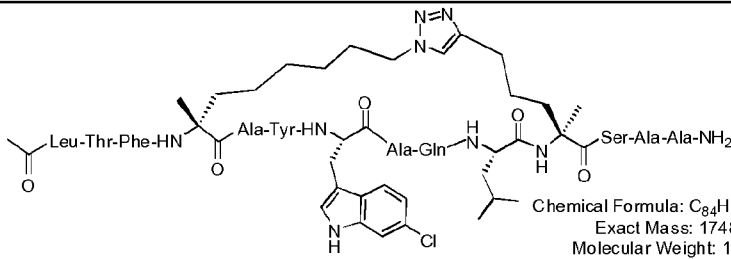
wherein “Cyc” is a suitable aryl, cycloalkyl, cycloalkenyl, heteroaryl, or heterocyclyl group, unsubstituted or optionally substituted with an R_a or R_b group as described above.

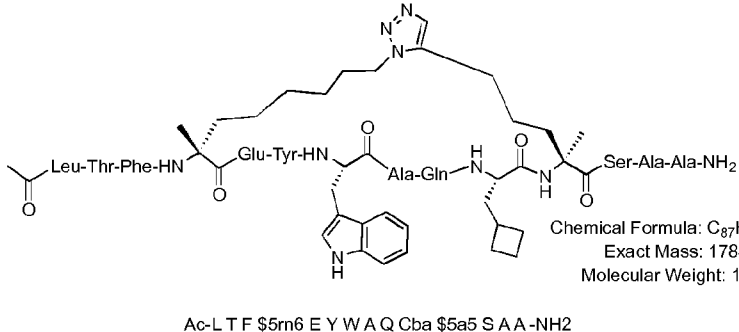
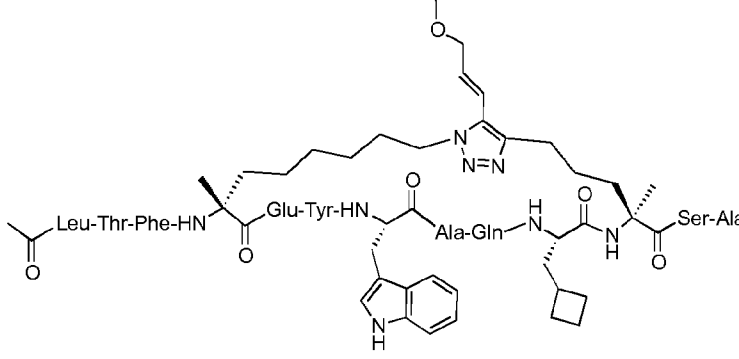
[00247] In some embodiments, the triazole substituent is chosen from the group consisting of:



[00248] Table 4 shows exemplary peptidomimetic macrocycles of Formula I:

Table 4b

	Structure
SP-449	 Chemical Formula: $C_{90}H_{133}N_{21}O_{19}$ Exact Mass: 1812.01 Molecular Weight: 1813.15 Ac- L T F \$4rn6 A Y W A Q L \$4a5 A A Nle A -NH₂
SP-64	 Chemical Formula: $C_{90}H_{133}N_{21}O_{19}$ Exact Mass: 1812.01 Molecular Weight: 1813.15 Ac- L T F \$4rn6 A Y W A Q L \$4a5 A A I a -NH₂
SP-153	 Chemical Formula: $C_{87}H_{124}N_{20}O_{21}$ Exact Mass: 1784.92 Molecular Weight: 1786.04 Ac- L T F \$4rn6 E Y W A Q Cba \$4a5 S A A -NH₂
SP-98	 Chemical Formula: $C_{84}H_{121}ClN_{20}O_{19}$ Exact Mass: 1748.88 Molecular Weight: 1750.44 Ac- L T F \$4rn6 A Y 6cIW A Q L \$4a5 S A A -NH₂

SP-456	 Chemical Formula: C₈₇H₁₂₄N₂₀O₂₁ Exact Mass: 1784.92 Molecular Weight: 1786.04 Ac-L T F \$5r n6 E Y W A Q Cba \$5a5 S A A -NH₂
SP-470	

[00249] In some embodiments, peptidomimetic macrocycles exclude peptidomimetic macrocycles shown in Table 5:

Table 5

#	Sequence
1	Ac-QSQQTF\$5r n6 NLWRLL\$5a5QN-NH ₂
2	Ac-QSQQTF\$4r n5 NLWRLL\$4a5QN-NH ₂
3	Ac-QSQQTF#5r n6 NLWRLL#5a5QN-NH ₂
4	Ac-QSQQTF#4r n5 NLWRLL#4a5QN-NH ₂
5	Ac-QSQQTF\$5r n5 NLWRLL\$5a5QN-NH ₂
6	Ac-QSQQTF\$5r a5 NLWRLL\$5n5QN-NH ₂
7	Ac-QSQQTF\$5r a5 NLWRLL\$5n6QN-NH ₂
8	Ac-QSQQTF\$4r a5 NLWRLL\$4n5QN-NH ₂
9	Ac-QSQQTF\$4r a5 NLWRLL\$4n6QN-NH ₂
10	Ac-QSQQTF\$4r n6 NLWRLL\$4a5QN-NH ₂
11	Ac-QSQQTF\$5r n6 NLWRLL\$5a6QN-NH ₂
12	Ac-QSQQTF\$5r a6 NLWRLL\$5n6QN-NH ₂
13	Ac-QSQQTF\$4r n6 NLWRLL\$4a6QN-NH ₂
14	Ac-QSQQTF\$4r a6 NLWRLL\$4n6QN-NH ₂
15	Ac-QSQQTF\$4r n5 NLWRLL\$4a6QN-NH ₂
16	Ac-QSQQTF4Me\$5r n6 NLWRLL4Me\$5a5QN-NH ₂
17	Ac-LTF\$4r a5 HYWAQL\$4n6S-NH ₂
18	H-F\$4r n6 HYWAQL\$4a5S-NH ₂
19	Ac-LTF\$4r n6 HYWAQL\$4a5S-NH ₂
20	Ac-F\$4r n6 HYWAQL\$4a5S-NH ₂
21	Ac-LTF\$4r n6 HYWAQL\$4a6S-NH ₂

22	Ac-LTF\$5ra5HYWAQL\$5n6S-NH2
23	Ac-LTF\$4rn6AYWAQL\$4a5A-NH2
24	Ac-LTF\$5ra5HYWAQL\$5n6S-NH2
25	Ac-LTF\$4rn6AYWAQL\$4a5A-NH2
26	Ac-LTFEHYWAQLTS-NH2

[00250] Peptides shown can comprise an N-terminal capping group such as acetyl or an additional linker such as beta-alanine between the capping group and the start of the peptide sequence.

[00251] In some embodiments, peptidomimetic macrocycles do not comprise a peptidomimetic macrocycle structure as shown in Table 5.

Example 3: Peptidomimetic macrocycles of Formula II

[00252] Peptidomimetic macrocycles were designed by replacing two or more naturally occurring amino acids with the corresponding synthetic amino acids. Substitutions were made at i and i+4, and i and i+7 positions. Macrocycles were generated by solid phase peptide synthesis followed by crosslinking the peptides via their thiol-containing side chains. Peptide synthesis is performed either manually or on an automated peptide synthesizer (Applied Biosystems, model 433A), using solid phase conditions, rink amide AM resin (Novabiochem), and Fmoc main-chain protecting group chemistry. The N-termini of the synthetic peptides are acetylated, while the C-termini are amidated.

[00253] The fully protected resin-bound peptides are synthesized on a Rink amide MBHA resin (loading 0.62 mmol/g) on a 0.1 mmol scale. Deprotection of the temporary Fmoc group is achieved by 2 × 20 min treatments of the resin bound peptide with 25% (v/v) piperidine in NMP. After extensive flow washing with NMP and dichloromethane, coupling of each successive amino acid was achieved with 1 × 60 min incubation with the appropriate preactivated Fmoc-amino acid derivative. All protected amino acids (1 mmol) were dissolved in NMP and activated with HCTU (1 mmol) and DIEA (1 mmol) prior to transfer of the coupling solution to the deprotected resin-bound peptide. After coupling was completed, the resin was extensively flow washed in preparation for the next deprotection/coupling cycle. Acetylation of the amino terminus was carried out in the presence of acetic anhydride/DIEA in NMP /NMM. The LC-MS analysis of a cleaved and deprotected sample obtained from an aliquot of the fully assembled resin-bound peptide was accomplished in order to verifying the completion of each coupling.

[00254] Purification of cross-linked compounds is achieved by high performance liquid chromatography (HPLC) (Varian ProStar) on a reverse phase C18 column (Varian) to yield the pure compounds. Chemical composition of the pure products was confirmed by LC/MS mass spectrometry (Micromass LCT interfaced with Agilent 1100 HPLC system) and amino acid analysis (Applied Biosystems, model 420A).

[00255] In a typical example, a peptide resin (0.1 mmol) was washed with DCM. Deprotection of the temporary Mmt group was achieved by 3 × 3 min treatments of the resin bound peptide with 2%

TFA/DCM 5% TIPS, then 30min treatments until no orange color is observed in the filtrate. In between treatments the resin was extensively flow washed with DCM. After complete removal of Mmt, the resin was washed with 5% DIEA/NMP solution 3X and considered ready for bisthioether coupling. Resin was loaded into a reaction vial. DCM/DMF 1/1 was added to the reaction vessel, followed by DIEA (2.4eq). After mixing well for 5 minutes, 4,4'-Bis(bromomethyl)biphenyl (1.05 eq) (TCI America B1921) was added. The reaction was then mechanically agitated at room temperature overnight. Where needed, the reaction was allowed additional time to reach completion. A similar procedure may be used in the preparation of five-methylene, six-methylene or seven-methylene crosslinkers (“%c7”, “%c6”, or “%c5”).

[00256] The bisthioether resin-bound peptides were deprotected and cleaved from the solid support by treatment with TFA/H₂O/TIS (94/3/3 v/v) for 3 h at room temperature. After filtration of the resin the TFA solution was precipitated in cold diethyl ether and centrifuged to yield the desired product as a solid. The crude product was purified by preparative HPLC.

[00257] Table 6 show a list of peptidomimetic macrocycles.

Table 6

SP	Sequence
471	Ac-F%cs7AYWEAc3cL%c7AAA-NH2
472	Ac-F%cs7AYWEAc3cL%c7AAibA-NH2
473	Ac-LTF%cs7AYWAQL%c7SANle-NH2
474	Ac-LTF%cs7AYWAQL%c7SAL-NH2
475	Ac-LTF%cs7AYWAQL%c7SAM-NH2
476	Ac-LTF%cs7AYWAQL%c7SAhL-NH2
477	Ac-LTF%cs7AYWAQL%c7SAF-NH2
478	Ac-LTF%cs7AYWAQL%c7SAI-NH2
479	Ac-LTF%cs7AYWAQL%c7SACHg-NH2
480	Ac-LTF%cs7AYWAQL%c7SAAib-NH2
481	Ac-LTF%cs7AYWAQL%c7SAA-NH2
482	Ac-LTF%cs7AYWA%7L%c7S%c7Nle-NH2
483	Ac-LTF%cs7AYWA%7L%c7S%c7A-NH2
484	Ac-F%cs7AYWEAc3cL%c7AANle-NH2
485	Ac-F%cs7AYWEAc3cL%c7AAL-NH2
486	Ac-F%cs7AYWEAc3cL%c7AAM-NH2
487	Ac-F%cs7AYWEAc3cL%c7AAhL-NH2
488	Ac-F%cs7AYWEAc3cL%c7AAF-NH2
489	Ac-F%cs7AYWEAc3cL%c7AAI-NH2
490	Ac-F%cs7AYWEAc3cL%c7AACHg-NH2
491	Ac-F%cs7AYWEAc3cL%c7AACha-NH2
492	Ac-F%cs7AYWEAc3cL%c7AAAib-NH2
493	Ac-LTF%cs7AYWAQL%c7AAAibV-NH2
494	Ac-LTF%cs7AYWAQL%c7AAAibV-NH2
495	Ac-LTF%cs7AYWAQL%c7SAibAA-NH2
496	Ac-LTF%cs7AYWAQL%c7SAibAA-NH2
497	Ac-HLTF%cs7HHWHQL%c7AANleNle-NH2
498	Ac-DLTF%cs7HHWHQL%c7RRLV-NH2
499	Ac-HHTF%cs7HHWHQL%c7AAML-NH2
500	Ac-F%cs7HHWHQL%c7RRDCha-NH2
501	Ac-F%cs7HHWHQL%c7HRFV-NH2
502	Ac-HLTF%cs7HHWHQL%c7AAhLA-NH2
503	Ac-DLTF%cs7HHWHQL%c7RRChgl-NH2

SP	Sequence
504	Ac-DLTF%cs7HHWHQL%c7RRChgl-NH2
505	Ac-HHTF%cs7HHWHQL%c7AAChav-NH2
506	Ac-F%cs7HHWHQL%c7RRDa-NH2
507	Ac-F%cs7HHWHQL%c7HRAibG-NH2
508	Ac-F%cs7AYWAQL%c7HHNleL-NH2
509	Ac-F%cs7AYWSAL%c7HQANle-NH2
510	Ac-F%cs7AYWVQL%c7QHChgl-NH2
511	Ac-F%cs7AYWTAL%c7QQNlev-NH2
512	Ac-F%cs7AYWYQL%c7HAibAa-NH2
513	Ac-LTF%cs7AYWAQL%c7HHLA-NH2
514	Ac-LTF%cs7AYWAQL%c7HHLA-NH2
515	Ac-LTF%cs7AYWAQL%c7HQNlev-NH2
516	Ac-LTF%cs7AYWAQL%c7HQNlev-NH2
517	Ac-LTF%cs7AYWAQL%c7QQM1-NH2
518	Ac-LTF%cs7AYWAQL%c7QQM1-NH2
519	Ac-LTF%cs7AYWAQL%c7HAibhLV-NH2
520	Ac-LTF%cs7AYWAQL%c7AHFA-NH2
521	Ac-HLTF%cs7HHWHQL%c7AANlel-NH2
522	Ac-DLTF%cs7HHWHQL%c7RRLa-NH2
523	Ac-HHTF%cs7HHWHQL%c7AAMv-NH2
524	Ac-F%cs7HHWHQL%c7RRDA-NH2
525	Ac-F%cs7HHWHQL%c7HRFCha-NH2
526	Ac-F%cs7AYWEAL%c7AA-NHAm
527	Ac-F%cs7AYWEAL%c7AA-NHiAm
528	Ac-F%cs7AYWEAL%c7AA-NHnPr3Ph
529	Ac-F%cs7AYWEAL%c7AA-NHnBu33Me
530	Ac-F%cs7AYWEAL%c7AA-NHnPr
531	Ac-F%cs7AYWEAL%c7AA-NHnEt2Ch
532	Ac-F%cs7AYWEAL%c7AA-NHnEt2Cp
533	Ac-F%cs7AYWEAL%c7AA-NHHex
534	Ac-LTF%cs7AYWAQL%c7AAIA-NH2
535	Ac-LTF%cs7AYWAQL%c7AAIA-NH2
536	Ac-LTF%cs7AYWAAL%c7AAMA-NH2
537	Ac-LTF%cs7AYWAAL%c7AAMA-NH2
538	Ac-LTF%cs7AYWAQL%c7AANlea-NH2
539	Ac-LTF%cs7AYWAQL%c7AANlea-NH2
540	Ac-LTF%cs7AYWAQL%c7AAIa-NH2
541	Ac-LTF%cs7AYWAQL%c7AAIa-NH2
542	Ac-LTF%cs7AYWAAL%c7AAMa-NH2
543	Ac-LTF%cs7AYWAAL%c7AAMa-NH2
544	Ac-LTF%cs7AYWAQL%c7AANlea-NH2
545	Ac-LTF%cs7AYWAQL%c7AANlea-NH2
546	Ac-LTF%cs7AYWAAL%c7AAIv-NH2
547	Ac-LTF%cs7AYWAAL%c7AAIv-NH2
548	Ac-LTF%cs7AYWAQL%c7AAMv-NH2
549	Ac-LTF%cs7AYWAAL%c7AANlev-NH2
550	Ac-LTF%cs7AYWAAL%c7AANlev-NH2
551	Ac-LTF%cs7AYWAQL%c7AAI1-NH2
552	Ac-LTF%cs7AYWAQL%c7AAI1-NH2
553	Ac-LTF%cs7AYWAAL%c7AAM1-NH2
554	Ac-LTF%cs7AYWAQL%c7AANlel-NH2
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556	Ac-F%cs7AYWEAL%c7AAMA-NH2
557	Ac-F%cs7AYWEAL%c7AANlea-NH2
558	Ac-F%cs7AYWEAL%c7AAIa-NH2
559	Ac-F%cs7AYWEAL%c7AAMa-NH2
560	Ac-F%cs7AYWEAL%c7AANlea-NH2
561	Ac-F%cs7AYWEAL%c7AAIv-NH2

SP	Sequence
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564	Ac-F%cs7AYWEAL%c7AAIl-NH2
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566	Ac-F%cs7AYWEAL%c7AANlel-NH2
567	Ac-F%cs7AYWEAL%c7AANlel-NH2
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569	Ac-LTF%cs7AY6clWAQL%c7SAA-NH2
570	Ac-WTF%cs7FYWSQL%c7AVAA-NH2
571	Ac-WTF%cs7FYWSQL%c7AVAA-NH2
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619	Ac-LTF%cs7EYWAQCpg%c7SAA-NH2

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623	Ac-LTF%cs7EYWAQCba%c7SAA-NH2
624	Ac-LTF%cs7EYWAQCba%c7SAA-NH2
625	Ac-LTF3C1%cs7EYWAQL%c7SAA-NH2
626	Ac-LTF3C1%cs7EYWAQL%c7SAA-NH2
627	Ac-LTF34F2%cs7EYWAQL%c7SAA-NH2
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629	Ac-LTF34F2%cs7EYWAQhL%c7SAA-NH2
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631	Ac-ETF%cs7EYWAQL%c7SAA-NH2
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SP	Sequence
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740	Ac-ETF%cs7AYWAQCba%c7AWNlea-NH2
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SP	Sequence
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SP	Sequence
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883	Ac-LTF%cs7Cou4YWAQL%c7AANleA-NH2
884	Ac-LTF%cs7Cou4YWAQL%c7AANleA-NH2
885	Ac-LTF%cs7AYWCou4QL%c7AANleA-NH2
886	Ac-LTF%cs7AYWAQL%c7Cou4ANleA-NH2
887	Ac-LTF%cs7AYWAQL%c7Cou4ANleA-NH2
888	Ac-LTF%cs7AYWAQL%c7ACou4NleA-NH2
889	Ac-LTF%cs7AYWAQL%c7ACou4NleA-NH2
890	Ac-LTF%cs7AYWAQL%c7AANleA-OH
891	Ac-LTF%cs7AYWAQL%c7AANleA-OH
892	Ac-LTF%cs7AYWAQL%c7AANleA-NHnPr
893	Ac-LTF%cs7AYWAQL%c7AANleA-NHnPr
894	Ac-LTF%cs7AYWAQL%c7AANleA-NHnBu33Me
895	Ac-LTF%cs7AYWAQL%c7AANleA-NHnBu33Me
896	Ac-LTF%cs7AYWAQL%c7AANleA-NHHex
897	Ac-LTF%cs7AYWAQL%c7AANleA-NHHex
898	Ac-LTA%cs7AYWAQL%c7AANleA-NH2
899	Ac-LThL%cs7AYWAQL%c7AANleA-NH2
900	Ac-LTF%cs7AYAAQL%c7AANleA-NH2
901	Ac-LTF%cs7AY2Na1AQL%c7AANleA-NH2
902	Ac-LTF%cs7EYWCou4QCba%c7SAA-NH2
903	Ac-LTF%cs7EYWCou7QCba%c7SAA-NH2
904	Dmaac-LTF%cs7EYWAQCba%c7SAA-NH2
905	Dmaac-LTF%cs7AYWAQL%c7AAAAAa-NH2
906	Dmaac-LTF%cs7AYWAQL%c7AAAAAa-NH2
907	Dmaac-LTF%cs7EYWAQL%c7AAAAAa-NH2
908	Dmaac-LTF%cs7EYWAQL%c7AAAAAa-NH2
909	Dmaac-LTF%cs7EF4coohWAQCba%c7AAIa-NH2

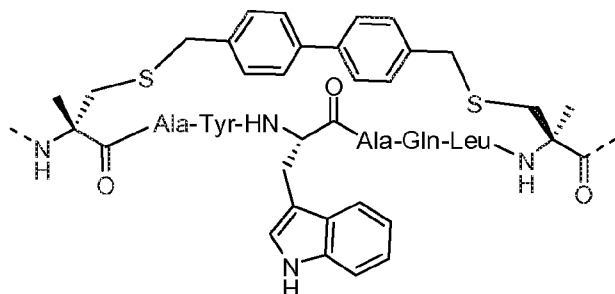
SP	Sequence
910	Dmaac-LTF%cs7EF4coohWAQCba%c7AAIa-NH2
911	Dmaac-LTF%cs7AYWAQL%c7AANleA-NH2
912	Dmaac-LTF%cs7AYWAQL%c7AANleA-NH2
913	Cou6BaLTF%cs7EYWAQhL%c7SAA-NH2
914	Cou8BaLTF%cs7EYWAQhL%c7SAA-NH2
915	Ac-LTF4I%cs7EYWAQL%c7AAAAAa-NH2

Table 6a shows exemplary peptidomimetic macrocycles:

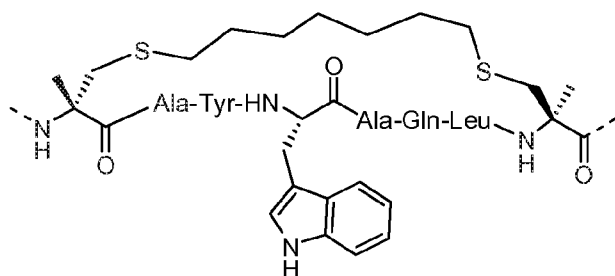
Table 6a

SP	Sequence	Exact Mass	Found Mass	Calc (M+1)/1	Calc (M+2)/2	Calc (M+3)/3
916	Ac-LTF%cs7AYWAQL%c7AANleA-NH2	1808.94		1809.95	905.48	603.99
917	Ac-LTF%cs7AYWAQL%c7AAAAAa-NH2	1908.96		1909.97	955.49	637.33
918	Ac-LTF%csBphAYWAQL%cBphAANleA-NH2	1890.92		1909.97	955.49	637.33
919	Ac-LTF%csBphAYWAQL%cBphAAAAAa-NH2	1990.92	996.88			
920	Ac-LTF%csBphEYWAQCba#cBphSAA-NH2	1865.16	933.45		933.58	
921	Ac-LTF%cs7EYWAQCba#c7SAA-NH2	1753.82		1754.83	877.92	585.61
922	Ac-LTF%csBphEYWAQCba#cBphSAA-NH2	1835.81		1836.82	918.91	612.94
923	Ac-LTF%csBphEYWAQL%cBphAAAAAa-NH2					
924	Ac-LTF%cs5AYWAQL%c5AANleA-NH2					
925	Ac-LTF%cs5AYWAQL%c5AAAAAa-NH2					
926	Ac-LTF%cs6AYWAQL%c6AANleA-NH2					
927	Ac-LTF%cs6AYWAQL%c6AAAAAa-NH2					
928	Ac-LTF%cs6EYWAQL%c6AAAAAa-NH2	1894.94		1895.96	948.48	632.66
929	Ac-LTF%cs5EYWAQL%c5AAAAAa-NH2	1880.93		1881.94	941.47	627.98
930	Ac-LTF%cs6EYWAQCba#c6SAANH2	1709.83		1710.84	855.92	570.95
931	Ac-LTF%cs5EYWAQCba#c5SAANH2	1695.81		1696.82	848.92	566.28

[00258] Partial structures of selected exemplary peptidomimetic macrocycles are shown below:

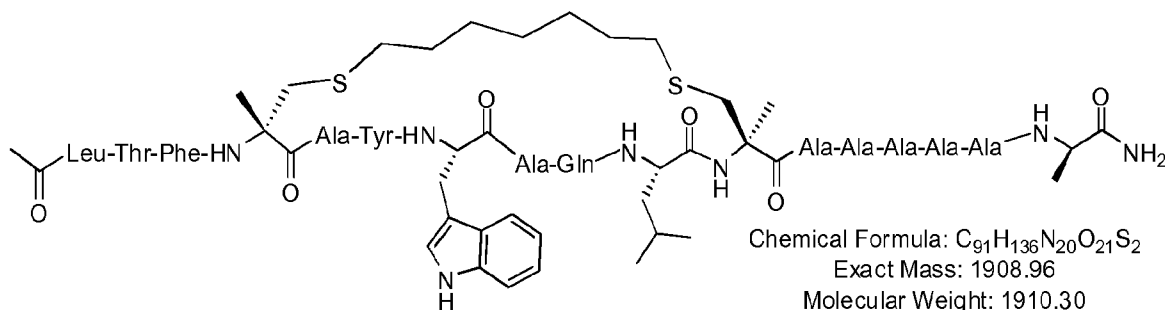


SP-918



SP-916/917

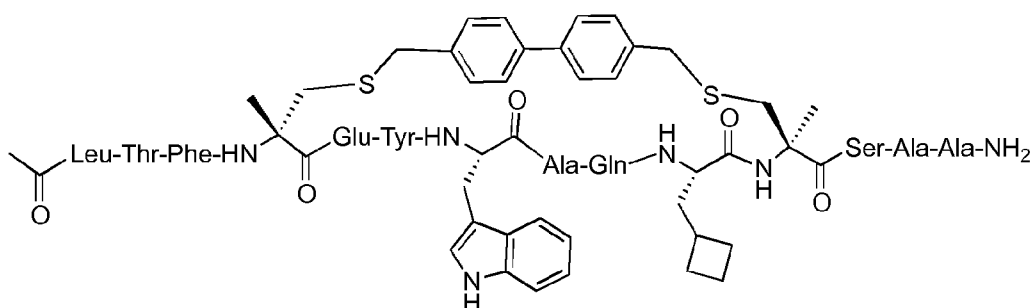
[00259] A structure of an exemplary peptidomimetic macrocycle is shown below:



Ac- L T F %cs7 A Y W A Q L %c7 A A A A a -NH₂

SP-917

[00260] Another structure of an exemplary peptidomimetic macrocycle is shown below:



SP-920

[00261] Amino acids represented as “#cs5” are D-cysteine connected by an i to i+7, five-methylene crosslinker to another thiol-containing amino acid. Amino acids represented as “#c5” are L-cysteine connected by an i to i+7, five- methylene crosslinker to another thiol-containing amino acid. Amino acids represented as “#cs6” are D-cysteine connected by an i to i+7, six- methylene crosslinker to another thiol-containing amino acid. Amino acids represented as “#c6” are L-cysteine connected by an i to i+7, six- methylene crosslinker to another thiol-containing amino acid. Amino acids represented as “#cs7” are D-cysteine connected by an i to i+7, seven- methylene crosslinker to another thiol-containing amino acid. Amino acids represented as “#c7” are L-cysteine connected by an i to i+7, seven- methylene crosslinker to another thiol-containing amino acid. Amino acids represented as “#cs8” are D-cysteine connected by an i to i+7, eight- methylene crosslinker to another thiol-containing amino acid. Amino acids represented as “#c8” are L-cysteine connected by an i to i+7, eight- methylene crosslinker to another thiol-containing amino acid. Amino acids represented as “%cs7” are alpha-methyl-D-cysteine connected by an i to i+7, seven-methylene crosslinker to another thiol-containing amino acid. Amino acids represented as “%c7” are alpha-methyl-L-cysteine connected by an i to i+7, seven-methylene crosslinker to another thiol-containing amino acid. Amino acids represented as “%cs8” are alpha-

methyl-D-cysteine connected by an i to i+7, eight-methylene crosslinker to another thiol-containing amino acid. Amino acids represented as “%c8” are alpha-methyl-L-cysteine connected by an i to i+7, eight- methylene crosslinker to another thiol-containing amino acid. Amino acids represented as “%cs9” are alpha-methyl-D-cysteine connected by an i to i+7, nine-methylene crosslinker to another thiol-containing amino acid. Amino acids represented as “%c9” are alpha-methyl-L-cysteine connected by an i to i+7, nine- methylene crosslinker to another thiol-containing amino acid. Amino acids represented as “%cs10” are alpha-methyl-D-cysteine connected by an i to i+7, ten-methylene crosslinker to another thiol-containing amino acid. Amino acids represented as “%c10” are alpha-methyl-L-cysteine connected by an i to i+7, ten-methylene crosslinker to another thiol-containing amino acid. Amino acids represented as “pen8” are D-penicillamine connected by an i to i+7, eight-methylene crosslinker to another thiol-containing amino acid. Amino acids represented as “Pen8” are L-penicillamine connected by an i to i+7, eight- methylene crosslinker to another thiol-containing amino acid. Amino acids represented as “#csBph” are D-cysteine connected by an i to i+7, Bph (4,4'-bismethyl-biphenyl) crosslinker to another thiol-containing amino acid. Amino acids represented as “#cBph” are L-cysteine connected by an i to i+7, Bph (4,4'-bismethyl-biphenyl) crosslinker to another thiol-containing amino acid. Amino acids represented as “%csBph” are alpha-methyl-D-cysteine connected by an i to i+7, Bph (4,4'-bismethyl-biphenyl) crosslinker to another thiol-containing amino acid. Amino acids represented as “%cBph” are alpha-methyl-L-cysteine connected by an i to i+7, Bph (4,4'-bismethyl-biphenyl) crosslinker to another thiol-containing amino acid. Amino acids represented as “#csBpy” are D-cysteine connected by an i to i+7, Bpy (6,6'-bismethyl-[3,3']bipyridine) crosslinker to another thiol-containing amino acid. Amino acids represented as “#cBpy” are L-cysteine connected by an i to i+7, Bpy (6,6'-bismethyl-[3,3']bipyridine) crosslinker to another thiol-containing amino acid. Amino acids represented as “%csBpy” are alpha-methyl-D-cysteine connected by an i to i+7, Bpy (6,6'-bismethyl-[3,3']bipyridine) crosslinker to another thiol-containing amino acid. Amino acids represented as “%cBpy” are alpha-methyl-L-cysteine connected by an i to i+7, Bpy (6,6'-bismethyl-[3,3']bipyridine) crosslinker to another thiol-containing amino acid. The number of methylene units indicated above refers to the number of methylene units between the two thiol groups of the crosslinker.

[00262] In some embodiments, a peptidomimetic macrocycle is obtained in more than one isomer, for example due to the configuration of a double bond within the structure of the crosslinker (*E* vs *Z*). Such isomers can or can not be separable by conventional chromatographic methods. In some embodiments, one isomer has improved biological properties relative to the other isomer. In one embodiment, an *E* crosslinker olefin isomer of a peptidomimetic macrocycle has better solubility, better target affinity, better in vivo or in vitro efficacy, higher helicity, or improved cell permeability relative to its *Z* counterpart. In another embodiment, a *Z* crosslinker olefin isomer of

a peptidomimetic macrocycle has better solubility, better target affinity, better in vivo or in vitro efficacy, higher helicity, or improved cell permeability relative to its *E* counterpart.

[00263] In some embodiments, peptidomimetic macrocycles exclude peptidomimetic macrocycles shown in Table 7:

Table 7

	Sequence
1	QSQQTF%csNLWLL%cs6QN
2	QSQQTF%csNLWLL%cs7QN
3	QSQQTF%csNLWLL%cs8QN
4	QSQQTF%csNLWLL%cs9QN

[00264] Peptides shown can comprise an N-terminal capping group such as acetyl or an additional linker such as beta-alanine between the capping group and the start of the peptide sequence.

[00265] In some embodiments, peptidomimetic macrocycles do not comprise a peptidomimetic macrocycle structure as shown in Table 7.

[00266] In other embodiments, peptidomimetic macrocycles exclude peptidomimetic macrocycles shown in Table 7a:

Table 7a

Number	Sequence
1	Ac-QSQQTF#cs5NLWRLL#c5QN-NH2
2	Ac-QSQQTF#cs6NLWRLL#c6QN-NH2
3	Ac-QSQQTF#cs7NLWRLL#c7QN-NH2
4	Ac-QSQQTF#cs8NLWRLL#c8QN-NH2
5	Ac-QSQQTF#cs9NLWRLL#c9QN-NH2
6	Ac-QSQQTF%cs8NLWRLL#c8QN-NH2
7	Ac-QSQQTF#cs8NLWRLLPen8QN-NH2
8	Ac-QSQQTF#c8NLWRLL#c8QN-NH2
9	Ac-QSQQTF#c8NLWRLL#c8QN-NH2
10	Ac-QSQQTF#cs8NLWALL#c8AN-NH2
11	Ac-QAibQQTF#cs8NLWALL#c8AN-NH2
12	Ac-QAibQQTF#cs8ALWALL#c8AN-NH2
13	Ac-QSQQTFpen8NLWRLLPen8QN-NH2
14	Ac-QSQQTFpen8NLWRLL#c8QN-NH2
15	Ac-QSQQTF%cs9NLWRLL#c9QN-NH2
16	Ac-LTF#cs8HYWAQL#c8S-NH2
17	Ac-LTF#cs8HYWAQI#c8S-NH2
18	Ac-LTF#cs8HYWAQNI#c8S-NH2
19	Ac-LTF#cs8HYWAQL#c8A-NH2
20	Ac-LTF#cs8HYWAbuQL#c8S-NH2

21	Ac-LTF#cs8AYWAQL#c8S-NH2
22	Ac-LTF#cs8AYWAQL#c8A-NH2
23	Ac-LTF#cs8HYWAQLPen8S-NH2
24	Ac-LTFpen8HYWAQLPen8S-NH2
25	Ac-LTFpen8HYWAQL#c8S-NH2
26	Ac-LTF#cs7HYWAQL#hc7S-NH2
27	Ac-LTF%cs8HYWAQL%c8S-NH2
28	Ac-LTF%cs9HYWAQL%c9S-NH2
29	Ac-LTF%cs10HYWAQL%c10S-NH2
30	Ac-LTF%cs7HYWAQL%c7S-NH2
31	Ac-LTF%cs4BEBHYWAQL%c4BEB-S-NH2
32	Ac-Fpen8AYWEAc3cL#c8A-NH2
33	Ac-F#cs8AYWEAc3cL#c8A-NH2
34	Ac-F%cs8AYWEAc3cL%c8A-NH2
35	Ac-LTFEHYWAQLTS-NH2

[00267] In some embodiments, peptidomimetic macrocycles do not comprise a peptidomimetic macrocycle structure as shown in Table 7a.

[00268] In other embodiments, peptidomimetic macrocycles exclude peptidomimetic macrocycles shown in Table 7b and disclosed in Muppidi et al., Chem. Commun. (2011) DOI: 10.1039/c1cc13320a:

Table 7b

Number	Sequence
1	LTFEHYWAQLTS
2	LTFCHYWAQLCS
3	LTF#cBphHYWAQL#cBphS
4	LTF#cBpyHYWAQL#cBpyS
5	LTFCRYWARLCS
6	LTF#cBphRYWARL#cBphS
7	LTF#cBpyRYWARL#cBpyS
8	LTFcHYWAQLCS
9	LTF#csBphHYWAQL#cBphS
10	LTF#csBpyHYWAQL#csBpyS
11	LTF#csBphRYWARL#cBphS
12	LTF#csBpyRYWARL#cBpyS

wherein C denotes L-cysteine and c denotes D-cysteine in Table 7b; and #cBph, #cBpy, #csBph, and #csBpy are as defined herein.

[00269] In some embodiments, peptidomimetic macrocycles do not comprise a peptidomimetic macrocycle structure as shown in Table 7b.

Example 4: Circular Dichroism (CD) analysis of alpha-helicity

[00270] Peptide solutions are analyzed by CD spectroscopy using a Jasco J-815 spectropolarimeter (Jasco Inc., Easton, MD) with the Jasco Spectra Manager Ver.2 system software. A Peltier temperature controller is used to maintain temperature control of the optical cell. Results are expressed as mean molar ellipticity $[\theta]$ (deg cm² dmol⁻¹) as calculated from the equation $[\theta] = \theta_{\text{obs}} \cdot \text{MRW} / 10 \cdot l \cdot c$ where θ_{obs} is the observed ellipticity in millidegrees, MRW is the mean residue weight of the peptide (peptide molecular weight/number of residues), l is the optical path length of the cell in centimeters, and c is the peptide concentration in mg/ml. Peptide concentrations are determined by amino acid analysis. Stock solutions of peptides are prepared in benign CD buffer (20 mM phosphoric acid, pH 2). The stocks are used to prepare peptide solutions of 0.05 mg/ml in either benign CD buffer or CD buffer with 50% trifluoroethanol (TFE) for analyses in a 10 mm pathlength cell. Variable wavelength measurements of peptide solutions are scanned at 4 °C from 195 to 250 nm, in 0.2 nm increments, and a scan rate 50 nm per minute. The average of six scans is reported.

Example 5: Direct binding assay MDM2 with Fluorescence polarization (FP)

[00271] The assay is performed according to the following general protocol:

1. Dilute MDM2 (In-house, 41kD) into FP buffer (High salt buffer-200mM NaCl, 5mM CHAPS, pH 7.5) to make 10 μ M working stock solution.
2. Add 30 μ l of 10 μ M of protein stock solution into A1 and B1 well of 96-well black HE microplate (Molecular Devices).
3. Fill in 30 μ l of FP buffer into column A2 to A12, B2 to B12, C1 to C12, and D1 to D12.
4. 2 or 3 fold series dilution of protein stock from A1, B1 into A2, B2; A2, B2 to A3, B3; ... to reach the single digit nM concentration at the last dilution point.
5. Dilute 1mM (in 100% DMSO) of FAM labeled linear peptide with DMSO to 100 μ M (dilution 1: 10). Then, dilute from 100 μ M to 10 μ M with water (dilution 1:10) and then dilute with FP buffer from 10 μ M to 40nM (dilution 1:250). This is the working solution which will be a 10nM concentration in well (dilution 1:4). Keep the diluted FAM labeled peptide in the dark until use.
6. Add 10 μ l of 10nM of FAM labeled peptide into each well and incubate, and read at different time points. K_d with 5-FAM-BaLTFEHYWAQLTS-NH₂ is ~13.38 nM.

Example 6: Competitive Fluorescence polarization assay for MDM2

[00272] The assay is performed according to the following general protocol:

1. Dilute MDM2 (In-house, 41kD) into FP buffer (High salt buffer-200mM NaCl, 5mM CHAPS, pH 7.5) to make 84nM (2X) working stock solution.
2. Add 20 μ l of 84nM (2X) of protein stock solution into each well of 96-well black HE microplate (Molecular Devices)

3. Dilute 1mM (in 100% DMSO) of FAM labeled linear peptide with DMSO to 100 μ M (dilution 1: 10). Then, dilute from 100 μ M to 10 μ M with water (dilution 1:10) and then dilute with FP buffer from 10 μ M to 40nM (dilution 1:250). This is the working solution which will be a 10nM concentration in well (dilution 1:4). Keep the diluted FAM labeled peptide in the dark until use.
4. Make unlabeled peptide dose plate with FP buffer starting with 1 μ M (final) of peptide and making 5 fold serial dilutions for 6 points using following dilution scheme.
Dilute 10mM (in 100% DMSO) with DMSO to 5mM (dilution 1: 2). Then, dilute from 5mM to 500 μ M with H₂O (dilution 1:10) and then dilute with FP buffer from 500 μ M to 20 μ M (dilution 1:25). Making 5 fold serial dilutions from 4 μ M (4X) for 6 points.
5. Transfer 10 μ l of serial diluted unlabeled peptides to each well which is filled with 20 μ l of 84nM of protein.
6. Add 10 μ l of 10nM (4X) of FAM labeled peptide into each well and incubate for 3hr to read.

Example 7: Direct binding assay MDMX with Fluorescence polarization (FP)

[00273] The assay is performed according to the following general protocol:

1. Dilute MDMX (In-house, 40kD) into FP buffer (High salt buffer-200mM NaCl, 5mM CHAPS, pH 7.5) to make 10 μ M working stock solution.
 2. Add 30 μ l of 10 μ M of protein stock solution into A1 and B1 well of 96-well black HE microplate (Molecular Devices).
 3. Fill in 30 μ l of FP buffer into column A2 to A12, B2 to B12, C1 to C12, and D1 to D12.
 4. 2 or 3 fold series dilution of protein stock from A1, B1 into A2, B2; A2, B2 to A3, B3; ... to reach the single digit nM concentration at the last dilution point.
 5. Dilute 1mM (in 100% DMSO) of FAM labeled linear peptide with DMSO to 100 μ M (dilution 1: 10). Then, dilute from 100 μ M to 10 μ M with water (dilution 1:10) and then dilute with FP buffer from 10 μ M to 40nM (dilution 1:250). This is the working solution which will be a 10nM concentration in well (dilution 1:4). Keep the diluted FAM labeled peptide in the dark until use.
 6. Add 10 μ l of 10nM of FAM labeled peptide into each well and incubate, and read at different time points.
- K_d with 5-FAM-BaLTFEHYWAQLTS-NH₂ is ~51 nM.

Example 8: Competitive Fluorescence polarization assay for MDMX

[00274] The assay is performed according to the following general protocol:

1. Dilute MDMX (In-house, 40kD) into FP buffer (High salt buffer-200mM NaCl, 5mM CHAPS, pH 7.5.) to make 300nM (2X) working stock solution.
2. Add 20 μ l of 300nM (2X) of protein stock solution into each well of 96-well black HE microplate (Molecular Devices)

3. Dilute 1mM (in 100% DMSO) of FAM labeled linear peptide with DMSO to 100 μ M (dilution 1: 10). Then, dilute from 100 μ M to 10 μ M with water (dilution 1:10) and then dilute with FP buffer from 10 μ M to 40nM (dilution 1:250). This is the working solution which will be a 10nM concentration in well (dilution 1:4). Keep the diluted FAM labeled peptide in the dark until use.
 4. Make unlabeled peptide dose plate with FP buffer starting with 5 μ M (final) of peptide and making 5 fold serial dilutions for 6 points using following dilution scheme.
 5. Dilute 10mM (in 100% DMSO) with DMSO to 5mM (dilution 1: 2). Then, dilute from 5mM to 500 μ M with H₂O (dilution 1:10) and then dilute with FP buffer from 500 μ M to 20 μ M (dilution 1:25). Making 5 fold serial dilutions from 20 μ M (4X) for 6 points.
 6. Transfer 10 μ l of serial diluted unlabeled peptides to each well which is filled with 20 μ l of 300nM of protein.
 7. Add 10 μ l of 10nM (4X) of FAM labeled peptide into each well and incubate for 3hr to read.
- [00275] Results from Examples 4-7 are shown in Table 8. The following scale is used for IC₅₀ and K_i values: “+” represents a value greater than 1000 nM, “++” represents a value greater than 100 and less than or equal to 1000 nM, “+++” represents a value greater than 10 nM and less than or equal to 100 nM, and “++++” represents a value of less than or equal to 10 nM. Cell viability assay results (performed as in Example 9) are also included in Table 8 using the following scale: “+” represents a value greater than 30 μ M, “++” represents a value greater than 15 μ M and less than or equal to 30 μ M, “+++” represents a value greater than 5 μ M and less than or equal to 15 μ M, and “++++” represents a value of less than or equal to 5 μ M. “IC₅₀ ratio” represents the ratio of average IC₅₀ in p53+/+ cells relative to average IC₅₀ in p53-/- cells.

Table 8

SP	IC ₅₀ (MDM2)	IC ₅₀ (MDMX)	K _i (MDM2)	K _i (MDMX)	SJSA-1 EC ₅₀ (72h)	IC ₅₀ Ratio
449	++++	++++	++++	++++	++++	
450			++		+++	
451			+++		+++	
452					+	
456			++++	+++	+++	
457			++++	++++	++++	
461					+++	
459			+	+	+	
460			+	+	+	
463					++	
464					+	
153			++++	+++	++++	1-29
465			++++	++++		
466			++++	++++		
470			++++	++++		
916	+++	+++	++++	++++	++	
917	+++	+++	++++	+++	+	

919					+++	
-----	--	--	--	--	-----	--

Example 9: Competition Binding ELISA (MDM2 & MDMX)

[00276] p53-His6 protein (30 nM/well) is coated overnight at room temperature in the wells of a 96-well Immulon plates. On the day of the experiment, plates are washed with 1X PBS-Tween 20 (0.05%) using an automated ELISA plate washer, blocked with ELISA Micro well Blocking for 30 minutes at room temperature; excess blocking agent is washed off by washing plates with 1X PBS-Tween 20 (0.05%). Peptides are diluted from 10 mM DMSO stocks to 500 μ M working stocks in sterile water, further dilutions made in 0.5% DMSO to keep the concentration of DMSO constant across the samples. The peptides are added to wells at 2X desired concentrations in 50 μ l volumes, followed by addition of diluted GST-MDM2 or GST-HMDX protein (final concentration: 10nM). Samples are incubated at room temperature for 2h, plates are washed with PBS-Tween 20 (0.05%) prior to adding 100 μ l of HRP-conjugated anti-GST antibody [Hypromatrix, INC] diluted to 0.5 μ g/ml in HRP-stabilizing buffer. Post 30 min incubation with detection antibody, plates are washed and incubated with 100 μ l per well of TMB-E Substrate solution up to 30 minutes; reactions are stopped using 1M HCL and absorbance measured at 450 nm on micro plate reader. Data is analyzed using Graph Pad PRISM software.

Example 10: Cell Viability assay

[00277] The assay is performed according to the following general protocol:

Cell Plating: Trypsinize, count and seed cells at the pre-determined densities in 96-well plates a day prior to assay. Following cell densities are used for each cell line in use:

- SJSA-1: 7500 cells/ well
- RKO: 5000 cells/well
- RKO-E6: 5000 cells/well
- HCT-116: 5000 cells/well
- SW-480: 2000 cells/well
- MCF-7: 5000 cells/well

[00278] On the day of study, replace media with fresh media with 11% FBS (assay media) at room temperature. Add 180 μ L of the assay media per well. Control wells with no cells, receive 200 μ l media.

[00279] Peptide dilution: all dilutions are made at room temperature and added to cells at room temperature.

- Prepare 10 mM stocks of the peptides in DMSO. Serially dilute the stock using 1:3 dilution scheme to get 10, 3.3, 1.1, 0.33, 0.11, 0.03, 0.01mM solutions using DMSO as diluents. Dilute

the serially DMSO-diluted peptides 33.3 times using sterile water. This gives range of 10X working stocks. Also prepare DMSO/sterile water (3% DMSO) mix for control wells.

- Thus the working stocks concentration range μM will be 300, 100, 30, 10, 3, 1, 0.3 and 0 μM . Mix well at each dilution step using multichannel.
- Row H has controls. H1- H3 will receive 20 μl of assay media. H4-H9 will receive 20 μl of 3% DMSO-water vehicle. H10-H12 will have media alone control with no cells.
- Positive control: MDM2 small molecule inhibitor, Nutlin-3a (10 mM) is used as positive control. Nutlin was diluted using the same dilution scheme as peptides.

[00280] Addition of working stocks to cells:

- Add 20 μl of 10X desired concentration to appropriate well to achieve the final concentrations in total 200 μl volume in well. (20 μl of 300 μM peptide + 180 μl of cells in media = 30 μM final concentration in 200 μl volume in wells). Mix gently a few times using pipette. Thus final concentration range used will be 30, 10, 3, 1, 0.3, 0.1, 0.03 & 0 μM (for potent peptides further dilutions are included).
- Controls include wells that get no peptides but contain the same concentration of DMSO as the wells containing the peptides, and wells containing NO CELLS.
- Incubate for 72 hours at 37°C in humidified 5% CO₂ atmosphere.
- The viability of cells is determined using MTT reagent from Promega. Viability of SJSA-1, RKO, RKO-E6, HCT-116 cells is determined on day 3, MCF-7 cells on day 5 and SW-480 cells on day 6. At the end of designated incubation time, allow the plates to come to room temperature. Remove 80 μl of assay media from each well. Add 15 μl of thawed MTT reagent to each well.
- Allow plate to incubate for 2h at 37°C in humidified 5% CO₂ atmosphere and add 100 μl solubilization reagent as per manufacturer's protocol. Incubate with agitation for 1h at room temperature and read on Synergy Biotek multiplate reader for absorbance at 570nm.
- Analyze the cell viability against the DMSO controls using GraphPad PRISM analysis tools.

[00281] Reagents:

- Invitrogen cell culture Media
 - i. Falcon 96-well clear cell culture treated plates (Nunc 353072)
- DMSO (Sigma D 2650)
- RPMI 1640 (Invitrogen 72400)
- MTT (Promega G4000)

[00282] Instruments: Multiplate Reader for Absorbance readout (Synergy 2).

[00283] Results are shown in Table 8.

Example 11: P21 ELISA assay

[00284] The assay is performed according to the following general protocol:

Cell Plating:

- Trypsinize, count and seed SJSA1 cells at the density of 7500 cells/ 100 µl/well in 96-well plates a day prior to assay.
- On the day of study, replace media with fresh RPMI-11% FBS (assay media). Add 90µL of the assay media per well. Control wells with no cells, receive 100 µl media.

[00285] Peptide dilution:

- Prepare 10 mM stocks of the peptides in DMSO. Serially dilute the stock using 1:3 dilution scheme to get 10, 3.3, 1.1, 0.33, 0.11, 0.03, 0.01mM solutions using DMSO as diluents. Dilute the serially DMSO-diluted peptides 33.3 times using sterile water. This gives range of 10X working stocks. Also prepare DMSO/sterile water (3% DMSO) mix for control wells.
- Thus the working stocks concentration range µM will be 300, 100, 30, 10, 3, 1, 0.3 and 0 µM. Mix well at each dilution step using multichannel.
- Row H has controls. H1- H3 will receive 10 µl of assay media. H4-H9 will receive 10 µl of 3% DMSO-water vehicle. H10-H12 will have media alone control with no cells.
- Positive control: MDM2 small molecule inhibitor, Nutlin-3a (10 mM) is used as positive control. Nutlin was diluted using the same dilution scheme as peptides.

[00286] Addition of working stocks to cells:

- Add 10 µl of 10X desired concentration to appropriate well to achieve the final concentrations in total 100 µl volume in well. (10 µl of 300 µM peptide + 90 µl of cells in media = 30 µM final concentration in 100 µl volume in wells). Thus final concentration range used will be 30, 10, 3, 1, 0.3 & 0 µM.
- Controls will include wells that get no peptides but contain the same concentration of DMSO as the wells containing the peptides, and wells containing NO CELLS.
- 20h-post incubation, aspirate the media; wash cells with 1X PBS (without Ca⁺⁺/Mg⁺⁺) and lyse in 60 µl of 1X Cell lysis buffer (Cell Signaling technologies 10X buffer diluted to 1X and supplemented with protease inhibitors and Phosphatase inhibitors) on ice for 30 min.
- Centrifuge plates in at 5000 rpm speed in at 4°C for 8 min; collect clear supernatants and freeze at -80 °C till further use.

[00287] Protein Estimation:

- Total protein content of the lysates is measured using BCA protein detection kit and BSA standards from Thermofisher. Typically about 6-7 µg protein is expected per well.
- Use 50 µl of the lysate per well to set up p21 ELISA.

[00288] Human Total p21 ELISA: The ELISA assay protocol is followed as per the manufacturer's instructions. 50 µl lysate is used for each well, and each well is set up in triplicate.

[00289] Reagents:

- -Cell-Based Assay (-)-Nutlin-3 (10 mM): Cayman Chemicals, catalog # 600034
- - OptiMEM, Invitrogen catalog # 51985

- -Cell Signaling Lysis Buffer (10X), Cell signaling technology, Catalog # 9803
- -Protease inhibitor Cocktail tablets(mini), Roche Chemicals, catalog # 04693124001
- -Phosphatase inhibitor Cocktail tablet, Roche Chemicals, catalog # 04906837001
- -Human total p21 ELISA kit, R&D Systems, DYC1047-5
- -STOP Solution (1M HCL), Cell Signaling Technologies, Catalog # 7002

[00290] Instruments: Micro centrifuge- Eppendorf 5415D and Multiplate Reader for Absorbance readout (Synergy 2).

Example 12: Caspase 3 Detection assay:

[00291] The assay is performed according to the following general protocol:

Cell Plating: Trypsinize, count and seed SJSA1 cells at the density of 7500 cells/ 100 µl/well in 96-well plates a day prior to assay. On the day of study, replace media with fresh RPMI-11% FBS (assay media). Add 180µL of the assay media per well. Control wells with no cells, receive 200 µl media.

[00292] Peptide dilution:

- Prepare 10 mM stocks of the peptides in DMSO. Serially dilute the stock using 1:3 dilution scheme to get 10, 3.3, 1.1, 0.33, 0.11, 0.03, 0.01mM solutions using DMSO as diluents. Dilute the serially DMSO-diluted peptides 33.3 times using sterile water This gives range of 10X working stocks. Also prepare DMSO/sterile water (3% DMSO) mix for control wells.
- Thus the working stocks concentration range µM will be 300, 100, 30, 10, 3, 1, 0.3 and 0 µM. Mix well at each dilution step using multichannel. Add 20 ul of 10X working stocks to appropriate wells.
- Row H has controls. H1- H3 will receive 20 ul of assay media. H4-H9 will receive 20 ul of 3% DMSO-water vehicle. H10-H12 will have media alone control with no cells.
- Positive control: MDM2 small molecule inhibitor, Nutlin-3a (10 mM) is used as positive control. Nutlin was diluted using the same dilution scheme as peptides.

[00293] Addition of working stocks to cells:

- **Add 10 µl of 10X desired concentration to appropriate well to achieve the final concentrations in total 100 µl volume in well.** (10 µl of 300 µM peptide + 90 µl of cells in media = 30 µM final concentration in 100 µl volume in wells). Thus final concentration range used will be 30, 10, 3, 1, 0.3& 0 µM.
- Controls will include wells that get no peptides but contain the same concentration of DMSO as the wells containing the peptides, and wells containing NO CELLS.
- 48 h-post incubation, aspirate 80 µl media from each well; add 100 µl Caspase3/7Glo assay reagent (Promega Caspase 3/7 glo assay system, G8092)per well, incubate with gentle shaking for 1h at room temperature.
- read on Synergy Biotek multiplate reader for luminescence.

- Data is analyzed as Caspase 3 activation over DMSO-treated cells.

Example 13. Cell Lysis by Peptidomimetic Macrocycles

- [00294] SJSA-1 cells are plated out one day in advance in clear flat-bottom plates (Costar, catalog number 353072) at 7500cells/well with 100ul/well of growth media, leaving row H columns 10-12 empty for media alone. On the day of the assay, media was exchanged with RPMI 1% FBS media, 90uL of media per well.
- [00295] 10 mM stock solutions of the peptidomimetic macrocycles are prepared in 100% DMSO. Peptidomimetic macrocycles were then diluted serially in 100% DMSO, and then further diluted 20-fold in sterile water to prepare working stock solutions in 5% DMSO/water of each peptidomimetic macrocycle at concentrations ranging from 500 uM to 62.5 uM.
- [00296] 10 uL of each compound is added to the 90 uL of SJSA-1 cells to yield final concentrations of 50 uM to 6.25 uM in 0.5% DMSO-containing media. The negative control (non-lytic) sample was 0.5% DMSO alone and positive control (lytic) samples include 10 uM Melittin and 1% Triton X-100.
- [00297] Cell plates are incubated for 1 hour at 37C. After the 1 hour incubation, the morphology of the cells is examined by microscope and then the plates were centrifuged at 1200rpm for 5 minutes at room temperature. 40uL of supernatant for each peptidomimetic macrocycle and control sample is transferred to clear assay plates. LDH release is measured using the LDH cytotoxicity assay kit from Caymen, catalog# 1000882.

Example 14: p53 GRIP assay

- [00298] Thermo Scientific* BioImage p53-MDM2 Redistribution Assay monitors the protein interaction with MDM2 and cellular translocation of GFP-tagged p53 in response to drug compounds or other stimuli. Recombinant CHO-hIR cells stably express human p53(1-312) fused to the C-terminus of enhanced green fluorescent protein (EGFP) and PDE4A4-MDM2(1-124), a fusion protein between PDE4A4 and MDM2(1-124). They provide a ready-to-use assay system for measuring the effects of experimental conditions on the interaction of p53 and MDM2. Imaging and analysis is performed with a HCS platform.
- [00299] CHO-hIR cells are regularly maintained in Ham's F12 media supplemented with 1% Penicillin-Streptomycin, 0.5 mg/ml Geneticin, 1 mg/ml Zeocin and 10% FBS. Cells seeded into 96-well plates at the density of 7000 cells/ 100 µl per well 18-24 hours prior to running the assay using culture media. The next day, media is refreshed and PD177 is added to cells to the final concentration of 3µM to activate foci formation. Control wells are kept without PD-177 solution. 24h post stimulation with PD177, cells are washed once with Opti-MEM Media and 50 µL of the Opti-MEM Media supplemented with PD-177(6 µM) is added to cells. Peptides are diluted from 10 mM DMSO stocks to 500 µM working stocks in sterile water, further dilutions made in 0.5%

DMSO to keep the concentration of DMSO constant across the samples. Final highest DMSO concentration is 0.5% and is used as the negative control. Cayman Chemicals Cell-Based Assay (-)-Nutlin-3 (10 mM) is used as positive control. Nutlin was diluted using the same dilution scheme as peptides. 50 µl of 2X desired concentrations is added to the appropriate well to achieve the final desired concentrations. Cells are then incubated with peptides for 6 h at 37°C in humidified 5% CO₂ atmosphere. Post-incubation period, cells are fixed by gently aspirating out the media and adding 150 µl of fixing solution per well for 20 minutes at room temperature. Fixed cells are washed 4 times with 200 µl PBS per well each time. At the end of last wash, 100 µl of 1 µM Hoechst staining solution is added. Sealed plates incubated for at least 30 min in dark, washed with PBS to remove excess stain and PBS is added to each well. Plates can be stored at 4°C in dark up to 3 days. The translocation of p53/MDM2 is imaged using Molecular translocation module on Cellomics Arrayscan instrument using 10x objective, XF-100 filter sets for Hoechst and GFP. The output parameters was Mean- CircRINGAveIntenRatio (the ratio of average fluorescence intensities of nucleus and cytoplasm, (well average)). The minimally acceptable number of cells per well used for image analysis was set to 500 cells.

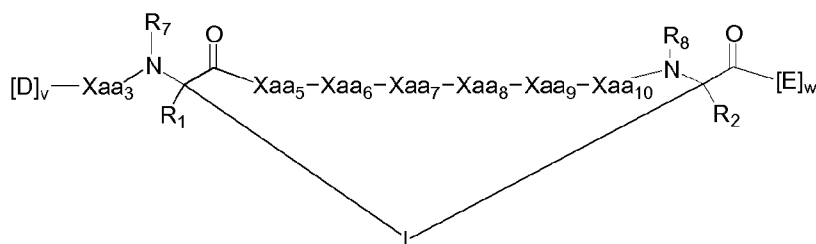
Example 15: Solubility Determination for Peptidomimetic Macrocycles

[00300] Peptidomimetic macrocycles are first dissolved in neat N, N-dimethylacetamide (DMA, Sigma-Aldrich, 38840-1L-F) to make 20X stock solutions over a concentration range of 20-140 mg/mL. The DMA stock solutions are diluted 20-fold in an aqueous vehicle containing 2% Solutol-HS-15, 25 mM Histidine, 45 mg/mL Mannitol to obtain final concentrations of 1-7 mg/ml of the peptidomimetic macrocycles in 5% DMA, 2% Solutol-HS-15, 25 mM Histidine, 45 mg/mL Mannitol. The final solutions are mixed gently by repeat pipetting or light vortexing, and then the final solutions are sonicated for 10 min at room temperature in an ultrasonic water bath. Careful visual observation is then performed under hood light using a 7x visual amplifier to determine if precipitate exists on the bottom or as a suspension. Additional concentration ranges are tested as needed to determine the maximum solubility limit for each peptidomimetic macrocycle.

CLAIMS

WHAT IS CLAIMED IS:

1. A peptidomimetic macrocycle of Formula:



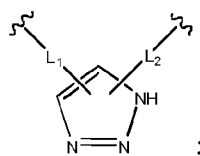
wherein:

each of Xaa₃, Xaa₅, Xaa₆, Xaa₇, Xaa₈, Xaa₉, and Xaa₁₀ is individually an amino acid, wherein at least three of Xaa₃, Xaa₅, Xaa₆, Xaa₇, Xaa₈, Xaa₉, and Xaa₁₀ are the same amino acid as the amino acid at the corresponding position of the sequence Phe₃-X₄-His₅-Tyr₆-Trp₇-Ala₈-Gln₉-Leu₁₀-X₁₁-Ser₁₂, where each X is an amino acid;

each D and E is independently an amino acid;

R₁ and R₂ are independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo-; or at least one of R₁ and R₂ forms a macrocycle-forming linker L' connected to the alpha position of one of said D or E amino acids;

each L and L' is independently a macrocycle-forming linker of the formula



L₁ and L₂ are independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, cycloarylene, heterocycloarylene, or [-R₄-K-R₄]_n, each being optionally substituted with R₅;

each R₃ is independently hydrogen, alkyl, alkenyl, alkynyl, arylalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, cycloalkylalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅;

each R₄ is independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene;

each K is independently O, S, SO, SO₂, CO, CO₂, or CONR₃;

each R₅ is independently halogen, alkyl, -OR₆, -N(R₆)₂, -SR₆, -SOR₆, -SO₂R₆, -CO₂R₆, 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;

each R_7 is independently $-H$, 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;

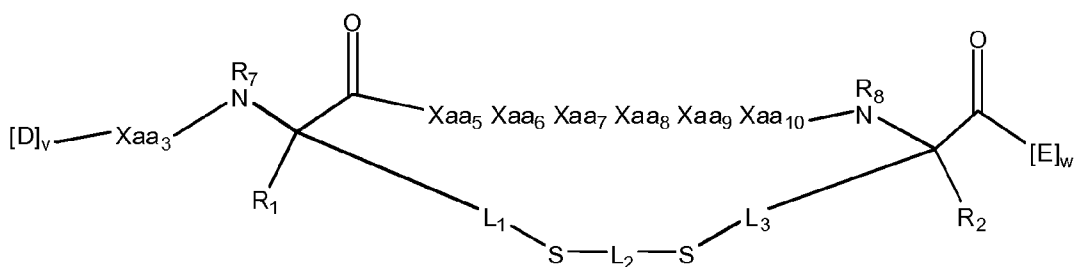
each R_8 is independently $-H$, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R_5 , or part of a cyclic structure with an E residue;

v is an integer from 1-1000, for example 1-500, 1-200, 1-100, 1-50, 1-30, 1-20 or 1-10;

w is an integer from 3-1000, for example 3-500, 3-200, 3-100, 3-50, 3-30, 3-20, or 3-10; and

n is an integer from 1-5.

2. A peptidomimetic macrocycle of Formula:



wherein:

each of Xaa_3 , Xaa_5 , Xaa_6 , Xaa_7 , Xaa_8 , Xaa_9 , and Xaa_{10} is individually an amino acid, wherein at least three of Xaa_3 , Xaa_5 , Xaa_6 , Xaa_7 , Xaa_8 , Xaa_9 , and Xaa_{10} are the same amino acid as the amino acid at the corresponding position of the sequence Phe₃-X₄-His₅-Tyr₆-Trp₇-Ala₈-Gln₉-Leu₁₀-X₁₁-Ser₁₂, where each X is an amino acid;

each D and E is independently an amino acid;

R_1 and R_2 are independently $-H$, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo-; or at least one of R_1 and R_2 forms a macrocycle-forming linker L' connected to the alpha position of one of said D or E amino acids;

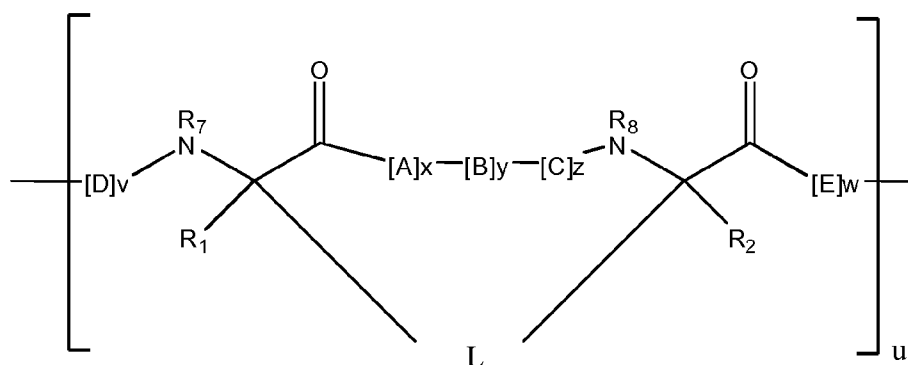
each L or L' is independently a macrocycle-forming linker of the formula $-L_1-L_2-$;

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 ;

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

- 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 unsubstituted or substituted with R_5 ;
- each 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, $-OR_6$, $-N(R_6)_2$, $-SR_6$, $-SOR_6$, $-SO_2R_6$, $-CO_2R_6$, 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;
- v is an integer from 1-1000, for example 1-500, 1-200, 1-100, 1-50, 1-30, 1-20 or 1-10;
- w is an integer from 3-1000, for example 3-500, 3-200, 3-100, 3-50, 3-30, 3-20, or 3-10; and
- n is an integer from 1-5.

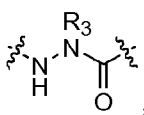
3. A peptidomimetic macrocycle comprising an amino acid sequence which is at least about 60% identical to an amino acid sequence chosen from the group consisting of the amino acid sequences in Tables 4, 4a, or 4b, wherein the peptidomimetic macrocycle has the formula:



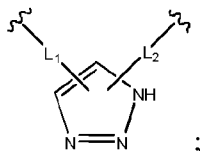
Formula (I)

wherein:

each A, C, D, and E is independently an amino acid;

B is an amino acid, , [-NH-L₃-CO-], [-NH-L₃-SO₂-], or [-NH-L₃-];

R₁ and R₂ are independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo-; or at least one of R₁ and R₂ forms a macrocycle-forming linker L' connected to the alpha position of one of said D or E amino acids; each L and L' is independently a macrocycle-forming linker of the formula



L₁, L₂ and L₃ are independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, cycloarylene, heterocycloarylene, or [-R₄-K-R₄]_n, each being optionally substituted with R₅;

each R₃ is independently hydrogen, alkyl, alkenyl, alkynyl, arylalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, cycloalkylalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅;

each R₄ is independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene;

each K is independently O, S, SO, SO₂, CO, CO₂, or CONR₃;

each R₅ is independently halogen, alkyl, -OR₆, -N(R₆)₂, -SR₆, -SOR₆, -SO₂R₆, -CO₂R₆, a fluorescent moiety, a radioisotope or a therapeutic agent;

each R₆ is independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heterocycloalkyl, a fluorescent moiety, a radioisotope or a therapeutic agent;

each R₇ is independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅, or part of a cyclic structure with a D residue;

each R₈ is independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅, or part of a cyclic structure with an E residue;

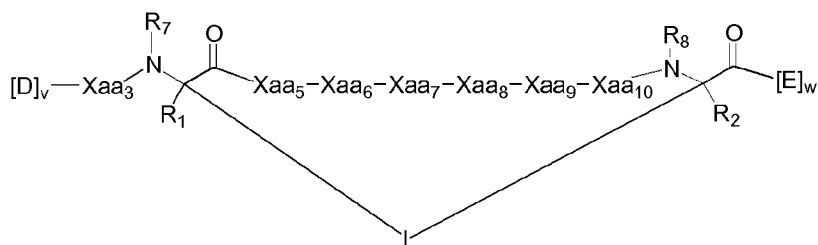
v and w are independently integers from 1-1000, for example 1-500, 1-200, 1-100, 1-50, 1-30, 1-20 or 1-10;

u is an integer from 1-10, for example 1-5, 1-3 or 1-2;

x, y and z are independently integers from 0-10, for example the sum of x+y+z is 2, 3, or 6; and

n is an integer from 1-5.

4. A peptidomimetic macrocycle of Formula:



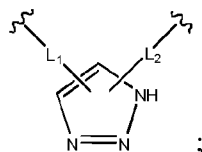
wherein:

each of Xaa₃, Xaa₅, Xaa₆, Xaa₇, Xaa₈, Xaa₉, and Xaa₁₀ is individually an amino acid, wherein at least three of Xaa₃, Xaa₅, Xaa₆, Xaa₇, Xaa₈, Xaa₉, and Xaa₁₀ are the same amino acid as the amino acid at the corresponding position of the sequence Phe₃-X₄-Glu₅-Tyr₆-Trp₇-Ala₈-Gln₉-Leu₁₀/Cba₁₀-X₁₁-Ala₁₂, where each X is an amino acid;

each D and E is independently an amino acid;

R₁ and R₂ are independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo-; or at least one of R₁ and R₂ forms a macrocycle-forming linker L' connected to the alpha position of one of said D or E amino acids;

each L and L' is independently a macrocycle-forming linker of the formula



L₁ and L₂ are independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, cycloarylene, heterocycloarylene, or [-R₄-K-R₄]_n, each being optionally substituted with R₅;

R₃ is hydrogen, alkyl, alkenyl, alkynyl, arylalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, cycloalkylalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅;

each R₄ is alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene;

each K is O, S, SO, SO₂, CO, CO₂, or CONR₃;

each R₅ is independently halogen, alkyl, -OR₆, -N(R₆)₂, -SR₆, -SOR₆, -SO₂R₆, -CO₂R₆, a fluorescent moiety, a radioisotope or a therapeutic agent;

each R₆ is independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heterocycloalkyl, a fluorescent moiety, a radioisotope or a therapeutic agent;

R₇ is -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅, or part of a cyclic structure with a D residue;

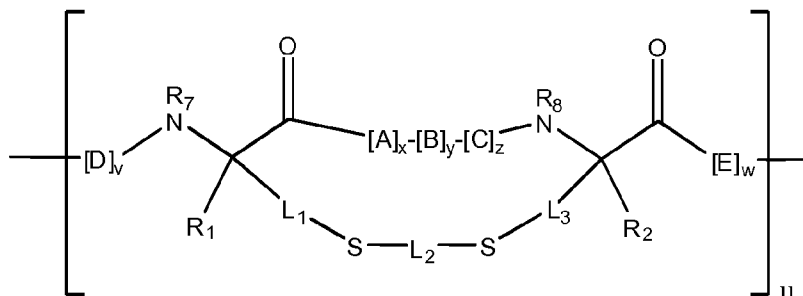
R_8 is $-H$, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R_5 , or part of a cyclic structure with an E residue;

v is an integer from 1-1000, for example 1-500, 1-200, 1-100, 1-50, 1-30, 1-20, or 1-10;

w is an integer from 3-1000, for example 3-500, 3-200, 3-100, 3-50, 3-30, 3-20, or 3-10; and

n is an integer from 1-5.

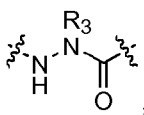
5. A peptidomimetic macrocycle comprising an amino acid sequence which is at least about 60% identical to an amino acid sequence chosen from the group consisting of the amino acid sequences in Tables 6 or 6a, wherein the peptidomimetic macrocycle has the formula:



Formula (I)

wherein:

each A, C, D, and E is independently an amino acid;

B is an amino acid, , $[-NH-L_4-CO-]$, $[-NH-L_4-SO_2-]$, 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-; or at least one of R_1 and R_2 forms a macrocycle-forming linker L' connected to the alpha position of one of said D or E amino acids;

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

L_1 , L_2 , L_3 and L_4 are independently alkylene, alkenylene, alkynylene, heteroalkylene,

cycloalkylene, heterocycloalkylene, cycloarylene, heterocycloarylene or $[-R_4-K-R_4-]_n$, each being unsubstituted or substituted with R_5 ;

each K is O, S, SO, SO₂, CO, CO₂, or CONR₃;

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

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;

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

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

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;

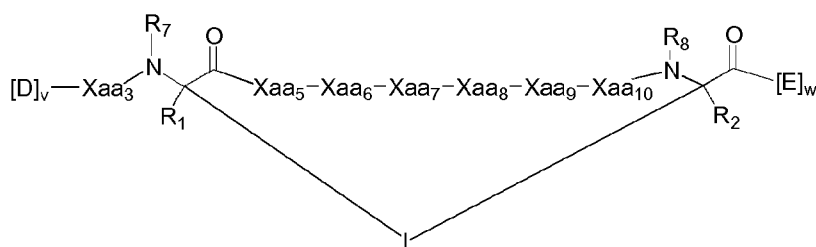
R_8 is $-H$, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R_5 , or part of a cyclic structure with an E residue;

v is an integer from 1-1000, for example 1-500, 1-200, 1-100, 1-50, 1-30, 1-20, or 1-10;

w is an integer from 3-1000, for example 3-500, 3-200, 3-100, 3-50, 3-30, 3-20, or 3-10; and

n is an integer from 1-5.

7. A peptidomimetic macrocycle of Formula:



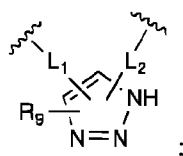
wherein:

each of Xaa_3 , Xaa_5 , Xaa_6 , Xaa_7 , Xaa_8 , Xaa_9 , and Xaa_{10} is individually an amino acid, wherein at least three of Xaa_3 , Xaa_5 , Xaa_6 , Xaa_7 , Xaa_8 , Xaa_9 , and Xaa_{10} are the same amino acid as the amino acid at the corresponding position of the sequence Phe₃-X₄-His₅-Tyr₆-Trp₇-Ala₈-Gln₉-Leu₁₀-X₁₁-Ser₁₂, where each X is an amino acid;

each D and E is independently an amino acid;

R_1 and R_2 are independently $-H$, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo-; or at least one of R_1 and R_2 forms a macrocycle-forming linker L' connected to the alpha position of one of said D or E amino acids;

each L and L' is independently a macrocycle-forming linker of the formula



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_3 is independently hydrogen, alkyl, alkenyl, alkynyl, arylalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, cycloalkylalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R_5 ;

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

each K is independently 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;

each R_7 is independently $-H$, 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;

each R_8 is independently $-H$, 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;

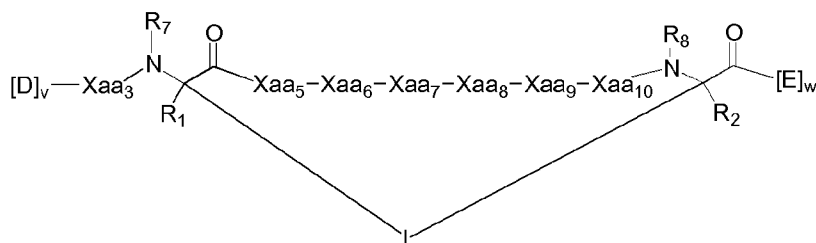
each R_9 is independently alkyl, alkenyl, alkynyl, aryl, cycloalkyl, cycloalkenyl, heteroaryl, or heterocyclyl group, unsubstituted or optionally substituted with R_a and/or R_b ;

v is an integer from 1-1000, for example 1-500, 1-200, 1-100, 1-50, 1-30, 1-20 or 1-10;

w is an integer from 3-1000, for example 3-500, 3-200, 3-100, 3-50, 3-30, 3-20, or 3-10; and

n is an integer from 1-5.

8. A peptidomimetic macrocycle of Formula:



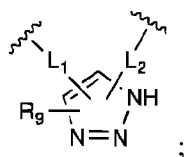
wherein:

each of Xaa₃, Xaa₅, Xaa₆, Xaa₇, Xaa₈, Xaa₉, and Xaa₁₀ is individually an amino acid, wherein at least three of Xaa₃, Xaa₅, Xaa₆, Xaa₇, Xaa₈, Xaa₉, and Xaa₁₀ are the same amino acid as the amino acid at the corresponding position of the sequence Phe₃-X₄-Glu₅-Tyr₆-Trp₇-Ala₈-Gln₉-Leu₁₀/Cba₁₀-X₁₁-Ala₁₂, where each X is an amino acid;

each D and E is independently an amino acid;

R₁ and R₂ are independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo-; or at least one of R₁ and R₂ forms a macrocycle-forming linker L' connected to the alpha position of one of said D or E amino acids;

each L and L' is independently a macrocycle-forming linker of the formula



L₁ and L₂ are independently alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, cycloarylene, heterocycloarylene, or [-R₄-K-R₄]_n, each being optionally substituted with R₅;

R₃ is hydrogen, alkyl, alkenyl, alkynyl, arylalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, cycloalkylalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅;

each R₄ is alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene;

each K is O, S, SO, SO₂, CO, CO₂, or CONR₃;

each R₅ is independently halogen, alkyl, -OR₆, -N(R₆)₂, -SR₆, -SOR₆, -SO₂R₆, -CO₂R₆, a fluorescent moiety, a radioisotope or a therapeutic agent;

each R₆ is independently -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heterocycloalkyl, a fluorescent moiety, a radioisotope or a therapeutic agent;

R₇ is -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅, or part of a cyclic structure with a D residue;

R₈ is -H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R₅, or part of a cyclic structure with an E residue;

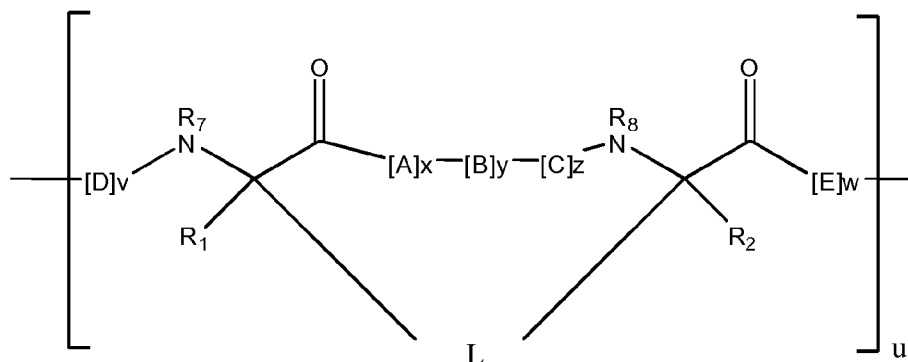
each R₉ is independently alkyl, alkenyl, alkynyl, aryl, cycloalkyl, cycloalkenyl, heteroaryl, or heterocyclyl group, unsubstituted or optionally substituted with R_a and/or R_b;

v is an integer from 1-1000, for example 1-500, 1-200, 1-100, 1-50, 1-30, 1-20, or 1-10;

w is an integer from 3-1000, for example 3-500, 3-200, 3-100, 3-50, 3-30, 3-20, or 3-10; and

n is an integer from 1-5.

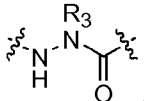
9. A peptidomimetic macrocycle comprising an amino acid sequence which is at least about 60% identical to an amino acid sequence chosen from the group consisting of the amino acid sequences in Tables 4, 4a, or 4b, wherein the peptidomimetic macrocycle has the formula:



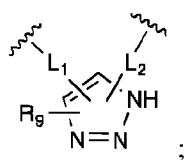
Formula (I)

wherein:

each A, C, D, and E is independently an amino acid;

B is an amino acid, , $[-NH-L_3-CO-]$, $[-NH-L_3-SO_2-]$, 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 at least one of R_1 and R_2 forms a macrocycle-forming linker L' connected to the alpha position of one of said D or E amino acids; each L and L' is independently a macrocycle-forming 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_3 is independently hydrogen, alkyl, alkenyl, alkynyl, arylalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, cycloalkylalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R_5 ;

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

each K is independently 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;

each R_7 is independently -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;

each R_8 is independently -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 R_9 is independently alkyl, alkenyl, alkynyl, aryl, cycloalkyl, cycloalkenyl, heteroaryl, or heterocyclyl group, unsubstituted or optionally substituted with R_a and/or R_b ;

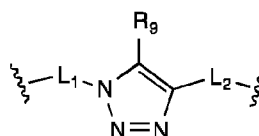
v and w are independently integers from 1-1000, for example 1-500, 1-200, 1-100, 1-50, 1-30, 1-20 or 1-10;

u is an integer from 1-10, for example 1-5, 1-3 or 1-2;

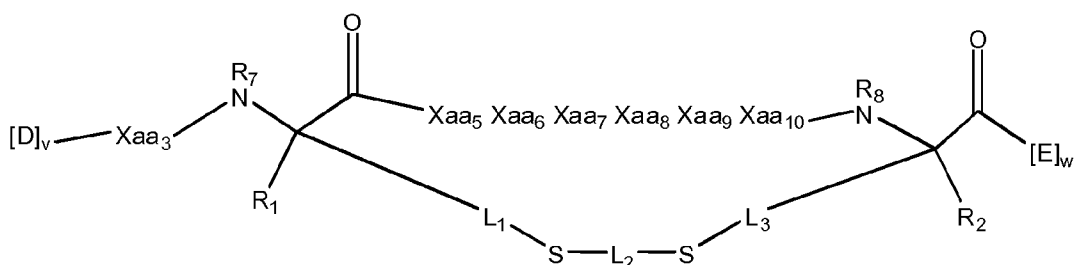
x, y and z are independently integers from 0-10, for example the sum of $x+y+z$ is 2, 3, or 6; and

n is an integer from 1-5.

10. The peptidomimetic macrocycle of claim 7, 8 or 9, wherein each L and L' is independently a macrocycle-forming linker of the formula:



11. A peptidomimetic macrocycle of Formula:



wherein:

each of Xaa_3 , Xaa_5 , Xaa_6 , Xaa_7 , Xaa_8 , Xaa_9 , and Xaa_{10} is individually an amino acid, wherein at least three of Xaa_3 , Xaa_5 , Xaa_6 , Xaa_7 , Xaa_8 , Xaa_9 , and Xaa_{10} are the same amino acid as the amino acid at the corresponding position of the sequence Phe₃-X₄-His₅-Tyr₆-Trp₇-Ala₈-Gln₉-Leu₁₀-X₁₁-Ser₁₂, where each X is an amino acid;

each D and E is independently an amino acid;

R_1 and R_2 are independently $-H$, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo-; or at least one of R_1 and R_2 forms a macrocycle-forming linker L' connected to the alpha position of one of said D or E amino acids;

each L or L' is independently 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 ;

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

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 unsubstituted or substituted with R_5 ;

each 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, $-OR_6$, $-N(R_6)_2$, $-SR_6$, $-SOR_6$, $-SO_2R_6$, $-CO_2R_6$, 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;

v is an integer from 1-1000, for example 1-500, 1-200, 1-100, 1-50, 1-30, 1-20 or 1-10;

w is an integer from 3-1000, for example 3-500, 3-200, 3-100, 3-50, 3-30, 3-20, or 3-10; and

n is an integer from 1-5.

12. The peptidomimetic macrocycle of any one of claims 1, 2, 4, 6, 7, 8, or 11, wherein Xaa_5 is Glu or an amino acid analog thereof.

13. The peptidomimetic macrocycle of claim 12, wherein Xaa_5 is Glu or an amino acid analog thereof and wherein the peptidomimetic macrocycle has an improved property, such as improved binding affinity, improved solubility, improved cellular efficacy, improved helicity, improved cell permeability, improved *in vivo* or *in vitro* anti-tumor efficacy, or improved induction of apoptosis relative to a corresponding peptidomimetic macrocycle where Xaa_5 is Ala.

14. The peptidomimetic macrocycle of any one of claims 1-13, wherein the peptidomimetic macrocycle has improved binding affinity to MDM2 or MDMX relative to a corresponding peptidomimetic macrocycle where w is 0, 1 or 2.
15. The peptidomimetic macrocycle of any one of claims 1-13, wherein the peptidomimetic macrocycle has a reduced ratio of binding affinities to MDMX versus MDM2 relative to a corresponding peptidomimetic macrocycle where w is 0, 1 or 2.
16. The peptidomimetic macrocycle of any one of claims 1-13, wherein the peptidomimetic macrocycle has improved *in vitro* anti-tumor efficacy against p53 positive tumor cell lines relative to a corresponding peptidomimetic macrocycle where w is 0, 1 or 2.
17. The peptidomimetic macrocycle of any one of claims 1-13, wherein the peptidomimetic macrocycle shows improved *in vitro* induction of apoptosis in p53 positive tumor cell lines relative to a corresponding peptidomimetic macrocycle where w is 0, 1 or 2.
18. The peptidomimetic macrocycle of any one of claims 1-13, wherein the peptidomimetic macrocycle has an improved *in vitro* anti-tumor efficacy ratio for p53 positive versus p53 negative or mutant tumor cell lines relative to a corresponding peptidomimetic macrocycle where w is 0, 1 or 2.
19. The peptidomimetic macrocycle of any one of claims 1-13, wherein the peptidomimetic macrocycle has improved *in vivo* anti-tumor efficacy against p53 positive tumors relative to a corresponding peptidomimetic macrocycle where w is 0, 1 or 2.
20. The peptidomimetic macrocycle of any one of claims 1-13, wherein the peptidomimetic macrocycle has improved *in vivo* induction of apoptosis in p53 positive tumors relative to a corresponding peptidomimetic macrocycle where w is 0, 1 or 2.
21. The peptidomimetic macrocycle of any one of claims 1-13, wherein the peptidomimetic macrocycle has improved cell permeability relative to a corresponding peptidomimetic macrocycle where w is 0, 1 or 2.
22. The peptidomimetic macrocycle of any one of claims 1-13, wherein the peptidomimetic macrocycle has improved solubility relative to a corresponding peptidomimetic macrocycle where w is 0, 1 or 2.
23. The peptidomimetic macrocycle of any preceding claim, wherein each E is independently an amino acid selected from Ala (alanine), D-Ala (D-alanine), Aib (α -aminoisobutyric acid), Sar (N-methyl glycine), and Ser (serine).

24. The peptidomimetic macrocycle of any preceding claim, wherein $[D]_v$ is $-\text{Leu}_1\text{-Thr}_2$.
25. The peptidomimetic macrocycle of any one of claims 12-24, wherein w is 3-10.
26. The peptidomimetic macrocycle of claim 25, wherein w is 3-6.
27. The peptidomimetic macrocycle of claim 25, wherein w is 6-10.
28. The peptidomimetic macrocycle of claim 27, wherein w is 6.
29. The peptidomimetic macrocycle of any one of claims 12-24, wherein v is 1-10.
30. The peptidomimetic macrocycle of claim 24, wherein v is 2-10.
31. The peptidomimetic macrocycle of claim 25, wherein v is 2-5.
32. The peptidomimetic macrocycle of claim 26, wherein v is 2.
33. The peptidomimetic macrocycle of any one of claims 1-24, wherein w is 3-1000, for example 3-500, 3-200, 3-100, 3-50, 3-30, 3-20, or 3-10.
34. The peptidomimetic macrocycle of any preceding claim, wherein the peptidomimetic macrocycle is not a macrocycle of Table 5, Table 7, Table 7a, or Table 7b.
35. The peptidomimetic macrocycle of any preceding claim, wherein each E is Ser or Ala or an analog thereof.
36. The peptidomimetic macrocycle of any preceding claim, comprising at least one amino acid which is an amino acid analog.
37. A method of treating cancer in a subject comprising administering to the subject a peptidomimetic macrocycle of any one of the preceding claims.
38. A method of modulating the activity of p53 and/or MDM2 and/or MDMX in a subject comprising administering to the subject a peptidomimetic macrocycle any one of the preceding claims.
39. A method of antagonizing the interaction between p53 and MDM2 and/or between p53 and MDMX proteins in a subject comprising administering to the subject a peptidomimetic macrocycle any one of the preceding claims.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 13/26241

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 38/12 (2013.01)

USPC - 514/19.9

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - A61K 38/12 (2013.01)

USPC - 514/19.9

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

IPC(8) - A61K 38/12 (2013.01)

USPC - 514/19.9;530/321,21.1

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

PatBase, GoogleScholar, Dialog

Peptidomimetic, macrocycle, MDM2, MDMX, Triazole, E3 ligase inhibitors, MDM murine double minute 2 protein, nutlin, TRIAZOLE-CROSSLINKED THIOETHER-CROSSLINKED

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2010/0298201 A1 (NASH et al.) 25 November 2010 (25.11.2010) para[0031], para[0074], para[0075], para[0081]-para[0097], para[0246], para[0257], para[0270]	1-13
Y	Kallen et al. Crystal Structures of Human MdmX (HdmX) in Complex with p53 Peptide Analogues Reveal Surprising Conformational Changes. March 27, 2009 The Journal of Biological Chemistry, 284, 8812-8821. page 8815, Fig. 1, cmp 2, R=Cl, page 8813, col 1, page 8819, col 2	1-13

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

20 April 2013 (20.04.2013)

Date of mailing of the international search report

23 MAY 2013

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-3201

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300

RCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 13/26241

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

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

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

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

3. ☒ Claims Nos.: 14-39
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.