Office de la Propriété Intellectuelle du Canada
Un organisme d'Industrie Canada

Canadian Intellectual Property Office
An agency of Industry Canada

CA 2743177 A1 2010/05/27

(21) 2 743 177

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2009/11/24
(87) Date publication PCT/PCT Publication Date: 2010/05/27
(85) Entrée phase nationale/National Entry: 2011/05/10
(86) N° demande PCT/PCT Application No.: US 2009/065824
(87) N° publication PCT/PCT Publication No.: 2010/060112
(30) Priorité/Priority: 2008/11/24 (US61/117,508)

(51) Cl.Int./Int.Cl. *C07K 2/00* (2006.01), *A61K 38/00* (2006.01), *C07K 1/107* (2006.01)

(71) Demandeur/Applicant:
AILERON THERAPEUTICS, INC., US

(72) Inventeurs/Inventors:
NASH, HUW M., US;
ANNIS, DAVID ALLEN, US;
KAPELLER-LIBERMANN, ROSANA, US;
SAWYER, TOMI K., US;
KAWAHATA, NORIYUKI, US

(74) Agent: GOWLING LAFLEUR HENDERSON LLP

(54) Titre : MACROCYCLES PEPTIDOMIMETIQUES AVEC PROPRIETES AMELIOREES
(54) Title: PEPTIDOMIMETIC MACROCYCLES WITH IMPROVED PROPERTIES

(57) Abrégé/Abstract:
The present invention provides biologically active peptidomimetic macrocycles with improved properties relative to their corresponding polypeptides. The invention additionally provides methods of preparing and using such macrocycles, for example in therapeutic applications.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 May 2010 (27.05.2010)

PCT

(10) International Publication Number
WO 2010/060112 A1

(51) International Patent Classification:
A61K 38/12 (2006.01) *A61K 38/00* (2006.01)

(21) International Application Number:
PCT/US2009/065824

(22) International Filing Date:
24 November 2009 (24.11.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/117,508 24 November 2008 (24.11.2008) US

(71) Applicant *(for all designated States except US)*: **AILERON THERAPEUTICS, INC.** [US/US]; 840 Memorial Drive, Cambridge, MA 02139 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **NASH, Huw, M.** [US/US]; 79 Ledge Rock Road, Concord, MA 01742 (US). **ANNIS, David, Allen** [US/US]; 508 Green Street, No. 3, Cambridge, MA 02139 (US). **KAPELLER-LIBERMANN, Rosana** [US/US]; 86 Beacon Street, Chestnut Hill, MA 02467 (US). **SAWYER, Tomi, K.** [US/US]; 8 Nathan Stone Road, Southborough, MA 01772 (US). **KAWAHATA, Noriyuki** [US/US]; 30 School Street, Unit #3, Somerville, MA 02143 (US).

(74) Agents: **ORBAI, Lucian** et al.; Wilson Sonsini Goodrich & Rosati, 650 Page Mill Road, Palo Alto, CA 94304-1050 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

WO 2010/060112 A1

(54) Title: PEPTIDOMIMETIC MACROCYCLES WITH IMPROVED PROPERTIES

(57) Abstract: The present invention provides biologically active peptidomimetic macrocycles with improved properties relative to their corresponding polypeptides. The invention additionally provides methods of preparing and using such macrocycles, for example in therapeutic applications.

PEPTIDOMIMETIC MACROCYCLES WITH IMPROVED PROPERTIES

CROSS REFERENCE

[0001] This application claims the benefit of U.S. Provisional Application No. 61/117,508, filed November 24, 2008, which is incorporated herein in its entirety by reference.

BACKGROUND OF THE INVENTION

[0002] Recombinant or synthetically produced polypeptides have important applications as pharmaceuticals. Polypeptides such as short peptides, however, often suffer from poor metabolic stability, poor cell penetrability, and promiscuous binding due to conformational flexibility. Various approaches to stabilizing helical peptides have been tried, for example by using intramolecular crosslinkers to maintain the peptide in a desired configuration by introducing disulfide bonds, amide bonds, or carbon-carbon bonds to link amino acid side chains. See, e.g., Jackson et al. (1991), J. Am. Chem. Soc. 113:9391-9392; Phelan et al. (1997), J. Am. Chem. Soc. 119:455-460; Taylor (2002), Biopolymers 66: 49-75; Brunel et al. (2005), Chem. Commun. (20):2552-2554; Hiroshige et al. (1995), J. Am. Chem. Soc. 117: 11590-11591; Blackwell et al. (1998), Angew. Chem. Int. Ed. 37:3281-3284; Schafmeister et al. (2000), J. Am. Chem. Soc. 122:5891-5892; Walensky et al. (2004), Science 305:1466-1470; Bernal et al. (2007), J. Am. Chem Soc. 129:2456-2457; United States Patent Application 2005/0250680, filed Nov. 5, 2004; United States Patent 7,192,713 B1 (Verdine et al); United States Patent Application No. 11/957,325 filed Dec. 14, 2007; US Patent Application No. 12/037,041 filed Feb. 25, 2008 and U.S. Patent 5,811,515, the contents of which patents and publications are incorporated herein by reference. There remains a significant need for therapeutic and pharmaceutically useful polypeptides with improved biological properties such as improved *in vivo* half-lives, efficacy at lower doses or reduced frequency of administration.

SUMMARY OF THE INVENTION

[0003] The present invention addresses these and other needs. In one aspect, the present invention provides helical peptidomimetic macrocycles with improved pharmacokinetic properties relative to their corresponding non-cross-linked counterparts.

[0004] For example, the present invention provides a method of increasing the *in vivo* half-life of a helical polypeptide by installing one or more cross-links. In some embodiments of the method, the *in vivo* half-life of said polypeptide is increased on average at least 50-fold relative to a corresponding polypeptide lacking said cross-links. In other embodiments of the method, the *in vivo* half-life of said polypeptide is increased at least 100-fold, 150-fold or 200-fold relative to a corresponding polypeptide lacking said cross-links. In some embodiments, the polypeptide is selected such that the apparent serum binding affinity (Kd*) of the crosslinked polypeptide is 1, 3, 10, 70 micromolar or greater. In other embodiments, the Kd* of the crosslinked polypeptide is 1 to 10, 70, or 700 micromolar. In other embodiments, the crosslinked polypeptides is selected such that it possesses an estimated free fraction in human blood of between 0.1 and 50%, or between 0.15 and 10%. In some embodiments, the polypeptide is selected such that the % helicity of the crosslinked polypeptide is greater than 25%, 50% or 75% at room temperature under aqueous conditions. In other embodiments, the % helicity of the crosslinked polypeptide is increased at least 2-fold, 5-fold or 10-fold relative to a corresponding polypeptide lacking said cross-links.

[0005] In some embodiments of the method, said polypeptide contains one crosslink. In other embodimentsof the method, said polypeptide contains two cross-links.

[0006] In some embodiments of the method, one crosslink connects two α-carbon atoms. In other embodiments of the method, one α-carbon atom to which one crosslink is attached is substituted with a substituent of formula R–. In another embodiment of the method, two α-carbon atoms to which one crosslink is attached are substituted with independent substituents of formula R–.

[0007] In one embodiment of the methods of the invention, R– is alkyl. For example, R– is methyl. Alternatively, R– and any portion of one crosslink taken together can form a cyclic structure. In another embodiment of the method, one crosslink is formed of consecutive carbon-carbon bonds. For example, one crosslink may comprise at least 8, 9, 10, 11, or 12 consecutive bonds. In other embodiments, one crosslink may comprise at least 7, 8, 9, 10, or 11 carbon atoms.

[0008] In another embodiment of the method, the crosslinked polypeptide comprises an α-helical domain of a BCL-2 family member. For example, the crosslinked polypeptide comprises a BH3 domain. In other embodiments, the crosslinked polypeptide comprises at least 60%, 70%, 80%, 85%, 90% or 95% of any of the sequences in Tables 1, 2, 3 and 4.

[0009] In some embodiments of the method, the crosslinked polypeptide penetrates cell membranes by an energy-dependent process and binds to an intracellular target.

[0010] In other embodiments, the present invention provides a helical polypeptide comprising one or more cross-links, wherein the cross-linked helical polypeptide has an *in vivo* half-life greater than 360 minutes. In other embodiments, the *in vivo* half-life of said polypeptide is greater than 500 minutes or 1,000 minutes. In another embodiment, the *in vivo* half-life of said polypeptide is between 500-5,000 minutes.

[0011] In some embodiments, said helical polypeptide contains one crosslink. In other embodiments, said helical polypeptide contains two cross-links.

[0012] In some embodiments, one crosslink connects two α-carbon atoms. In other embodiments, one α-carbon atom to which one crosslink is attached is substituted with a substituent of formula R–. In another embodiment, two α-carbon atoms to which one crosslink is attached are substituted with independent substituents of formula R–.

[0013] In one embodiment of the invention, R– is alkyl. For example, R– is methyl. Alternatively, R– and any portion of one crosslink taken together can form a cyclic structure. In another embodiment, one crosslink is formed of consecutive carbon-carbon bonds. For example, one crosslink may comprise at least 8, 9, 10, 11, or 12 consecutive bonds. In other embodiments, one crosslink may comprise at least 7, 8, 9, 10, or 11 carbon atoms.

[0014] In another embodiment, the crosslinked polypeptide comprises an α-helical domain of a BCL-2 family member. For example, the crosslinked polypeptide comprises a BH3 domain. In other embodiments, the crosslinked polypeptide comprises at least 60%, 70%, 80%, 85%, 90% or 95% of any of the sequences in Tables 1, 2, 3 and 4.

[0015] In some embodiments, the crosslinked polypeptide penetrates cell membranes by an energy-dependent process and binds to an intracellular target.

INCORPORATION BY REFERENCE

[0016] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0018] FIGURE 1 describes sequences of peptidomimetic macrocycles of the invention and sequences of the corresponding non-cross-linked counterparts.

[0019] FIGURE 2 lists the *in vivo* half-lives of peptidomimetic macrocycles of the invention. Half-lives were measured in Sprague Dawley rats after one intravenous bolus injection at 0.6 mg/mL or 2 mg/mL of each unlabeled peptidomimetic macrocycle at a 3 mg/kg or 10 mg/kg dose, respectively. Three animals were used per compound and concentrations were determined by mass-spectrometric analysis of blood levels (plasma).

[0020] FIGURES 3a-u illustrate blood plasma concentration curves for several peptidomimetic macrocycles of the invention.

[0021] FIGURE 4 shows the apparent rat serum binding affinity and estimated free fraction in rat blood of peptidomimetic marocycles of the invention.

[0022] FIGURE 5 illustrates the PK profile in rat and monkey for a peptidomimetic macrocycle of the invention.

[0023] FIGURE 6 illustrates actual and predicted PK properties such as clearance rates of peptidomimetic macrocycles of the invention in rodents and higher species.

[0024] FIGURE 7 shows the molar ellipticity at 222 nm and estimated % helicity of peptidomimetic marocycles of the invention.

DETAILED DESCRIPTION OF THE INVENTION

[0025] 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.

[0026] 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.

[0027] As used herein, the term "stability" refers to the maintenance of a defined secondary structure in solution by a peptidomimetic macrocycle of the invention as measured by circular dichroism, NMR or another biophysical measure, or resistance to proteolytic degradation *in vitro* or *in vivo*. Non-limiting examples of secondary structures contemplated in this invention are α-helices, β-turns, and β-pleated sheets.

[0028] As used herein, the term "helical stability" refers to the maintenance of α helical structure by a peptidomimetic macrocycle of the invention as measured by circular dichroism or NMR. For example, in some embodiments,

the peptidomimetic macrocycles of the invention exhibit at least a 1.25, 1.5, 1.75 or 2-fold increase in α-helicity as determined by circular dichroism compared to a corresponding uncrosslinked polypeptide.

[0029] The term "α-amino acid" or simply "amino acid" refers to a molecule containing both an amino group and a carboxyl group bound to a carbon which is designated the α-carbon. Suitable amino acids include, without limitation, both the D-and L-isomers of the naturally-occurring amino acids, as well as non-naturally occurring amino acids prepared by organic synthesis or other metabolic routes. Unless the context specifically indicates otherwise, the term amino acid, as used herein, is intended to include amino acid analogs.

[0030] 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.

[0031] The term "amino acid analog" or "non-natural amino acid" refers to a molecule which is structurally similar to an amino acid and which can be substituted for an amino acid in the formation of a peptidomimetic macrocycle. Amino acid analogs include, without limitation, compounds which are structurally identical to an amino acid, as defined herein, except for the inclusion of one or more additional methylene groups between the amino and carboxyl group (*e.g.*, α-amino β-carboxy acids), or for the substitution of the amino or carboxy group by a similarly reactive group (*e.g.*, substitution of the primary amine with a secondary or tertiary amine, or substitution or the carboxy group with an ester).

[0032] A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequence of a polypeptide (*e.g.*, a BH3 domain or the p53 MDM2 binding domain) without abolishing or substantially altering its essential biological or biochemical activity (*e.g.*, receptor binding or activation). An "essential" amino acid residue is a residue that, when altered from the wild-type sequence of the polypeptide, results in abolishing or substantially abolishing the polypeptide's essential biological or biochemical activity.

[0033] A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (*e.g.*, K, R, H), acidic side chains (*e.g.*, D, E), uncharged polar side chains (*e.g.*, G, N, Q, S, T, Y, C), nonpolar side chains (*e.g.*, A, V, L, I, P, F, M, W), beta-branched side chains (*e.g.*, T, V, I) and aromatic side chains (*e.g.*, Y, F, W, H). Thus, a predicted nonessential amino acid residue in a BH3 polypeptide, for example, is preferably replaced with another amino acid residue from the same side chain family. Other examples of acceptable substitutions are substitutions based on isosteric considerations (e.g. norleucine for methionine) or other properties (e.g. 2-thienylalanine for phenylalanine).

[0034] 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.

[0035] The symbol " " when used as part of a molecular structure refers to a single bond or a *trans* or *cis* double bond.

[0036] The term "amino acid side chain" refers to a moiety attached to the α-carbon in an amino acid. For example, the amino acid side chain for alanine is methyl, the amino acid side chain for phenylalanine is phenylmethyl, the amino acid side chain for cysteine is thiomethyl, the amino acid side chain for aspartate is carboxymethyl, the amino acid side chain for tyrosine is 4-hydroxyphenylmethyl, etc. Other non-naturally occurring amino acid side

chains are also included, for example, those that occur in nature (*e.g.*, an amino acid metabolite) or those that are made synthetically (*e.g.*, an α,α di-substituted amino acid).

[0037] 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.

[0038] 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).

[0039] The term "macrocyclization reagent" or "macrocycle-forming reagent" as used herein refers to any reagent which may be used to prepare a peptidomimetic macrocycle of the invention by mediating the reaction between two reactive groups. Reactive groups may be, for example, an azide and alkyne, in which case macrocyclization reagents include, without limitation, Cu reagents such as reagents which provide a reactive Cu(I) species, such as CuBr, CuI or CuOTf, as well as Cu(II) salts such as $Cu(CO_2CH_3)_2$, $CuSO_4$, and $CuCl_2$ that can be converted in situ to an active Cu(I) reagent by the addition of a reducing agent such as ascorbic acid or sodium ascorbate. Macrocyclization reagents may additionally include, for example, Ru reagents known in the art such as $Cp^*RuCl(PPh_3)_2$, $[Cp^*RuCl]_4$ or other Ru reagents which may provide a reactive Ru(II) species. In other cases, the reactive groups are terminal olefins. In such embodiments, the macrocyclization reagents or macrocycle-forming reagents are metathesis catalysts including, but not limited to, stabilized, late transition metal carbene complex catalysts such as Group VIII transition metal carbene catalysts. For example, such catalysts are Ru and Os metal centers having a +2 oxidation state, an electron count of 16 and pentacoordinated. Additional catalysts are disclosed in Grubbs et al., "Ring Closing Metathesis and Related Processes in Organic Synthesis" Acc. Chem. Res. 1995, 28, 446-452, and U.S. Pat. No. 5,811,515. In yet other cases, the reactive groups are thiol groups. In such embodiments, the macrocyclization reagent is, for example, a linker functionalized with two thiol-reactive groups such as halogen groups.

[0040] The term "halo" or "halogen" refers to fluorine, chlorine, bromine or iodine or a radical thereof.

[0041] 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, C_1-C_{10} 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.

[0042] The term "alkylene" refers to a divalent alkyl (*i.e.*, -R-).

[0043] The term "alkenyl" refers to a hydrocarbon chain that is a straight chain or branched chain having one or more carbon-carbon double bonds. The alkenyl moiety contains the indicated number of carbon atoms. For example, C_2-C_{10} indicates that the group has from 2 to 10 (inclusive) carbon atoms in it. The term "lower alkenyl" refers to a C_2-C_6 alkenyl chain. In the absence of any numerical designation, "alkenyl" is a chain (straight or branched) having 2 to 20 (inclusive) carbon atoms in it.

[0044] The term "alkynyl" refers to a hydrocarbon chain that is a straight chain or branched chain having one or more carbon-carbon triple bonds. The alkynyl moiety contains the indicated number of carbon atoms. For example, C_2-C_{10} indicates that the group has from 2 to 10 (inclusive) carbon atoms in it. The term "lower alkynyl" refers to a C_2-C_6 alkynyl chain. In the absence of any numerical designation, "alkynyl" is a chain (straight or branched) having 2 to 20 (inclusive) carbon atoms in it.

[0045] The term "aryl" refers to a 6-carbon monocyclic or 10-carbon bicyclic aromatic ring system wherein 0, 1, 2, 3, or 4 atoms of each ring are substituted by a substituent. Examples of aryl groups include phenyl, naphthyl and the like. The term "arylalkyl" or the term "aralkyl" refers to alkyl substituted with an aryl. The term "arylalkoxy" refers to an alkoxy substituted with aryl.

[0046] "Arylalkyl" refers to an aryl group, as defined above, wherein one of the aryl group's hydrogen atoms has been replaced with a C_1-C_5 alkyl group, as defined above. Representative examples of an arylalkyl group include, but are not limited to, 2-methylphenyl, 3-methylphenyl, 4-methylphenyl, 2-ethylphenyl, 3-ethylphenyl, 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.

[0047] "Arylamido" refers to an aryl group, as defined above, wherein one of the aryl group's hydrogen atoms has been replaced with one or more -$C(O)NH_2$ groups. Representative examples of an arylamido group include 2-C(O)NH2-phenyl, 3-$C(O)NH_2$-phenyl, 4-$C(O)NH_2$-phenyl, 2-$C(O)NH_2$-pyridyl, 3-$C(O)NH_2$-pyridyl, and 4-$C(O)NH_2$-pyridyl,

[0048] "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.

[0049] "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)-$CH_3$-CH3, and –$CH_2$-$CH_2$–NH-C(O)-$CH{=}CH_2$.

[0050] "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, -$CH_2CH_2CH_2OH$, -$CH_2CH_2CH_2CH_2OH$, -$CH_2CH_2CH_2$ CH_2CH_2OH, -$CH_2CH(OH)CH_3$, -$CH_2CH(OH)CH_2CH_3$, -$CH(OH)CH_3$ and -$C(CH_3)_2CH_2OH$.

[0051] "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 -$C(CH_3)_2CH_2COOH$.

[0052] 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.

[0053] 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.

[0054] 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.

[0055] 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.

[0056] 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.

[0057] 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.

[0058] In some embodiments, the compounds of this invention contain one or more asymmetric centers and thus occur as racemates and racemic mixtures, single enantiomers, individual diastereomers and diastereomeric mixtures. All such isomeric forms of these compounds are included in the present invention unless expressly provided otherwise. In some embodiments, the compounds of this invention are also represented in multiple tautomeric forms, in such instances, the invention includes all tautomeric forms of the compounds described herein (*e.g.*, if alkylation of a ring system results in alkylation at multiple sites, the invention includes all such reaction products). All such isomeric forms of such compounds are included in the present invention unless expressly provided otherwise. All crystal forms of the compounds described herein are included in the present invention unless expressly provided otherwise.

[0059] 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%.

[0060] As used herein, the recitation of a numerical range for a variable is intended to convey that the invention may be practiced with the variable equal to any of the values within that range. Thus, for a variable which is inherently discrete, the variable is equal to any integer value within the numerical range, including the end-points of the range. Similarly, for a variable which is inherently continuous, the variable is equal to any real value within the numerical range, including the end-points of the range. As an example, and without limitation, a variable which is described as having values between 0 and 2 takes the values 0, 1 or 2 if the variable is inherently discrete, and takes the values 0.0, 0.1, 0.01, 0.001, or any other real values ≥ 0 and ≤ 2 if the variable is inherently continuous.

[0061] 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."

[0062] The term "on average" represents the mean value derived from performing at least three independent replicates for each data point.

[0063] The term "biological activity" encompasses structural and functional properties of a macrocycle of the invention. 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.

[0064] 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.

Biological Properties of the Peptidomimetic Macrocycles of the Invention

[0065] In one aspect, the invention provides a method of increasing the *in vivo* half-life of a helical polypeptide by installing one or more cross-links. For example, the *in vivo* half-life of said polypeptide is increased on average at least 50-fold relative to a corresponding polypeptide lacking said cross-links. In other embodiments of the method, the *in vivo* half-life of said polypeptide is increased at least 100-fold, 150-fold or 200-fold relative to a corresponding polypeptide lacking said cross-links. In other embodiments, the present invention provides a helical polypeptide comprising one or more cross-links, wherein the cross-linked helical polypeptide has an *in vivo* half-life greater than 360 minutes. In other embodiments, the *in vivo* half-life of said polypeptide is greater than 500 minutes or 1,000 minutes. In another embodiment, the *in vivo* half-life of said polypeptide is between 500-5,000 minutes. In another embodiment, the *in vivo* half-life of said polypeptide is determined after intravenous administration.

[0066] In some embodiments, the polypeptide is selected such that the apparent serum binding affinity (Kd*) of the crosslinked polypeptide is 1, 3, 10, 70 micromolar or greater. In other embodiments, the Kd* of the crosslinked polypeptide is 1 to 10, 70, or 700 micromolar. In other embodiments, the crosslinked polypeptides is selected such that it possesses an estimated free fraction in human blood of between 0.1 and 50%, or between 0.15 and 10%.

[0067] The present invention provides a method of identifying cross-linked polypeptides with the desired serum binding affinities, comprising the steps of synthesizing analogs of the parent cross-linked polypeptide and performing cellular assays in the absence of serum proteins and also in the presence of two or more concentrations of serum, so as to determine the apparent affinity of each cross-linked polypeptide for serum proteins and to calculate an EC_{50} in whole blood by mathematical extrapolation.

[0068] In one embodiment, the apparent Kd values for serum protein by EC50 shift analysis is used to provide a simple and rapid means of quantifying the propensity of experimental compounds to bind HSA and other serum proteins. A linear relationship exists between the apparent EC_{50} in the presence of serum protein (EC'_{50}) and the amount of serum protein added to an *in vitro* assay. This relationship is defined by the binding affinity of the compound for serum proteins, expressed as K_d*. This term is an experimentally determined, *apparent* dissociation constant that may result from the cumulative effects of multiple, experimentally indistinguishable, binding events. The form of this relationship is presented here in Eq. 1, and its derivation can be found in Copeland et al, *Biorg. Med Chem Lett.* ***2004,*** 14:2309-2312,the contents of which are incorporated herein by reference.

(1) $$EC'_{50} = EC_{50} + P\left(\frac{n}{1+\frac{K_d^*}{EC_{50}}}\right)$$

[0069] A significant proportion of serum protein binding can be ascribed to drug interactions with serum albumin, due to the very high concentration of this protein in serum (35– 50 g/L or 530–758 μM). To calculate the K_d value for these compounds we have assumed that the shift in EC_{50} upon protein addition can be ascribed fully to the serum albumin present in the added serum, where P is 700 μM for 100% serum, P is 70 μM for 10% serum, etc. We further make the simplifying assumption that all of the compounds bind serum albumin with a 1:1 stoichiometry, so that the term *n* in Eq. (1) is fixed at unity. With these parameters in place we calculate the K_d* value for each stapled peptide from the changes in EC_{50} values with increasing serum (and serum protein) concentrations by nonlinear regression analysis using Mathematica 4.1 (Wolfram Research, Inc., www.wolfram.com). The free fraction in blood is estimated per the following equation, where [*serum albumin*]$_{total}$ is set at 700 μM, as derived by Trainor, *Expert Opin. Drug Disc.*, **2007**, *2*(1):51-64, the contents of which are incorporated herein by reference. The formula below shows an embodiment where the serum albumin is human serum albumin.

(2) $$FreeFraction = \frac{K_d^*}{K_d^* + [HSA]_{total}}$$

[0070] In one embodiment, the improved biological activity is measured as increased cell penetrability or an increased ability to induce apoptosis. In yet other embodiments, the biological activity is measured as the percentage of the number of cells killed in an in vitro assay in which cultured cells are exposed to an effective concentration of said polypeptide.

[0071] In some embodiments, the polypeptide is selected such that the percent helicity of the crosslinked polypeptide is greater than 25%, 50% or 75% at room temperature under aqueous conditions. In other embodiments, the percent helicity at room temperature under aqueous conditions of the crosslinked polypeptide is increased at least 2-fold, 5-fold or 10-fold relative to a corresponding polypeptide lacking said cross-links.

Design of the Peptidomimetic Macrocycles of the Invention

[0072] Any protein or polypeptide with a known primary amino acid sequence which contains a helical structure believed to impart biological activity is the subject of the present invention. For example, the sequence of the polypeptide can be analyzed and amino acid analogs containing groups reactive with macrocyclization reagents can be substituted at the appropriate positions. The appropriate positions are determined by ascertaining which molecular surface(s) of the secondary structure is (are) required for biological activity and, therefore, across which other surface(s) the macrocycle forming linkers of the invention can form a macrocycle without sterically blocking the surface(s) required for biological activity. Such determinations are made using methods such as X-ray crystallography of complexes between the secondary structure and a natural binding partner to visualize residues (and surfaces) critical for activity; by sequential mutagenesis of residues in the secondary structure to functionally identify residues (and surfaces) critical for activity; or by other methods. By such determinations, the appropriate amino acids are substituted with the amino acids analogs and macrocycle-forming linkers of the invention. For example, for an α-helical secondary structure, one surface of the helix (*e.g.*, a molecular surface extending longitudinally along the axis of the helix and radially 45-135° about the axis of the helix) may be required to make contact with another biomolecule *in vivo* or *in vitro* for biological activity. In such a case, a macrocycle-forming linker is designed to link two α-carbons of the helix while extending longitudinally along the surface of the helix in the portion of that surface not directly required for activity.

[0073] In some embodiments of the invention, the peptide sequence is derived from the BCL-2 family of proteins. The BCL-2 family is defined by the presence of up to four conserved BCL-2 homology (BH) domains designated BH1, BH2, BH3, and BH4, all of which include α-helical segments (Chittenden *et al.* (1995), *EMBO* 14:5589; Wang *et al.* (1996), *Genes Dev.* 10:2859). Anti-apoptotic proteins, such as BCL-2 and BCL-X_L, display sequence conservation in all BH domains. Pro-apoptotic proteins are divided into "multidomain" family members (*e.g.*, BAK, BAX), which possess homology in the BH1, BH2, and BH3 domains, and "BH3-domain only" family members (*e.g.*, BID, BAD, BIM, BIK, NOXA, PUMA), that contain sequence homology exclusively in the BH3 amphipathic α-helical segment. BCL-2 family members have the capacity to form homo- and heterodimers, suggesting that competitive binding and the ratio between pro- and anti-apoptotic protein levels dictates susceptibility to death stimuli. Anti-apoptotic proteins function to protect cells from pro-apoptotic excess, *i.e.*, excessive programmed cell death. Additional "security" measures include regulating transcription of pro-apoptotic proteins and maintaining them as inactive conformers, requiring either proteolytic activation, dephosphorylation, or ligand-induced conformational change to activate pro-death functions. In certain cell types, death signals received at the plasma membrane trigger apoptosis via a mitochondrial pathway. The mitochondria can serve as a gatekeeper of cell death by sequestering cytochrome c, a critical component of a cytosolic complex which activates caspase 9, leading to fatal downstream proteolytic events. Multidomain proteins such as BCL-2/BCL-X_L and BAK/BAX play dueling roles of guardian and executioner at the mitochondrial membrane, with their activities further regulated by upstream BH3-only members of the BCL-2 family. For example, BID is a member of the BH3-domain only family of pro-apoptotic proteins, and transmits death signals received at the plasma membrane to effector pro-apoptotic proteins at the mitochondrial membrane. BID has the capability of interacting with both pro- and anti-apoptotic proteins, and upon activation by caspase 8, triggers cytochrome c release and mitochondrial apoptosis. Deletion and mutagenesis studies determined that the amphipathic α-helical BH3 segment of pro-apoptotic family members may function as a death domain and thus may represent a critical structural motif for interacting with multidomain apoptotic proteins. Structural studies have shown that the BH3 helix can interact with anti-apoptotic proteins by inserting into a hydrophobic groove formed by the interface of BH1, 2 and 3 domains. Activated BID can be bound and sequestered by anti-apoptotic proteins (*e.g.*, BCL-2 and BCL-X_L) and can trigger activation of the pro-apoptotic proteins BAX and BAK, leading to cytochrome c release and a mitochondrial apoptosis program. BAD is also a BH3-domain only pro-apoptotic family member whose expression triggers the activation of BAX/BAK. In contrast to BID, however, BAD displays preferential binding to anti-apoptotic family members, BCL-2 and BCL-X_L. Whereas the BAD BH3 domain exhibits high affinity binding to BCL-2, BAD BH3 peptide is unable to activate cytochrome c release from mitochondria *in vitro*, suggesting that BAD is not a direct activator of BAX/BAK. Mitochondria that over-express BCL-2 are resistant to BID-induced cytochrome c release, but co-treatment with BAD can restore BID sensitivity. Induction of mitochondrial apoptosis by BAD appears to result from either: (1) displacement of BAX/BAK activators, such as BID and BID-like proteins, from the BCL-2/BCL-XL binding pocket, or (2) selective occupation of the BCL-2/BCL-XL binding pocket by BAD to prevent sequestration of BID-like proteins by anti-apoptotic proteins. Thus, two classes of BH3-domain only proteins have emerged, BID-like proteins that directly activate mitochondrial apoptosis, and BAD-like proteins, that have the capacity to sensitize mitochondria to BID-like pro-apoptotics by occupying the binding pockets of multidomain anti-apoptotic proteins. Various α-helical domains of BCL-2 family member proteins amendable to the methodology disclosed herein have been disclosed (Walensky *et al.* (2004), *Science* 305:1466; and

Walensky *et al.*, U.S. Patent Publication No. 2005/0250680, the entire disclosures of which are incorporated herein by reference).

[0074] In other embodiments, the peptide sequence is derived from the tumor suppressor p53 protein which binds to the oncogene protein MDM2. The MDM2 binding site is localized within a region of the p53 tumor suppressor that forms an α helix. In U.S. Pat. No. 7,083,983, the entire contents of which are incorporated herein by reference, Lane *et al.* disclose that the region of p53 responsible for binding to MDM2 is represented approximately by amino acids 13-31 (PLSQETFSDLWKLLPENNV) of mature human P53 protein. Other modified sequences disclosed by Lane are also contemplated in the instant invention. Furthermore, the interaction of p53 and MDM2 has been discussed by Shair *et al.* (1997), *Chem. & Biol.* 4:791, the entire contents of which are incorporated herein by reference, and mutations in the p53 gene have been identified in virtually half of all reported cancer cases. As stresses are imposed on a cell, p53 is believed to orchestrate a response that leads to either cell-cycle arrest and DNA repair, or programmed cell death. As well as mutations in the p53 gene that alter the function of the p53 protein directly, p53 can be altered by changes in MDM2. The MDM2 protein has been shown to bind to p53 and disrupt transcriptional activation by associating with the transactivation domain of p53. For example, an 11 amino-acid peptide derived from the transactivation domain of p53 forms an amphipathic α-helix of 2.5 turns that inserts into the MDM2 crevice. Thus, in some embodiments, novel α-helix structures generated by the method of the present invention are engineered to generate structures that bind tightly to the helix acceptor and disrupt native protein-protein interactions. These structures are then screened using high throughput techniques to identify optimal small molecule peptides. The novel structures that disrupt the MDM2 interaction are useful for many applications, including, but not limited to, control of soft tissue sarcomas (which over-expresses MDM2 in the presence of wild type p53). These cancers are then, in some embodiments, held in check with small molecules that intercept MDM2, thereby preventing suppression of p53. Additionally, in some embodiments, small molecules disrupters of MDM2-p53 interactions are used as adjuvant therapy to help control and modulate the extent of the p53 dependent apoptosis response in conventional chemotherapy.

[0075] A non-limiting exemplary list of suitable peptide sequences for use in the present invention is given below:

TABLE 1

Name	Sequence (bold = critical residues)	Cross-linked Sequence (X = x-link residue)
BH3 peptides		
BID-BH3	QEDIIRNIARHLAQV**GD**SMDRSIPP	QEDIIRNIARHLA**X**VGD**X**MDRSIPP
BIM-BH3	DNRPEIWIAQELRR**IGD**EFNAYYAR	DNRPEIWIAQELR**X**IGD**X**FNAYYAR
BAD-BH3	NLWAAQRYGR**EL**RRM**SD**EFVDSFKK	NLWAAQRYGRELR**X**MSD**X**FVDSFKK
PUMA-BH3	EEQWAREIGAQLRRMA**DD**LNAQYER	EEQWAREIGAQLR**X**MAD**X**LNAQYER
Hrk-BH3	RSSAAQLTAARLKAL**GD**ELHQRTM	RSSAAQLTAARLK**X**LGD**X**LHQRTM
NOXAA-BH3	AELPPEFAAQLRKI**GD**KVYCTW	AELPPEFAAQLR**X**IGD**X**VYCTW
NOXAB-BH3	VPADLKDECAQLRRI**GD**KVNLRQKL	VPADLKDECAQLR**X**IGD**X**VNLRQKL
BMF-BH3	QHRAEVQIARKLQCIA**D**QFHRLHT	QHRAEVQIARKLQ**X**IAD**X**FHRLHT
BLK-BH3	SSAAQLTAAR**L**KAL**GD**ELHQRT	SSAAQLTAARLK**X**LGD**X**LHQRT
BIK-BH3	CMEGSDALALRLACI**GD**EMDVSLRA	CMEGSDALALRLA**X**IGD**X**MDVSLRA
Bnip3	DIERRKEVESILKKN**SD**WIWDWSS	DIERRKEVESILK**X**NSD**X**IWDWSS

Name	Sequence (bold = critical residues)	Cross-linked Sequence (X = x-link residue)
BOK-BH3	GRLAEVCAV**L**LRL**GD**ELEMIRP	GRLAEVCAV**L**L**X**L**GD**X**L**EMIRP
BAX-BH3	PQDASTKKSEC**L**KRI**GD**ELDSNMEL	PQDASTKKSEC**L**K**X**I**GD**X**L**DSNMEL
BAK-BH3	PSSTMGQVGRQ**L**AII**GD**DINRR	PSSTMGQVGRQ**L**A**X**I**GD**X**I**NRR
BCL2L1-BH3	KQA**L**REA**GD**EFELR	KQA**L**R**X**A**GD**X**F**ELR
BCL2-BH3	LSPPVVHLALA**L**RQA**GD**DFSRR	LSPPVVHLALA**L**R**X**A**GD**X**F**SRR
BCL-XL-BH3	EVIPMAAVKQA**L**REA**GD**EFELRY	EVIPMAAVKQA**L**R**X**A**GD**X**F**ELRY
BCL-W-BH3	PADPLHQAMRAA**GD**EFETRF	PADPLHQAMR**X**A**GD**X**F**ETRF
MCL1-BH3	ATSRKLET**L**RRV**GD**GVQRNHETA	ATSRKLET**L**R**X**V**GD**X**V**QRNHETA
MTD-BH3	LAEVCTV**L**LRL**GD**ELEQIR	LAEVCTV**L**L**X**L**GD**X**L**EQIR
MAP-1-BH3	MTVGELSRA**L**GHENGSLDP	MTVGELSRA**L**G**X**ENG**X**LDP
NIX-BH3	VVEGEKEVEA**L**KKSA**D**WVSDWS	VVEGEKEVEA**L**K**X**SA**D**X**V**SDWS
4ICD(ERBB4)-BH3	SMARDPQRY**L**VIQ**GD**DRMKL	SMARDPQRY**L**V**X**Q**GD**X**R**MKL

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

TABLE 2

Name	Sequence (bold = critical residues)	Cross-linked Sequence (X = x-link residue)
BH3 peptides		
BID-BH3	QEDIIRNIARHLAQV**GD**SMDRSIPP	QEDIIRNI**X**RHL**X**QV**GD**SMDRSIPP
BIM-BH3	DNRPEIWIAQE**L**RRI**GD**EFNAYYAR	DNRPEIWI**X**QE**L**X**RIGD**EFNAYYAR
BAD-BH3	NLWAAQRYG**R**E**L**RRM**SD**EFVDSFKK	NLWAAQRY**X**RE**L**X**R**M**SD**EFVDSFKK
PUMA-BH3	EEQWAREIGAQ**L**RRMA**D**DLNAQYER	EEQWAREI**X**AQ**L**X**R**MA**D**DLNAQYER
Hrk-BH3	RSSAAQLTAARLKAL**GD**ELHQRTM	RSSAAQLT**X**ARL**X**AL**GD**ELHQRTM
NOXAA-BH3	AELPPEFAAQLRKI**GD**KVYCTW	AELPPEF**X**AQL**X**KI**GD**KVYCTW
NOXAB-BH3	VPADLKDECAQLRRI**GD**KVNLRQKL	VPADLKDE**X**AQL**X**RI**GD**KVNLRQKL
BMF-BH3	QHRAEVQIARK**L**QCIA**D**QFHRLHT	QHRAEVQI**X**RK**L**X**C**IA**D**QFHRLHT
BLK-BH3	SSAAQLTAAR**L**KAL**GD**ELHQRT	SSAAQLT**X**AR**L**X**A**L**GD**ELHQRT
BIK-BH3	CMEGSDALALRLACI**GD**EMDVSLRA	CMEGSDAL**X**LRL**X**CI**GD**EMDVSLRA
Bnip3	DIERRKEVESILKKN**SD**WIWDWSS	DIERRKEV**X**SIL**X**KN**SD**WIWDWSS
BOK-BH3	GRLAEVCAV**L**LRL**GD**ELEMIRP	GRLAEV**X**AV**L**X**R**L**GD**ELEMIRP
BAX-BH3	PQDASTKKSEC**L**KRI**GD**ELDSNMEL	PQDASTKK**X**EC**L**X**RIGD**ELDSNMEL
BAK-BH3	PSSTMGQVGRQLAII**GD**DINRR	PSSTMGQV**X**RQL**X**II**GD**DINRR
BCL2L1-BH3	KQA**L**REA**GD**EFELR	**X**QA**L**X**E**A**GD**EFELR
BCL2-BH3	LSPPVVHLALA**L**RQA**GD**DFSRR	LSPPVVHL**X**LAL**X**QA**GD**DFSRR
BCL-XL-BH3	EVIPMAAVKQA**L**REA**GD**EFELRY	EVIPMAAV**X**QAL**X**EA**GD**EFELRY
BCL-W-BH3	PADPLHQAMRAA**GD**EFETRF	PADPL**X**QAM**X**AA**GD**EFETRF
MCL1-BH3	ATSRKLET**L**RRV**GD**GVQRNHETA	ATSRK**X**ET**L**X**R**V**GD**GVQRNHETA

Name	Sequence (bold = critical residues)	Cross-linked Sequence (X = x-link residue)
MTD-BH3	LAEVCTV**L**LRL**GD**ELEQIR	LAEV**X**TV**L**X**R**L**GD**ELEQIR
MAP-1-BH3	MTVGELSRA**L**GHENGSLDP	MTVGEL**X**RAL**X**HENGSLDP
NIX-BH3	VVEGEKEVEA**L**KKSA**D**WVSDWS	VVEGEKE**X**EA**L**X**K**SA**D**WVSDWS
4ICD(ERBB4)-BH3	SMARDPQRY**L**VIQ**GD**DRMKL	SMARDP**X**RY**L**X**I**Q**GD**DRMKL

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

TABLE 3

Name	Sequence (bold = critical residues)	Cross-linked Sequence (X = x-link residue)
P53 peptides		
hp53 peptide 1	LSQET**FSDLW**KLLPEN	LSQET**FSD**X**W**KLLPEX
hp53 peptide 2	LSQET**FSDLW**KLLPEN	LSQEX**FSDLW**KX**L**PEN
hp53 peptide 3	LSQET**FSDLW**KLLPEN	LSQXT**FSDLW**X**L**LPEN
hp53 peptide 4	LSQET**FSDLW**KLLPEN	LSQET**F**X**DLW**KLLX**E**N
hp53 peptide 5	LSQET**FSDLW**KLLPEN	QSQQT**F**X**NLW**RLLX**Q**N

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

TABLE 4

Name	Sequence (bold = critical residues)	Cross-linked Sequence (X = x-link residue)
GPCR peptide ligands		
Angiotensin II	DRV**YIHPF**	DR**X**Y**X**H**PF**
Bombesin	EQRLGNQ**W**AVG**H**LM	EQRLGN**X**WAVG**H**L**X**
Bradykinin	RPPGF**SPFR**	RPP**X**F**SPFR**X
C5a	ISHKDMQ**L**GR	ISHKDM**X**LGR**X**
C3a	ARASH**L**G**L**A**R**	ARASHL**X**LAR**X**
α-melanocyte stimulating hormone	SYSME**HFRW**GKPV	SYSM**X**H**FRW**X**K**PV

[0076] **Table 4** lists sequences which target human G protein-coupled receptors and are implicated in numerous human disease conditions (Tyndall *et al.* (2005), *Chem. Rev.* 105:793-826).

Peptidomimetic Macrocycles of the Invention

[0077] In some embodiments of the method, a polypeptide of the invention contains one crosslink. In other embodiments of the method, said polypeptide contains two cross-links. In some embodiments of the method, one crosslink connects two α-carbon atoms. In other embodiments of the method, one α-carbon atom to which one crosslink is attached is substituted with a substituent of formula R–. In another embodiment of the method, two α-carbon atoms to which one crosslink is attached are substituted with independent substituents of formula R–. In one embodiment of the methods of the invention, R– is alkyl. For example, R– is methyl. Alternatively, R– and any portion of one crosslink taken together can form a cyclic structure. In another embodiment of the method, one crosslink is formed of consecutive carbon-carbon bonds. For example, one crosslink may comprise

at least 8, 9, 10, 11, or 12 consecutive bonds. In other embodiments, one crosslink may comprise at least 7, 8, 9, 10, or 11 carbon atoms.

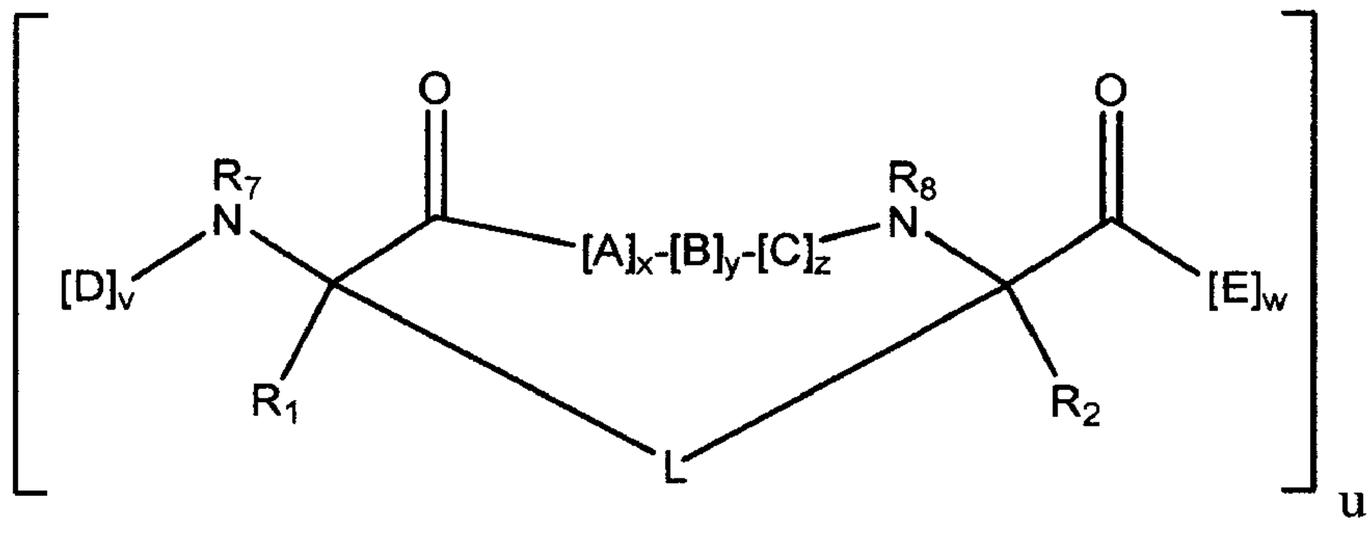
[0078] In another embodiment of the method, the crosslinked polypeptide comprises an α-helical domain of a BCL-2 family member. For example, the crosslinked polypeptide comprises a BH3 domain. In other embodiments, the crosslinked polypeptide comprises at least 60%, 70%, 80%, 85%, 90% or 95% of any of the sequences in Tables 1, 2, 3 and 4. In some embodiments of the method, the crosslinked polypeptide penetrates cell membranes by an energy-dependent process and binds to an intracellular target.

[0079] In some embodiments, said helical polypeptide contains one crosslink. In other embodiments, said helical polypeptide contains two cross-links.

[0080] In some embodiments, one crosslink connects two α-carbon atoms. In other embodiments, one α-carbon atom to which one crosslink is attached is substituted with a substituent of formula R–. In another embodiment, two α-carbon atoms to which one crosslink is attached are substituted with independent substituents of formula R–. In one embodiment of the invention, R– is alkyl. For example, R– is methyl. Alternatively, R– and any portion of one crosslink taken together can form a cyclic structure. In another embodiment, one crosslink is formed of consecutive carbon-carbon bonds. For example, one crosslink may comprise at least 8, 9, 10, 11, or 12 consecutive bonds. In other embodiments, one crosslink may comprise at least 7, 8, 9, 10, or 11 carbon atoms.

[0081] In another embodiment, the crosslinked polypeptide comprises an α-helical domain of a BCL-2 family member. For example, the crosslinked polypeptide comprises a BH3 domain. In other embodiments, the crosslinked polypeptide comprises at least 60%, 70%, 80%, 85%, 90% or 95% of any of the sequences in Tables 1, 2, 3 and 4. In some embodiments, the crosslinked polypeptide penetrates cell membranes by an energy-dependent process and binds to an intracellular target.

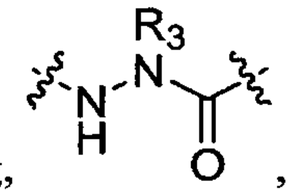
[0082] In some embodiments, the peptidomimetic macrocycles of the invention have the Formula (I):



Formula (I)

wherein:

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

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

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

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

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

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

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

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

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

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

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

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

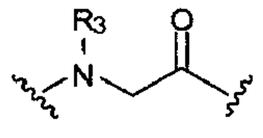
each of v and w is independently an integer from 1-1000;

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

n is an integer from 1-5.

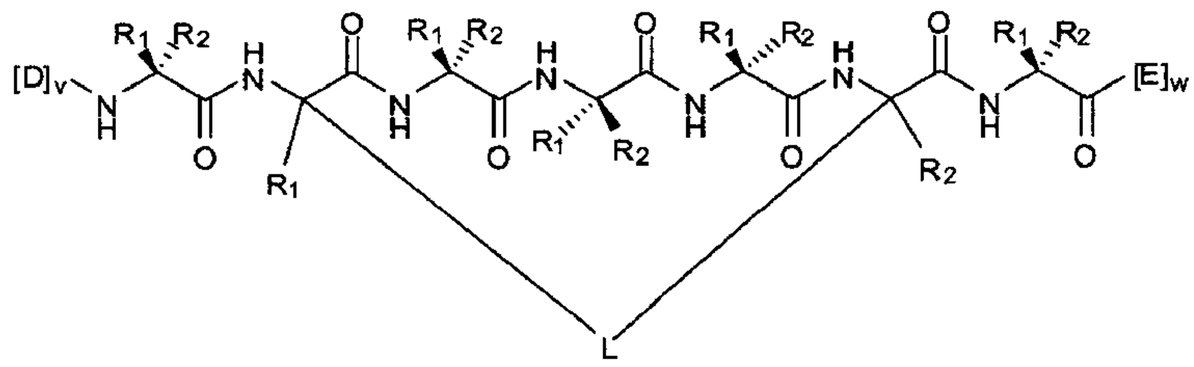
[0083] In one example, at least one of R_1 and R_2 is alkyl, unsubstituted or substituted with halo–. In another example, both R_1 and R_2 are independently alkyl, unsubstituted or substituted with halo–. In some embodiments, at least one of R_1 and R_2 is methyl. In other embodiments, R_1 and R_2 are methyl.

[0084] In some embodiments of the invention, x+y+z is at least 3. In other embodiments of the invention, x+y+z is 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10. Each occurrence of A, B, C, D or E in a macrocycle or macrocycle precursor of the invention is independently selected. For example, a sequence represented by the formula $[A]_x$, when x is 3, encompasses embodiments where the amino acids are not identical, e.g. 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.

[0085] In some embodiments, the peptidomimetic macrocycle of the invention comprises a secondary structure which is an α-helix and R_8 is –H, allowing intrahelical hydrogen bonding. In some embodiments, at least one of A, B, C, D or E is an α,α-disubstituted amino acid. In one example, B is an α,α-disubstituted amino acid. For instance, at least one of A, B, C, D or E is  .

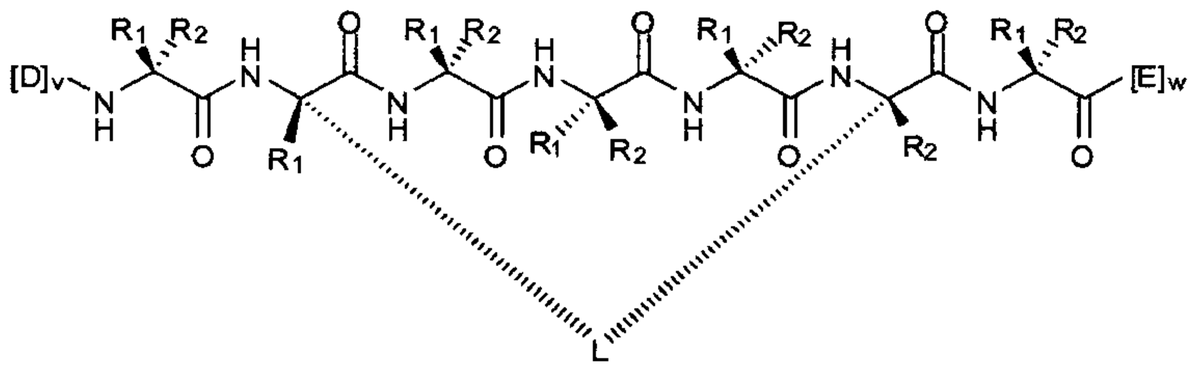
[0086] In other embodiments, the length of the macrocycle-forming linker L as measured from a first Cα to a second Cα is selected to stabilize a desired secondary peptide structure, such as an α-helix formed by residues of the peptidomimetic macrocycle including, but not necessarily limited to, those between the first Cα to a second Cα.

[0087] In one embodiment, the peptidomimetic macrocycle of Formula (I) is:

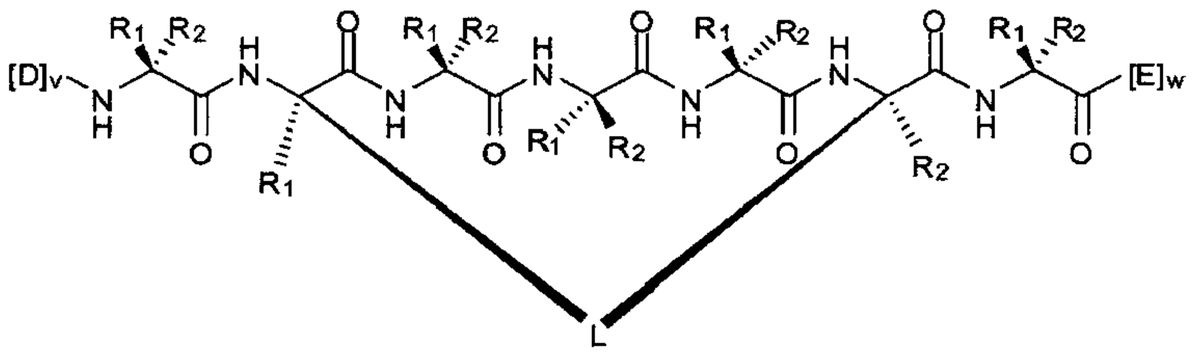


[0088] wherein each R_1 and R_2 is independently independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo–.

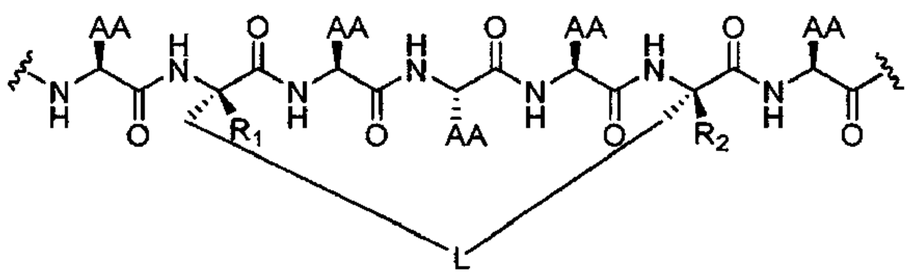
[0089] In related embodiments, the peptidomimetic macrocycle of Formula (I) is:

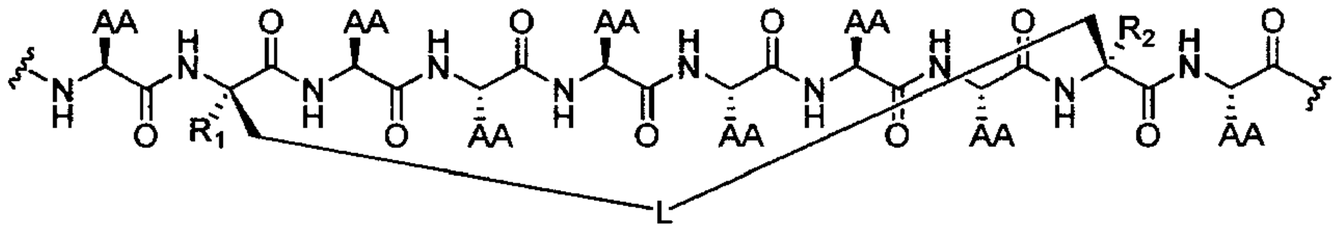


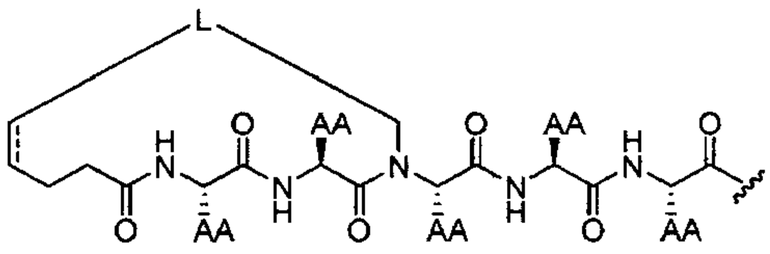
or

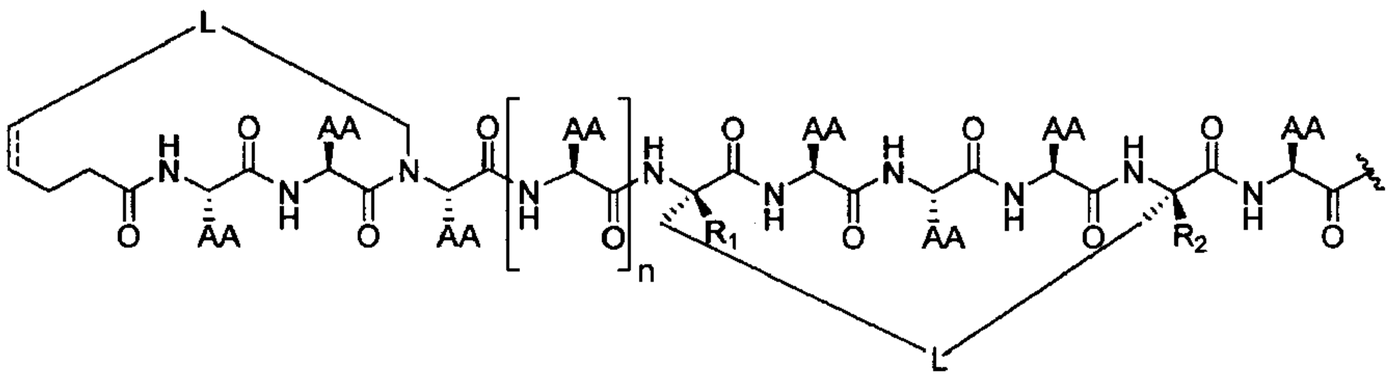


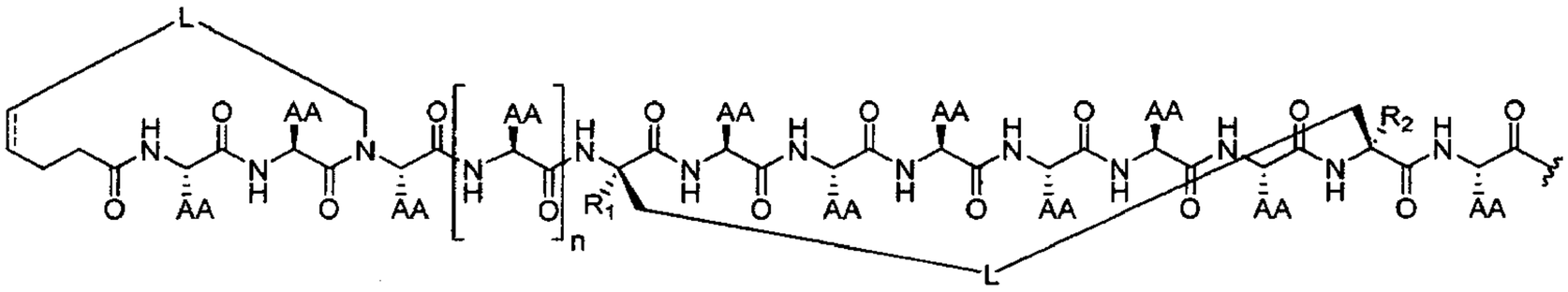
[0090] In other embodiments, the peptidomimetic macrocycle of Formula (I) is a compound of any of the formulas shown below:

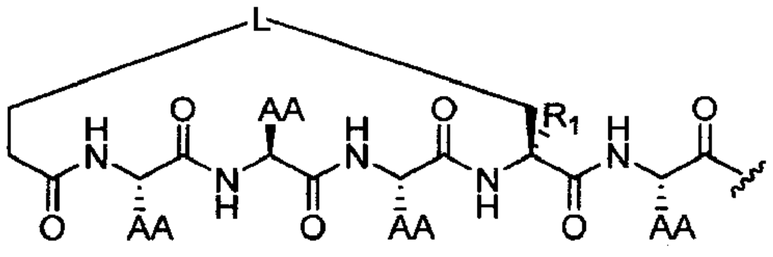


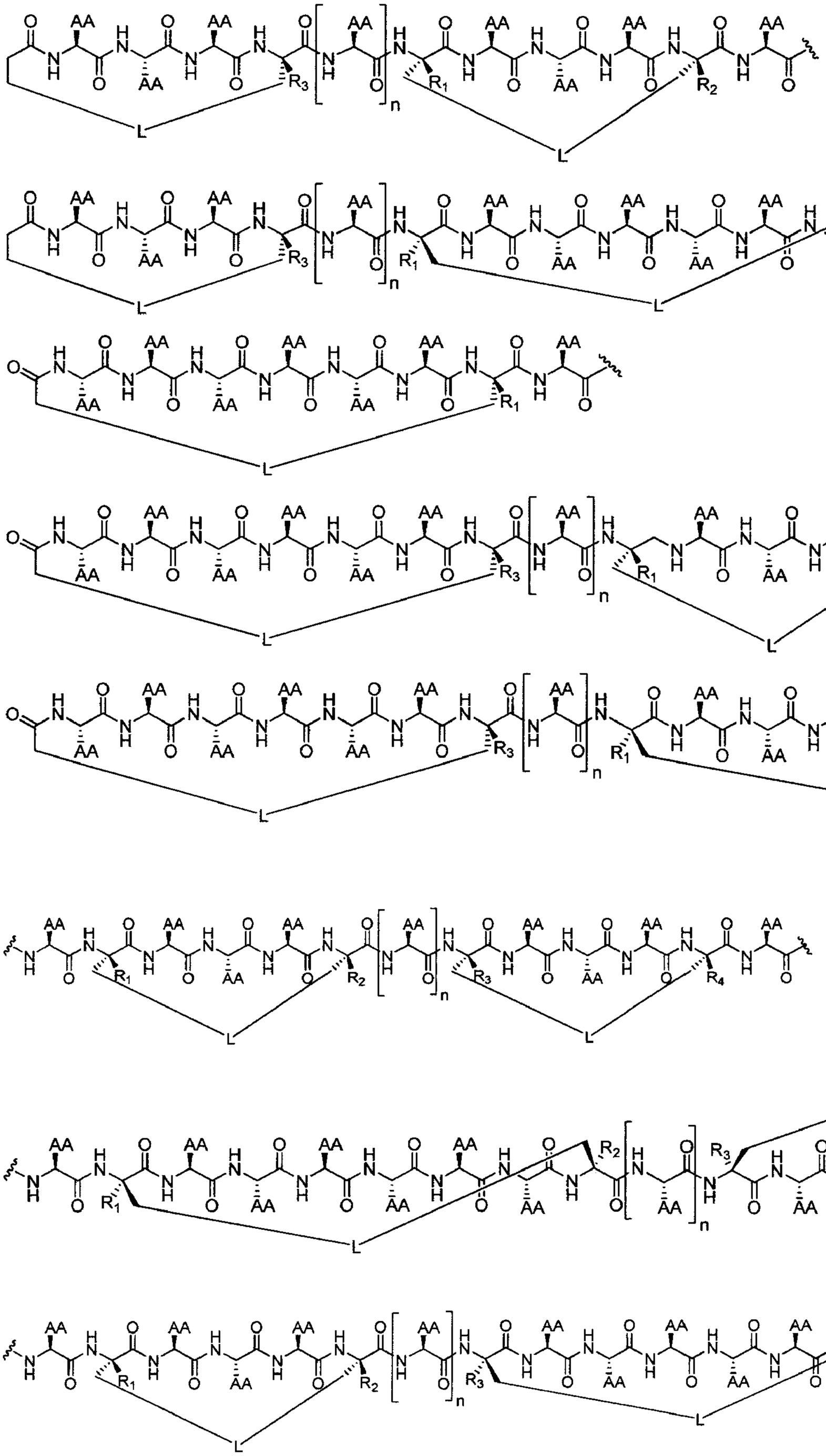


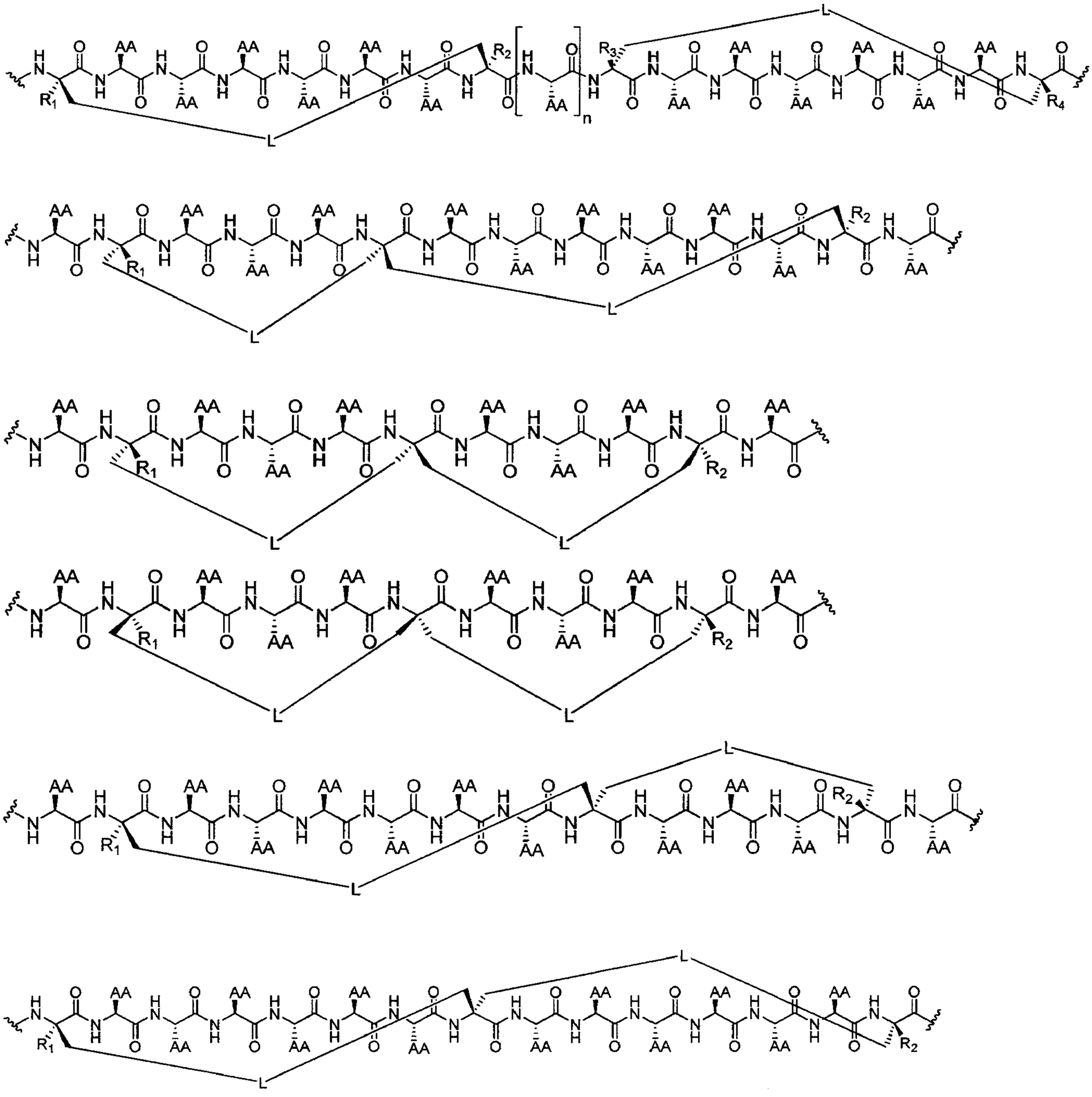






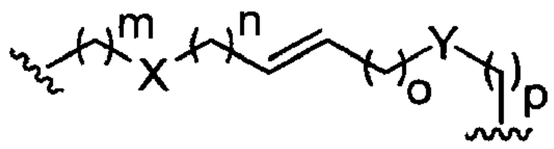




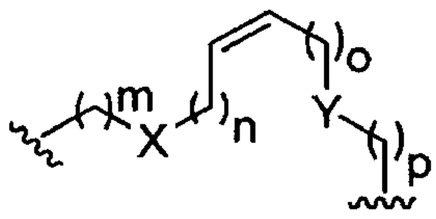


wherein "AA" represents any natural or non-natural amino acid side chain and "~~~" is $[D]_v$, $[E]_w$ as defined above, and n is an integer between 0 and 20, 50, 100, 200, 300, 400 or 500. In some embodiments, n is 0. In other embodiments, n is less than 50.

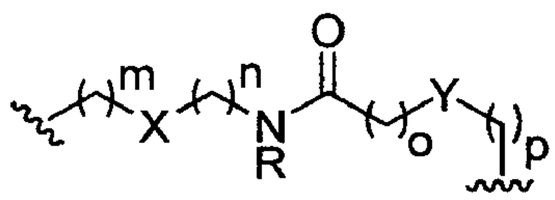
[0091] Exemplary embodiments of the macrocycle-forming linker L are shown below.



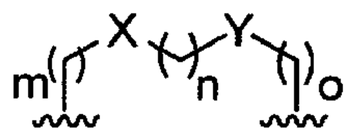
where X, Y = -CH_2-, O, S, or NH
m, n, o, p = 0-10



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

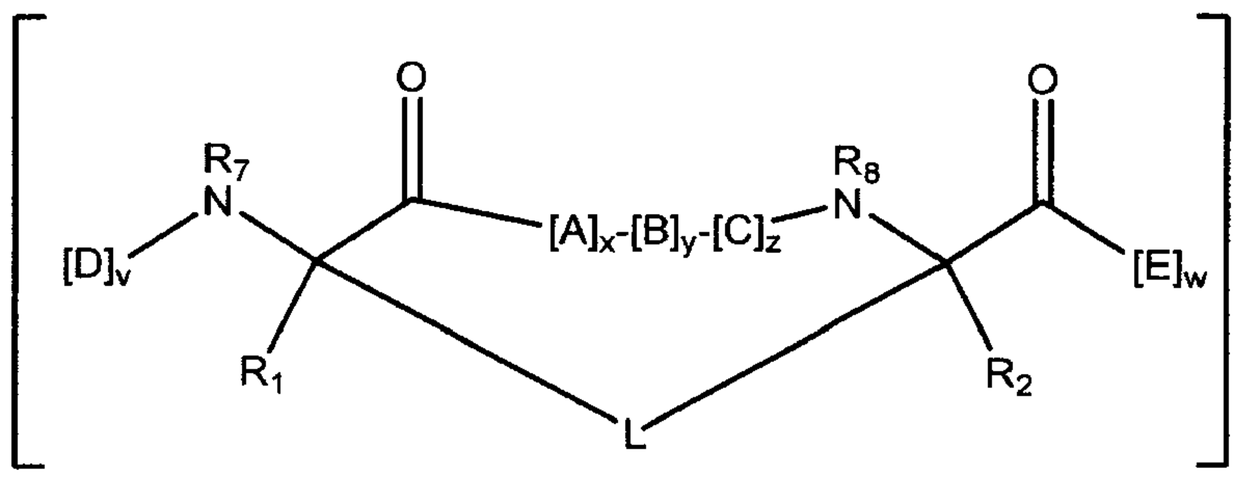


where X, Y = -CH_2-, O, S, or NH
m, n, o, p = 0-10
R = H, alkyl, other substituent



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

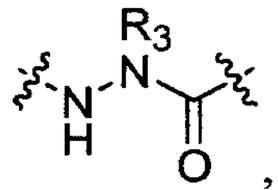
[0092] In some embodiments, the peptidomimetic macrocycles of the invention have the Formula (II):



Formula (II)

wherein:

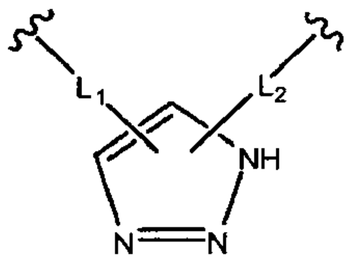
each A, C, D, and E is independently a natural or non-natural amino acid;

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

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

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

L is a macrocycle-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_4 is alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, arylene, or heteroarylene;

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

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

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

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

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

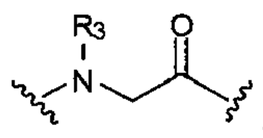
each of v and w is independently an integer from 1-1000;

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

n is an integer from 1-5.

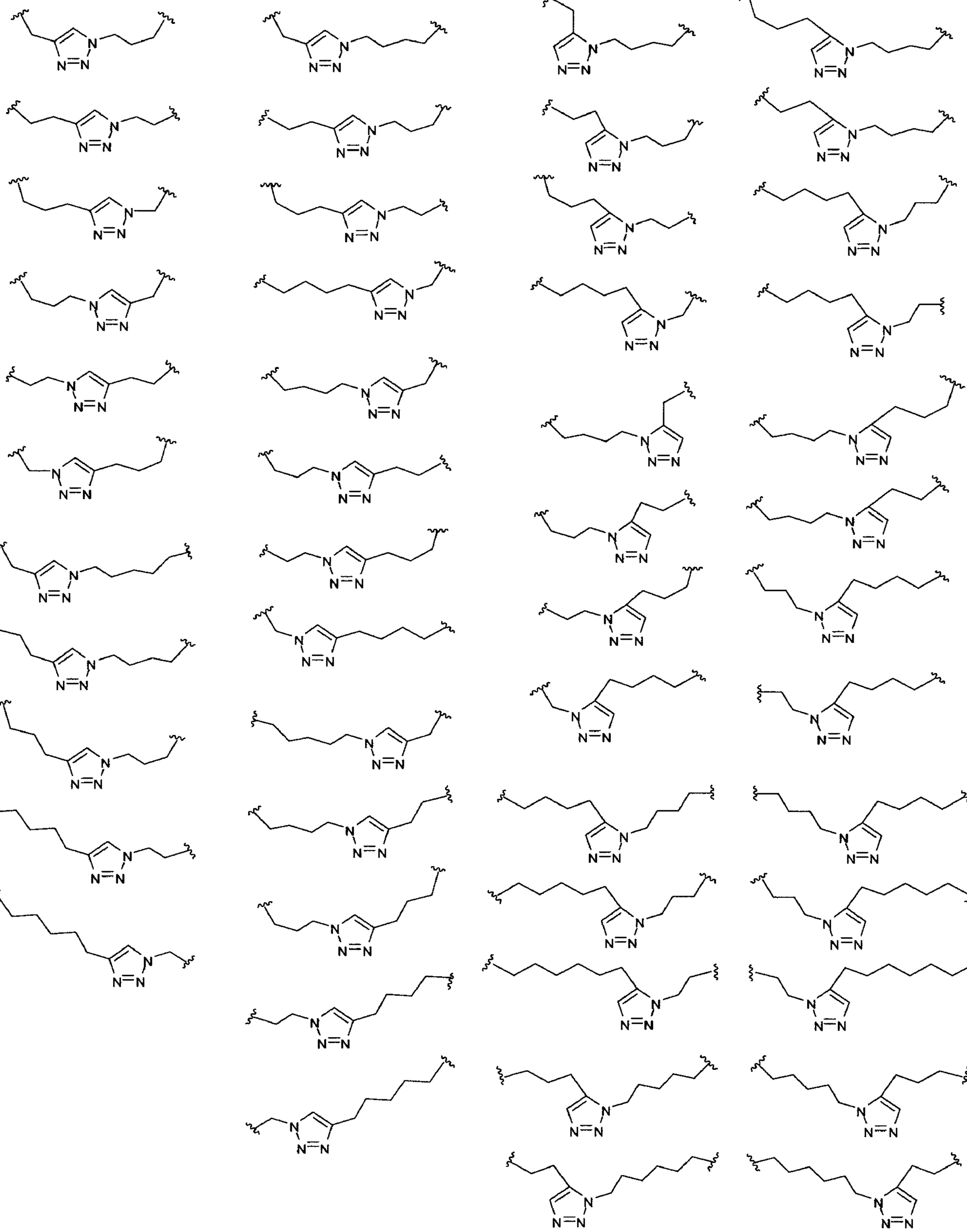
[0093] In one example, at least one of R_1 and R_2 is alkyl, unsubstituted or substituted with halo–. In another example, both R_1 and R_2 are independently alkyl, unsubstituted or substituted with halo–. In some embodiments, at least one of R_1 and R_2 is methyl. In other embodiments, R_1 and R_2 are methyl.

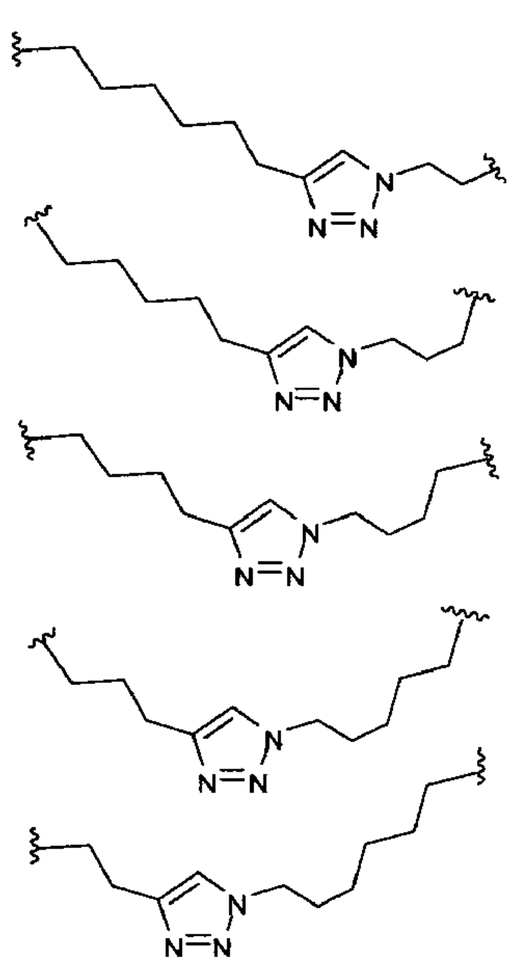
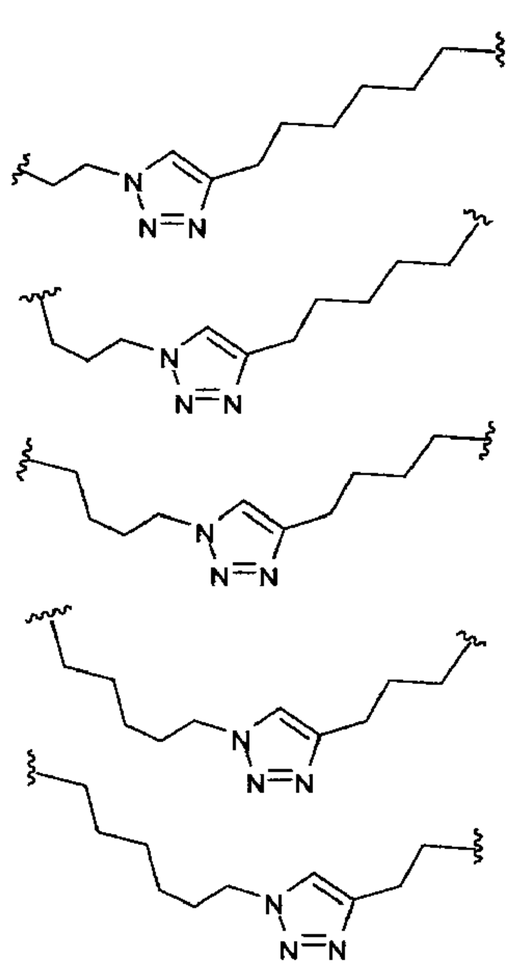
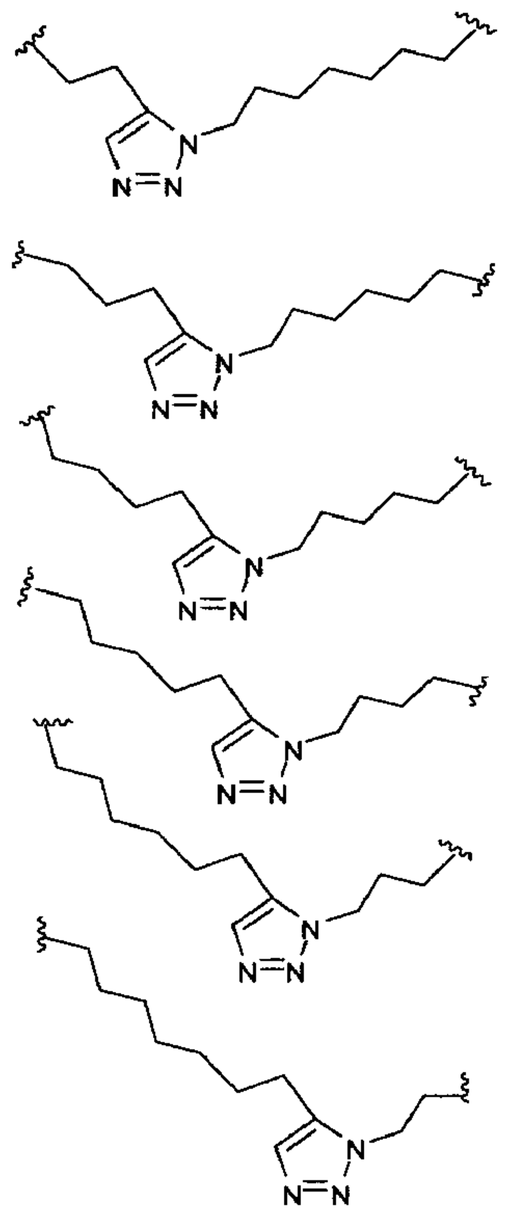
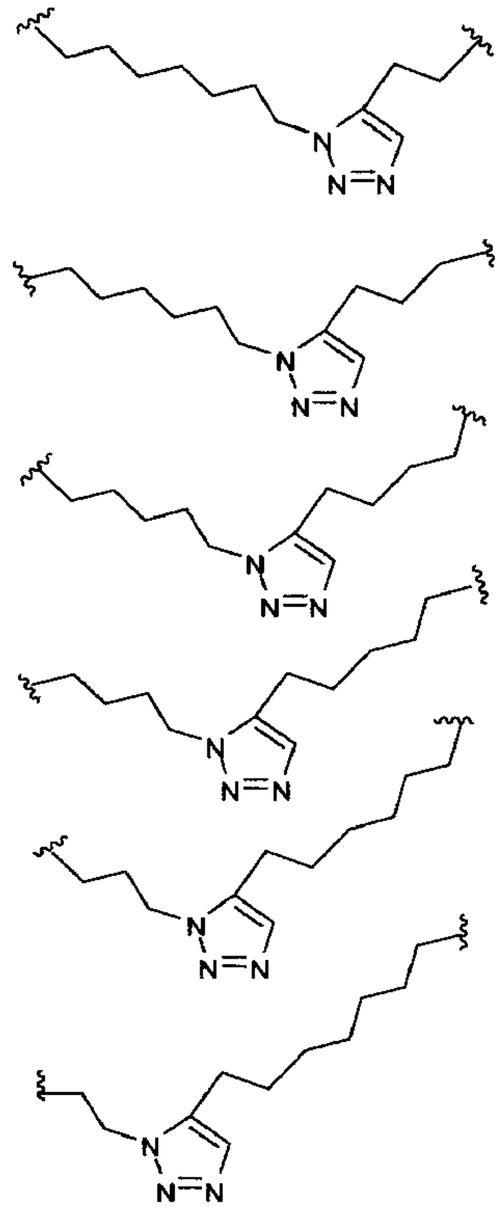
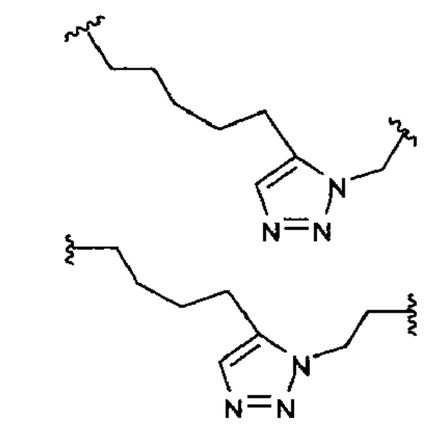
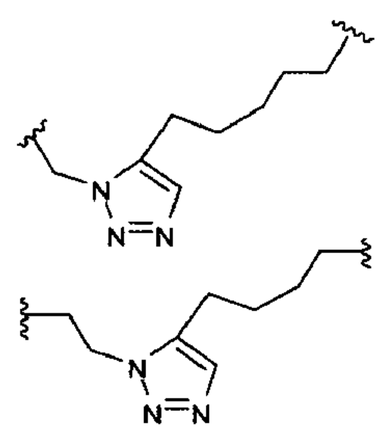
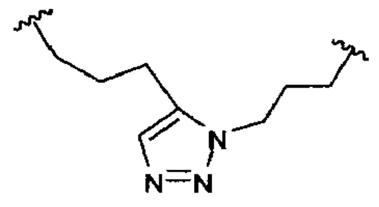
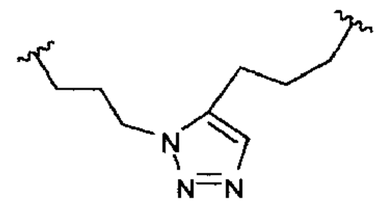
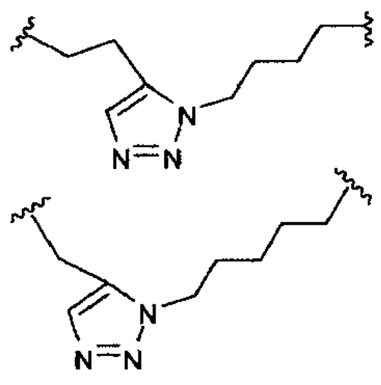
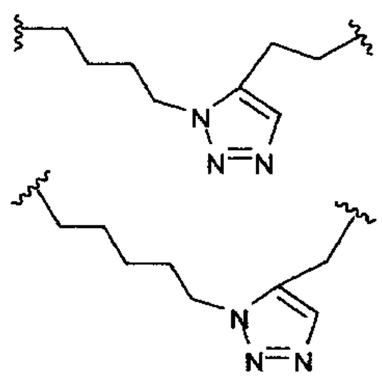
[0094] In some embodiments of the invention, x+y+z is at least 3. In other embodiments of the invention, x+y+z is 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10. Each occurrence of A, B, C, D or E in a macrocycle or macrocycle precursor of the invention is independently selected. For example, a sequence represented by the formula $[A]_x$, when x is 3, encompasses embodiments where the amino acids are not identical, e.g. 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.

[0095] In some embodiments, the peptidomimetic macrocycle of the invention comprises a secondary structure which is an α-helix and R_8 is –H, allowing intrahelical hydrogen bonding. In some embodiments, at least one of A, B, C, D or E is an α,α-disubstituted amino acid. In one example, B is an α,α-disubstituted amino acid. For instance, at least one of A, B, C, D or E is an α,α-disubstituted amino acid. In other embodiments, at least one of A, B, C, D or E is  .

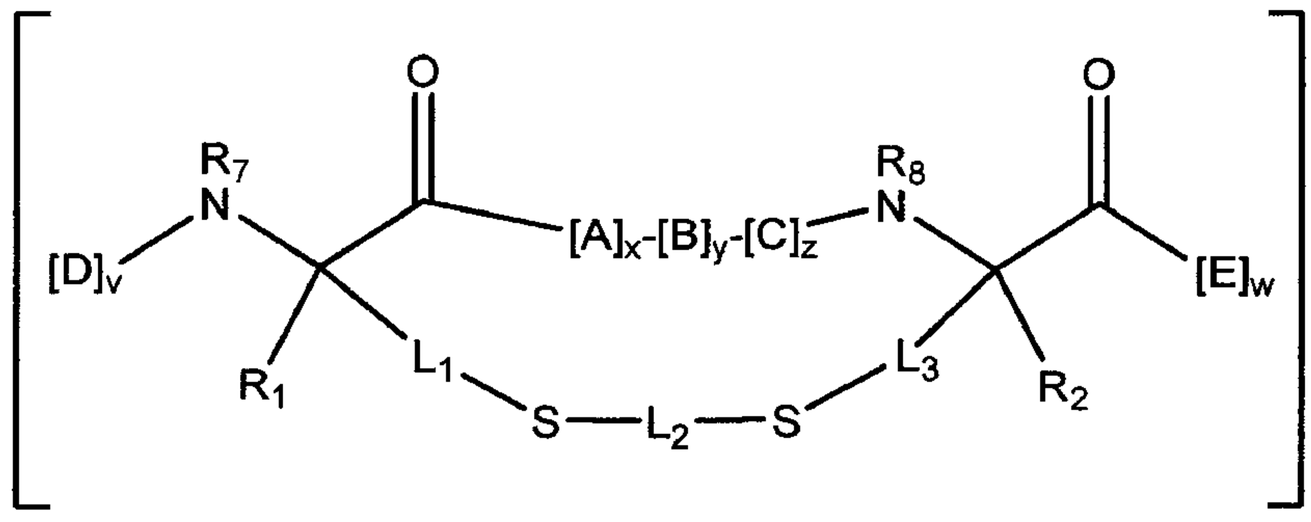
[0096] In other embodiments, the length of the macrocycle-forming linker L as measured from a first Cα to a second Cα is selected to stabilize a desired secondary peptide structure, such as an α-helix formed by residues of the peptidomimetic macrocycle including, but not necessarily limited to, those between the first Cα to a second Cα.

[0097] Exemplary embodiments of the macrocycle-forming linker L are shown below.



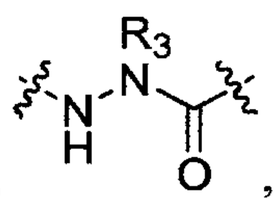
[0098] In other embodiments, the invention provides peptidomimetic macrocycles of Formula (III):



Formula (III)

wherein:

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

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

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

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

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

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

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

each R_5 is independently halogen, alkyl, -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, unsubstituted or substituted with R_5, or part of a cyclic structure with a D residue;

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

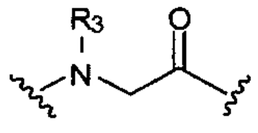
each of v and w is independently an integer from 1-1000;

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

n is an integer from 1-5.

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

[00101] In some embodiments, the peptidomimetic macrocycle of the invention comprises a secondary structure which is an α-helix and R_8 is –H, allowing intrahelical hydrogen bonding. In some embodiments, at least one of A, B, C, D or E is an α,α-disubstituted amino acid. In one example, B is an α,α-disubstituted amino acid. For instance, at least one of A, B, C, D or E is 2-aminoisobutyric acid. In other embodiments, at least one of A, B, C, D or E is  .

[00102] In other embodiments, the length of the macrocycle-forming linker $[-L_1\text{-}S\text{-}L_2\text{-}S\text{-}L_3-]$ as measured from a first Cα to a second Cα is selected to stabilize a desired secondary peptide structure, such as an α-helix formed by residues of the peptidomimetic macrocycle including, but not necessarily limited to, those between the first Cα to a second Cα.

[00103] Macrocycles or macrocycle precursors are synthesized, for example, by solution phase or solid-phase methods, and can contain both naturally-occurring and non-naturally-occurring amino acids. See, for example, Hunt, "The Non-Protein Amino Acids" in Chemistry and Biochemistry of the Amino Acids, edited by G.C. Barrett, Chapman and Hall, 1985. In some embodiments, the thiol moieties are the side chains of the amino acid residues L-cysteine, D-cysteine, α-methyl-L cysteine, α-methyl-D-cysteine, L-homocysteine, D-homocysteine, α-methyl-L-homocysteine or α-methyl-D-homocysteine. A bis-alkylating reagent is of the general formula $X\text{-}L_2\text{-}Y$ wherein L_2 is a linker moiety and X and Y are leaving groups that are displaced by -SH moieties to form bonds with L_2. In some embodiments, X and Y are halogens such as I, Br, or Cl.

[00104] In other embodiments, D and/or E in the compound of Formula I, II or III are further modified in order to facilitate cellular uptake. In some embodiments, lipidating or PEGylating a peptidomimetic macrocycle facilitates cellular uptake, increases bioavailability, increases blood circulation, alters pharmacokinetics, decreases immunogenicity and/or decreases the needed frequency of administration.

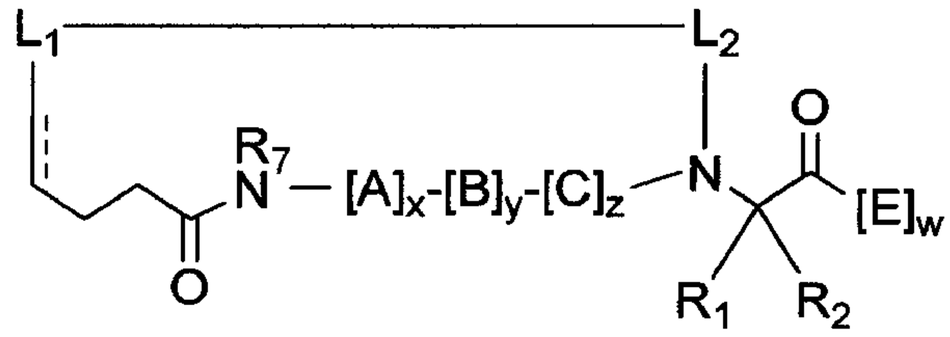
[00105] In other embodiments, at least one of [D] and [E] in the compound of Formula I, II or III represents a moiety comprising an additional macrocycle-forming linker such that the peptidomimetic macrocycle comprises at least two macrocycle-forming linkers. In a specific embodiment, a peptidomimetic macrocycle comprises two macrocycle-forming linkers.

[00106] In the peptidomimetic macrocycles of the invention, any of the macrocycle-forming linkers described herein may be used in any combination with any of the sequences shown in Tables 1-4 and also with any of the R–substituents indicated herein.

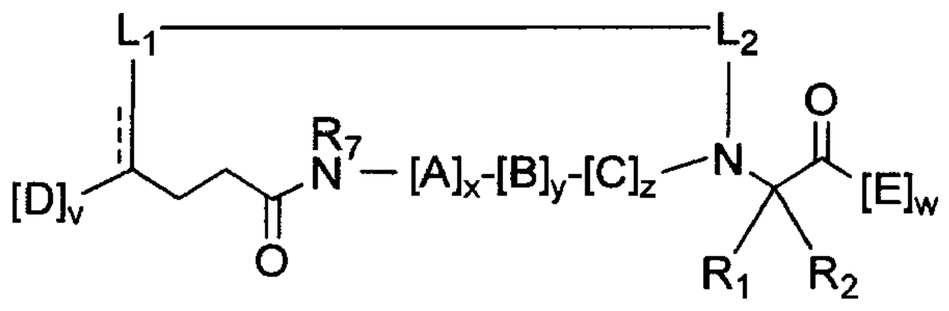
[00107] In some embodiments, the peptidomimetic macrocycle comprises at least one α-helix motif. For example, A, B and/or C in the compound of Formula I, II or III include one or more α-helices. As a general matter, α-helices include between 3 and 4 amino acid residues per turn. In some embodiments, the α-helix of the peptidomimetic macrocycle includes 1 to 5 turns and, therefore, 3 to 20 amino acid residues. In specific embodiments, the α-

helix includes 1 turn, 2 turns, 3 turns, 4 turns, or 5 turns. In some embodiments, the macrocycle-forming linker stabilizes an α-helix motif included within the peptidomimetic macrocycle. Thus, in some embodiments, the length of the macrocycle-forming linker L from a first Cα to a second Cα 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.

[00108] In other embodiments, the invention provides peptidomimetic macrocycles of Formula (IV) or (IVa):



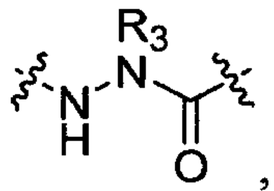
Formula (IV)



Formula (IVa)

wherein:

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

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

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

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

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

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

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

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

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

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

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

v is an integer from 1-1000;

w is an integer from 1-1000;

x is an integer from 0-10;

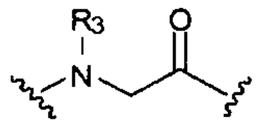
y is an integer from 0-10;

z is an integer from 0-10; and

n is an integer from 1-5.

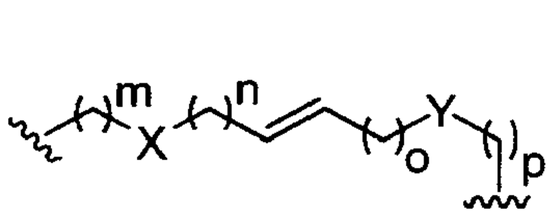
[00109] In one example, at least one of R_1 and R_2 is alkyl, unsubstituted or substituted with halo–. In another example, both R_1 and R_2 are independently alkyl, unsubstituted or substituted with halo–. In some embodiments, at least one of R_1 and R_2 is methyl. In other embodiments, R_1 and R_2 are methyl.

[00110] In some embodiments of the invention, x+y+z is at least 3. In other embodiments of the invention, x+y+z is 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10. Each occurrence of A, B, C, D or E in a macrocycle or macrocycle precursor of the invention is independently selected. For example, a sequence represented by the formula $[A]_x$, when x is 3, encompasses embodiments where the amino acids are not identical, e.g. 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.

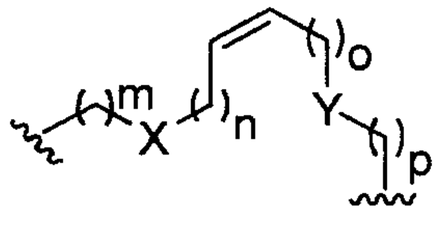
[00111] In some embodiments, the peptidomimetic macrocycle of the invention comprises a secondary structure which is an α-helix and R_8 is –H, allowing intrahelical hydrogen bonding. In some embodiments, at least one of A, B, C, D or E is an α,α-disubstituted amino acid. In one example, B is an α,α-disubstituted amino acid. For instance, at least one of A, B, C, D or E is 2-aminoisobutyric acid. In other embodiments, at least one of A, B, C, D or E is  .

[00112] In other embodiments, the length of the macrocycle-forming linker L as measured from a first Cα to a second Cα is selected to stabilize a desired secondary peptide structure, such as an α-helix formed by residues of the peptidomimetic macrocycle including, but not necessarily limited to, those between the first Cα to a second Cα.

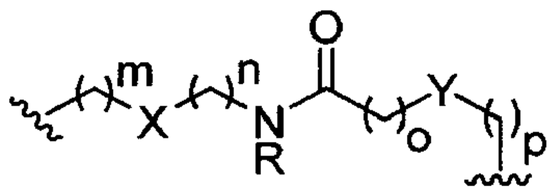
[00113] Exemplary embodiments of the macrocycle-forming linker L are shown below.



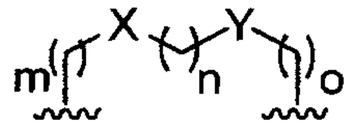
where X, Y = -CH_2-, O, S, or NH
m, n, o, p = 0-10



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

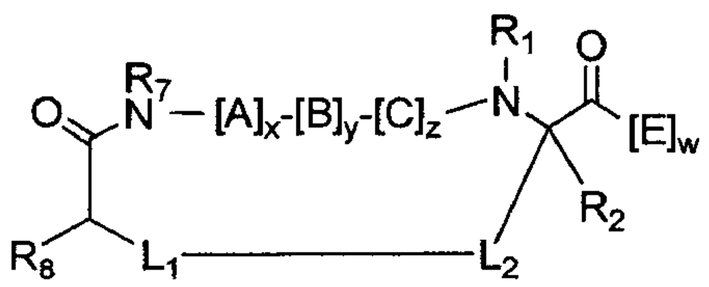


where X, Y = -CH_2-, O, S, or NH
m, n, o, p = 0-10
R = H, alkyl, other substituent



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

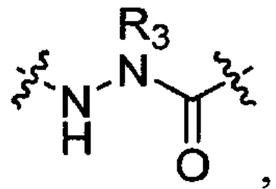
[00114] In other embodiments, the invention provides peptidomimetic macrocycles of Formula (V):



Formula (V)

wherein:

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

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

R_1, R_2 and R_8 are independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo–, or part of a cyclic structure with an E residue;

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

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

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

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

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

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

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

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

w is an integer from 1-1000;

x is an integer from 0-10;

y is an integer from 0-10;

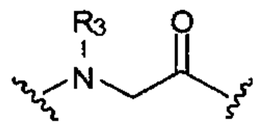
z is an integer from 0-10; and

n is an integer from 1-5.

[00115] 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 one embodiment, R_1 is H and R_2 is methyl. In another embodiment, R_1 and R_2 are methyl.

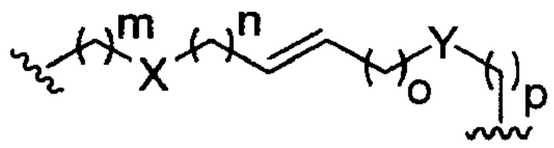
[00116] In some embodiments of the invention, x+y+z is at least 3. In other embodiments of the invention, x+y+z is 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10. Each occurrence of A, B, C, D or E in a macrocycle or macrocycle precursor of the invention is independently selected. For example, a sequence represented by the formula $[A]_x$, when x is 3, encompasses embodiments where the amino acids are not identical, e.g. 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.

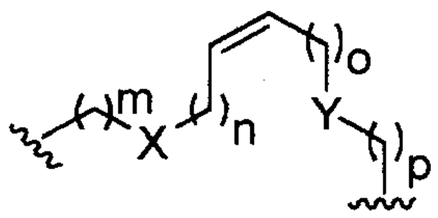
[00117] In some embodiments, the peptidomimetic macrocycle of the invention comprises a secondary structure which is an α-helix and R_8 is –H, allowing intrahelical hydrogen bonding. In some embodiments, at least one of A, B, C, D or E is an α,α-disubstituted amino acid. In one example, B is an α,α-disubstituted amino acid. For instance, at least one of A, B, C, D or E is 2-aminoisobutyric acid. In other embodiments, at least one of A, B, C, D or E is  .

[00118] In other embodiments, the length of the macrocycle-forming linker L as measured from a first Cα to a second Cα is selected to stabilize a desired secondary peptide structure, such as an α-helix formed by residues of the peptidomimetic macrocycle including, but not necessarily limited to, those between the first Cα to a second Cα.

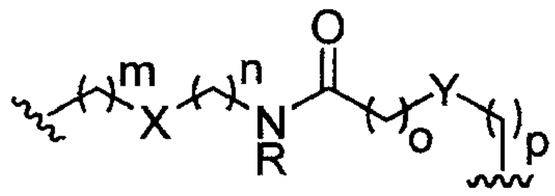
[00119] Exemplary embodiments of the macrocycle-forming linker L are shown below.



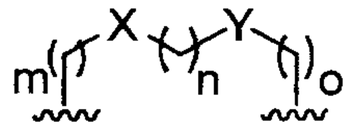
where X, Y = -CH_2-, O, S, or NH
m, n, o, p = 0-10



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

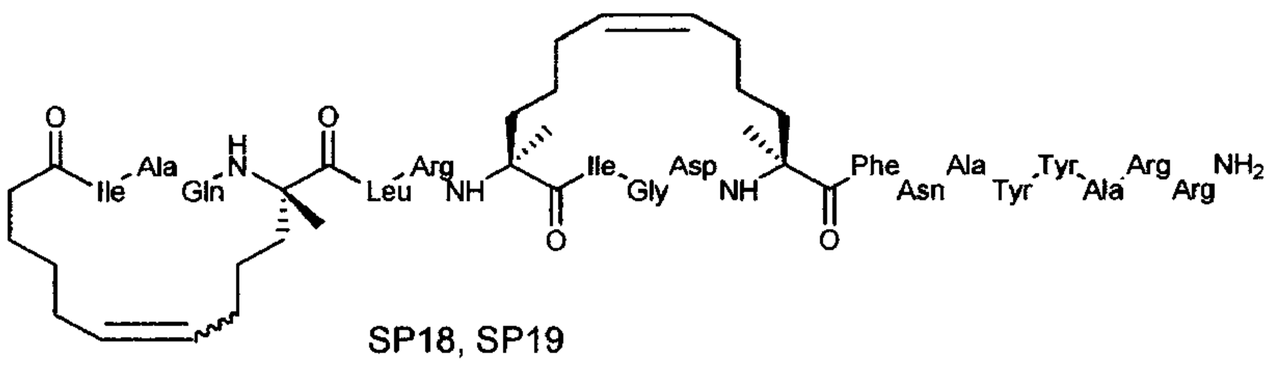


where X, Y = -CH_2-, O, S, or NH
m, n, o, p = 0-10
R = H, alkyl, other substituent

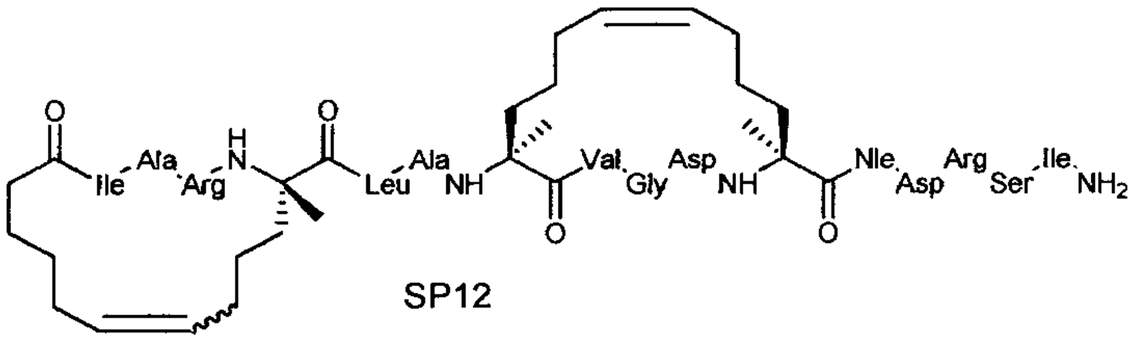


where X, Y = -CH_2-, O, S, or NH
m, n, o = 0-10

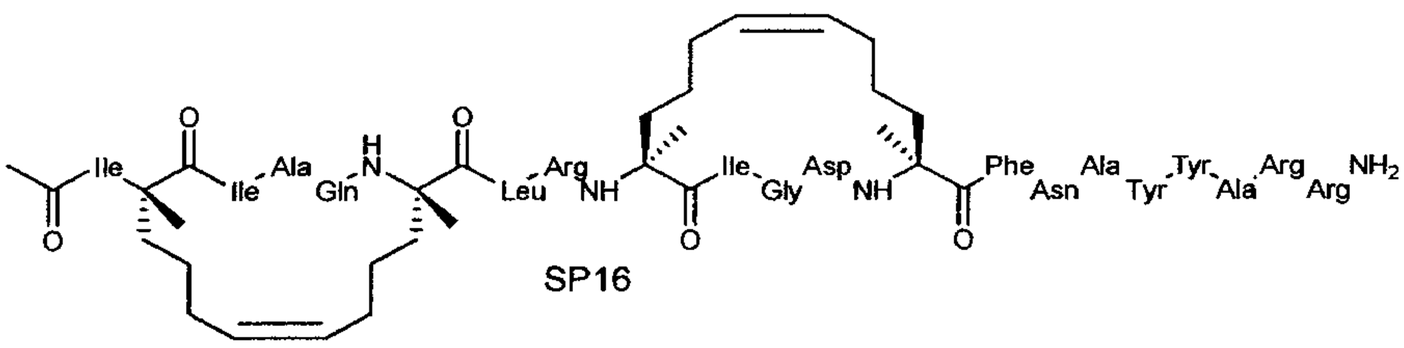
[00120] Exemplary embodiments of the peptidomimetic macrocycles are shown below.



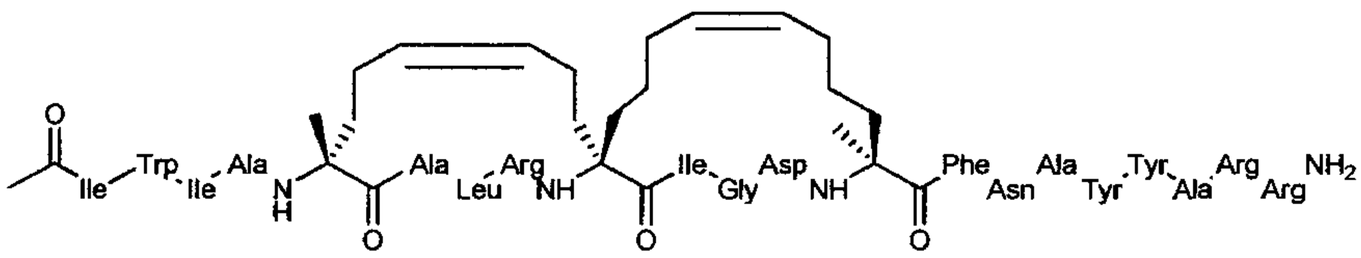
SP18, SP19



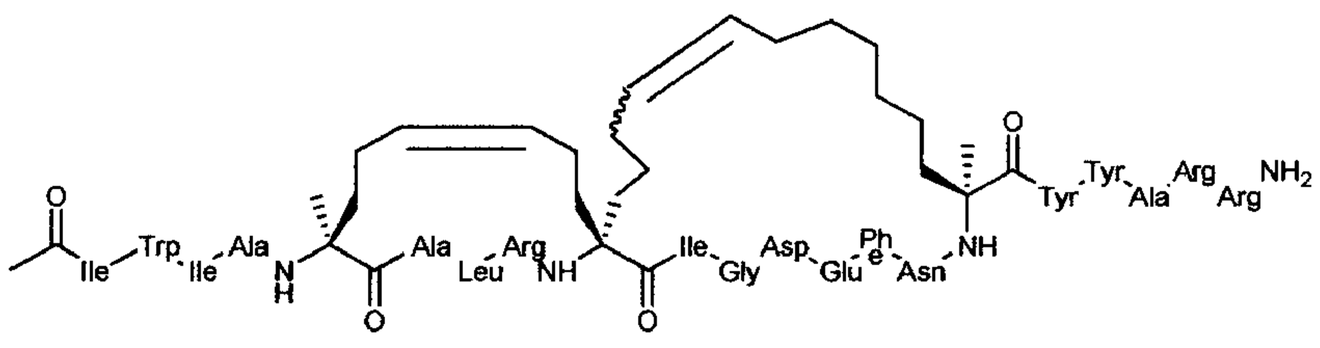
SP12



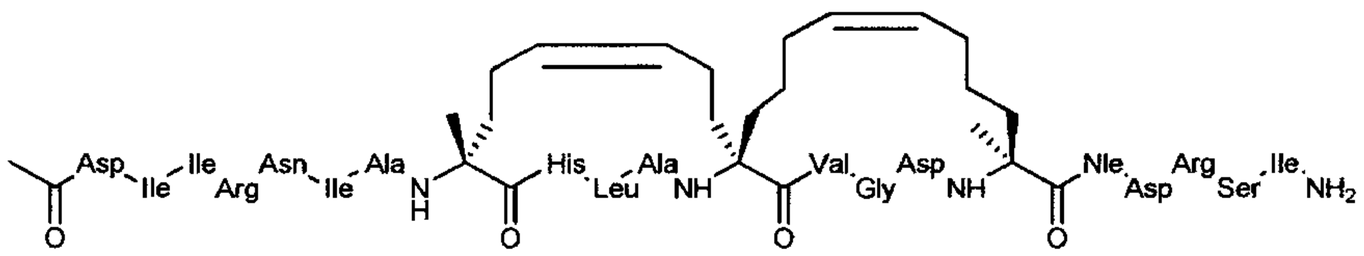
SP16



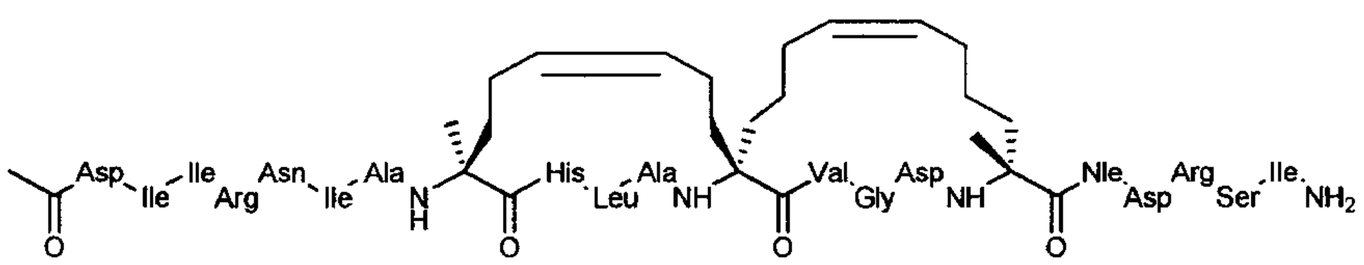
SP20



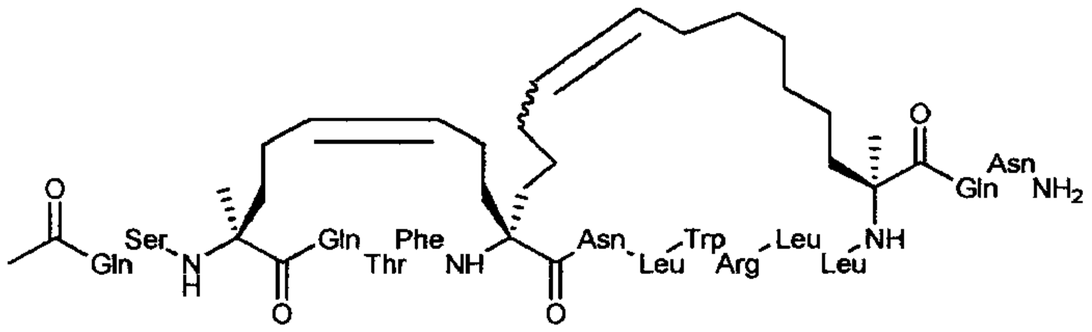
SP21



SP13



SP14



SP17

Preparation of Peptidomimetic Macrocycles

[00121] Peptidomimetic macrocycles of the invention may be prepared by any of a variety of methods known in the art. For example, any of the residues indicated by "X" in Tables 1, 2, 3 or 4 may be substituted with a residue capable of forming a crosslinker with a second residue in the same molecule or a precursor of such a residue.

[00122] Various methods to effect formation of peptidomimetic macrocycles are known in the art. For example, the preparation of peptidomimetic macrocycles of Formula I is described in Schafmeister et al., J. Am. Chem. Soc. 122:5891-5892 (2000); Schafmeister & Verdine, J. Am. Chem. Soc. 122:5891 (2005); Walensky et al., Science 305:1466-1470 (2004); and US Patent No. 7,192,713. The α,α-disubstituted amino acids and amino acid precursors disclosed in the cited references may be employed in synthesis of the peptidomimetic macrocycle precursor polypeptides. Following incorporation of such amino acids into precursor polypeptides, the terminal olefins are reacted with a metathesis catalyst, leading to the formation of the peptidomimetic macrocycle.

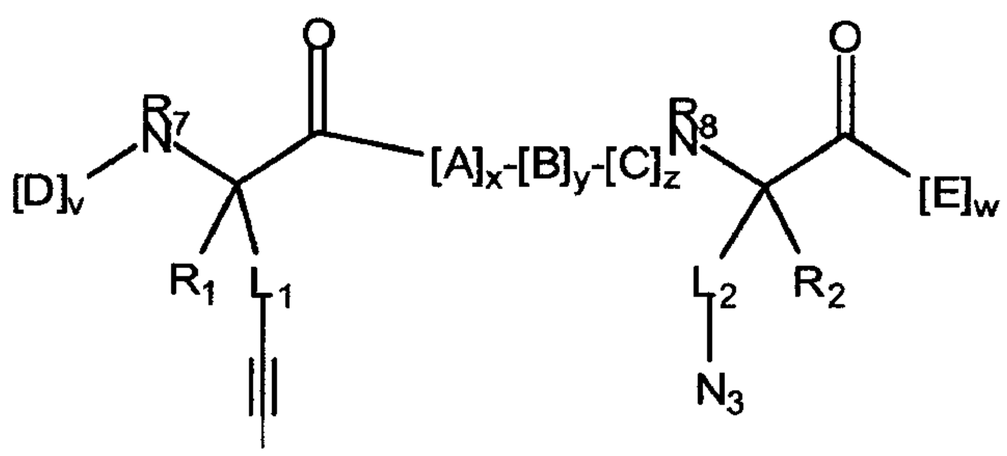
[00123] In other embodiments, the peptidomimetic macrocyles of the invention are of Formula IV or IVa. Methods for the preparation of such macrocycles are described, for example, in US Patent No. 7,202,332.

[00124] In some embodiments, the synthesis of these peptidomimetic macrocycles involves a multi-step process that features the synthesis of a peptidomimetic precursor containing an azide moiety and an alkyne moiety; followed by contacting the peptidomimetic precursor with a macrocyclization reagent to generate a triazole-linked peptidomimetic macrocycle. Macrocycles or macrocycle precursors are synthesized, for example, by solution

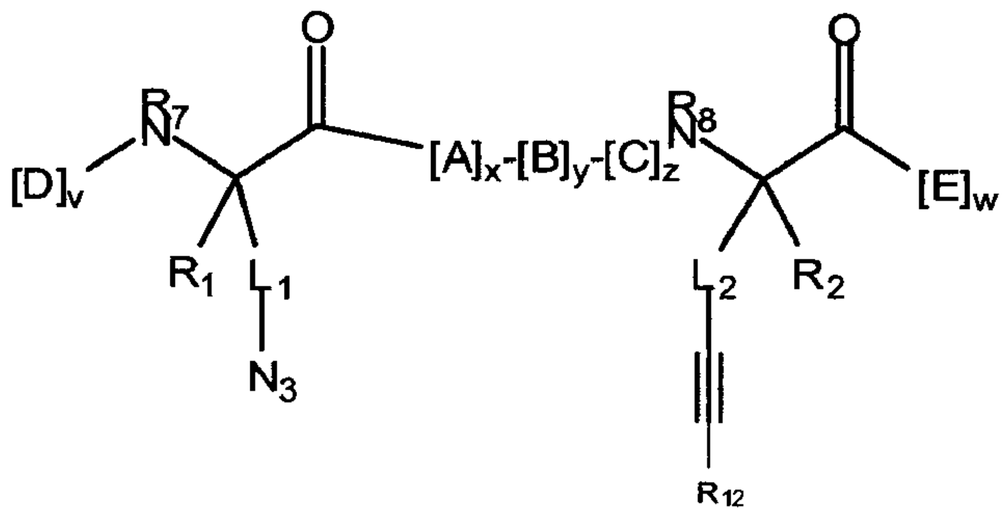
phase or solid-phase methods, and can contain both naturally-occurring and non-naturally-occurring amino acids. See, for example, Hunt, "The Non-Protein Amino Acids" in Chemistry and Biochemistry of the Amino Acids, edited by G.C. Barrett, Chapman and Hall, 1985.

[00125] In some embodiments, an azide is linked to the α-carbon of a residue and an alkyne is attached to the α-carbon of another residue. In some embodiments, the azide moieties are azido-analogs of amino acids L-lysine, D-lysine, alpha-methyl-L-lysine, alpha-methyl-D-lysine, L-ornithine, D-ornithine, alpha-methyl-L-ornithine or alpha-methyl-D-ornithine. In another embodiment, the alkyne moiety is L-propargylglycine. In yet other embodiments, the alkyne moiety is an amino acid selected from the group consisting of L-propargylglycine, D-propargylglycine, (S)-2-amino-2-methyl-4-pentynoic acid, (R)-2-amino-2-methyl-4-pentynoic acid, (S)-2-amino-2-methyl-5-hexynoic acid, (R)-2-amino-2-methyl-5-hexynoic acid, (S)-2-amino-2-methyl-6-heptynoic acid, (R)-2-amino-2-methyl-6-heptynoic acid, (S)-2-amino-2-methyl-7-octynoic acid, (R)-2-amino-2-methyl-7-octynoic acid, (S)-2-amino-2-methyl-8-nonynoic acid and (R)-2-amino-2-methyl-8-nonynoic acid.

[00126] In some embodiments, the invention provides a method for synthesizing a peptidomimetic macrocycle, the method comprising the steps of contacting a peptidomimetic precursor of Formula VI or Formula VII:



(Formula VI)



(Formula VII)

with a macrocyclization reagent;

wherein v, w, x, y, z, A, B, C, D, E, R_1, R_2, R_7, R_8, L_1 and L_2 are as defined for Formula (II); R_{12} is –H when the macrocyclization reagent is a Cu reagent and R_{12} is –H or alkyl when the macrocyclization reagent is a Ru reagent; and further wherein said contacting step results in a covalent linkage being formed between the alkyne and azide moiety in Formula III or Formula IV. For example, R_{12} may be methyl when the macrocyclization reagent is a Ru reagent.

[00127] In the peptidomimetic macrocycles of the invention, 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.

[00128] 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.

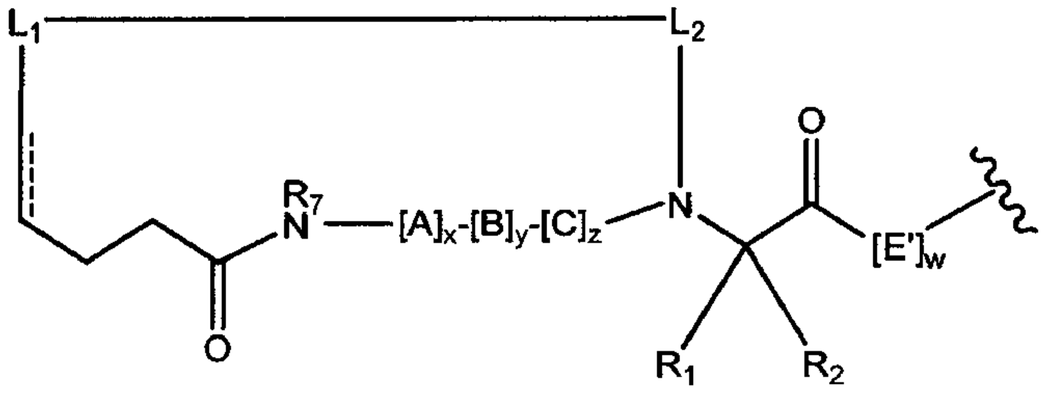
[00129] In some embodiments, the peptidomimetic precursor is purified prior to the contacting step. In other embodiments, the peptidomimetic macrocycle is purified after the contacting step. In still other embodiments, the peptidomimetic macrocycle is refolded after the contacting step. The method may be performed in solution, or, alternatively, the method may be performed on a solid support.

[00130] Also envisioned herein is performing the method of the invention in the presence of a target macromolecule that binds to the peptidomimetic precursor or peptidomimetic macrocycle under conditions that favor said binding. In some embodiments, the method is performed in the presence of a target macromolecule that binds preferentially to the peptidomimetic precursor or peptidomimetic macrocycle under conditions that favor said binding. The method may also be applied to synthesize a library of peptidomimetic macrocycles.

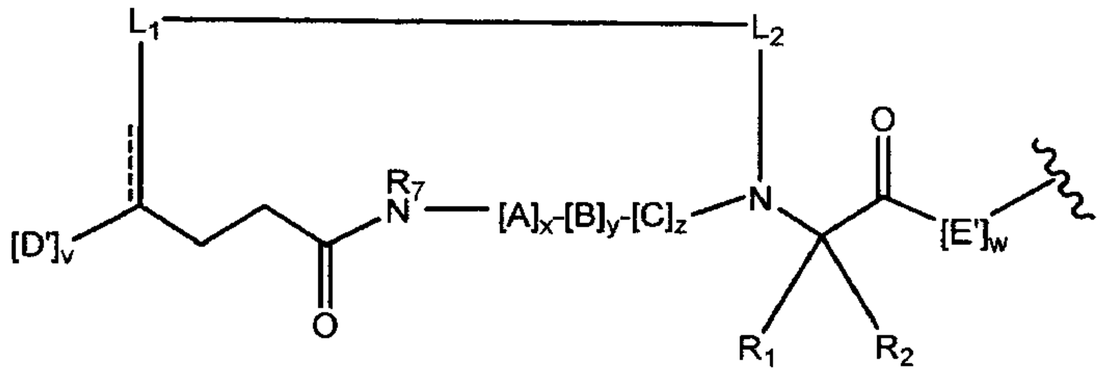
[00131] In some embodiments, the alkyne moiety of the peptidomimetic precursor of Formula VI or Formula VII is a sidechain of an amino acid selected from the group consisting of L-propargylglycine, D-propargylglycine, (S)-2-amino-2-methyl-4-pentynoic acid, (R)-2-amino-2-methyl-4-pentynoic acid, (S)-2-amino-2-methyl-5-hexynoic acid, (R)-2-amino-2-methyl-5-hexynoic acid, (S)-2-amino-2-methyl-6-heptynoic acid, (R)-2-amino-2-methyl-6-heptynoic acid, (S)-2-amino-2-methyl-7-octynoic acid, (R)-2-amino-2-methyl-7-octynoic acid, (S)-2-amino-2-methyl-8-nonynoic acid, and (R)-2-amino-2-methyl-8-nonynoic acid. In other embodiments, the azide moiety of the peptidomimetic precursor of Formula VI or Formula VII 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.

[00132] In some embodiments, x+y+z is 3, and and A, B and C are independently natural or non-natural amino acids. In other embodiments, x+y+z is 6, and and A, B and C are independently natural or non-natural amino acids.

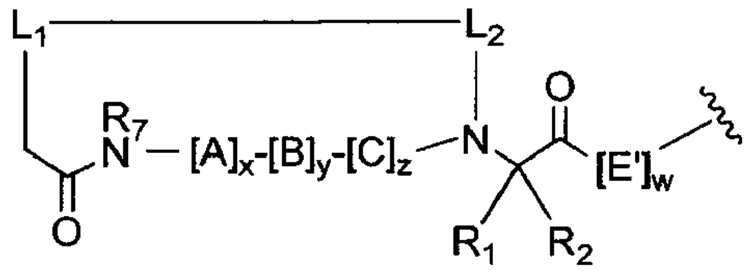
[00133] In some embodiments of peptidomimetic macrocycles of the invention, $[D]_v$ and/or $[E]_w$ comprise additional peptidomimetic macrocycles or macrocyclic structures. For example, $[D]_v$ may have the formula:



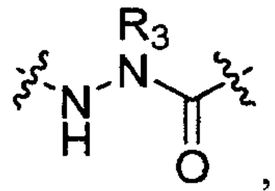
,



or



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

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

R_1, R_2 and R_8 are independently –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, cycloalkylalkyl, heteroalkyl, or heterocycloalkyl, unsubstituted or substituted with halo–, or part of a cyclic structure with an E residue;

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

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

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

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

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

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

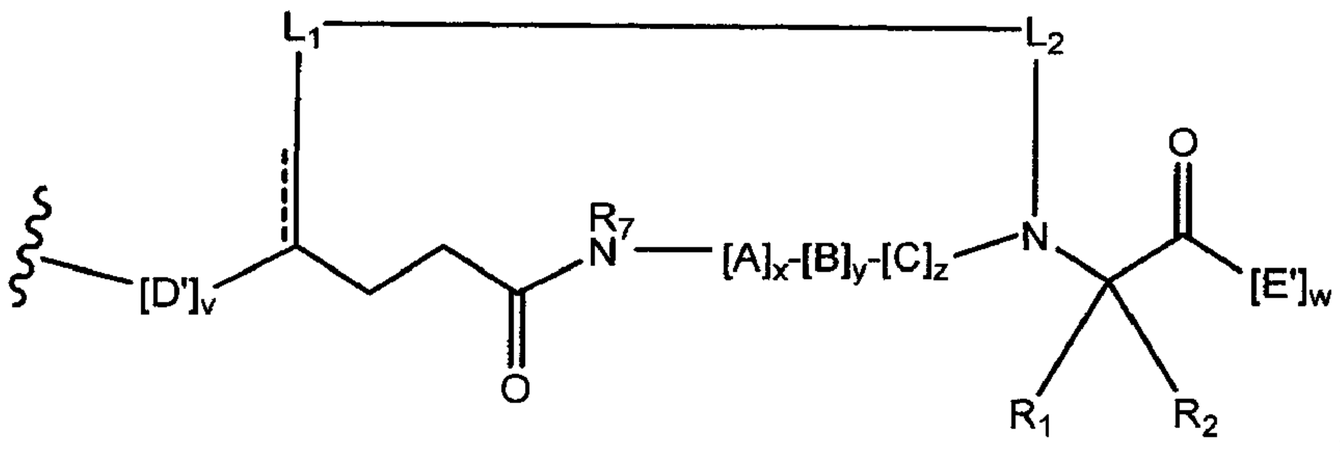
R_7 is –H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkyl, heteroalkyl, cycloalkylalkyl, heterocycloalkyl, cycloaryl, or heterocycloaryl, optionally substituted with R_5;

v is an integer from 1-1000;

w is an integer from 1-1000; and

x is an integer from 0-10.

[00134] In another embodiment, $[E]_w$ has the formula:



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

[00135] 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.

[00136] 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.

[00137] The peptidomimetic macrocycles of the invention 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).

[00138] 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.

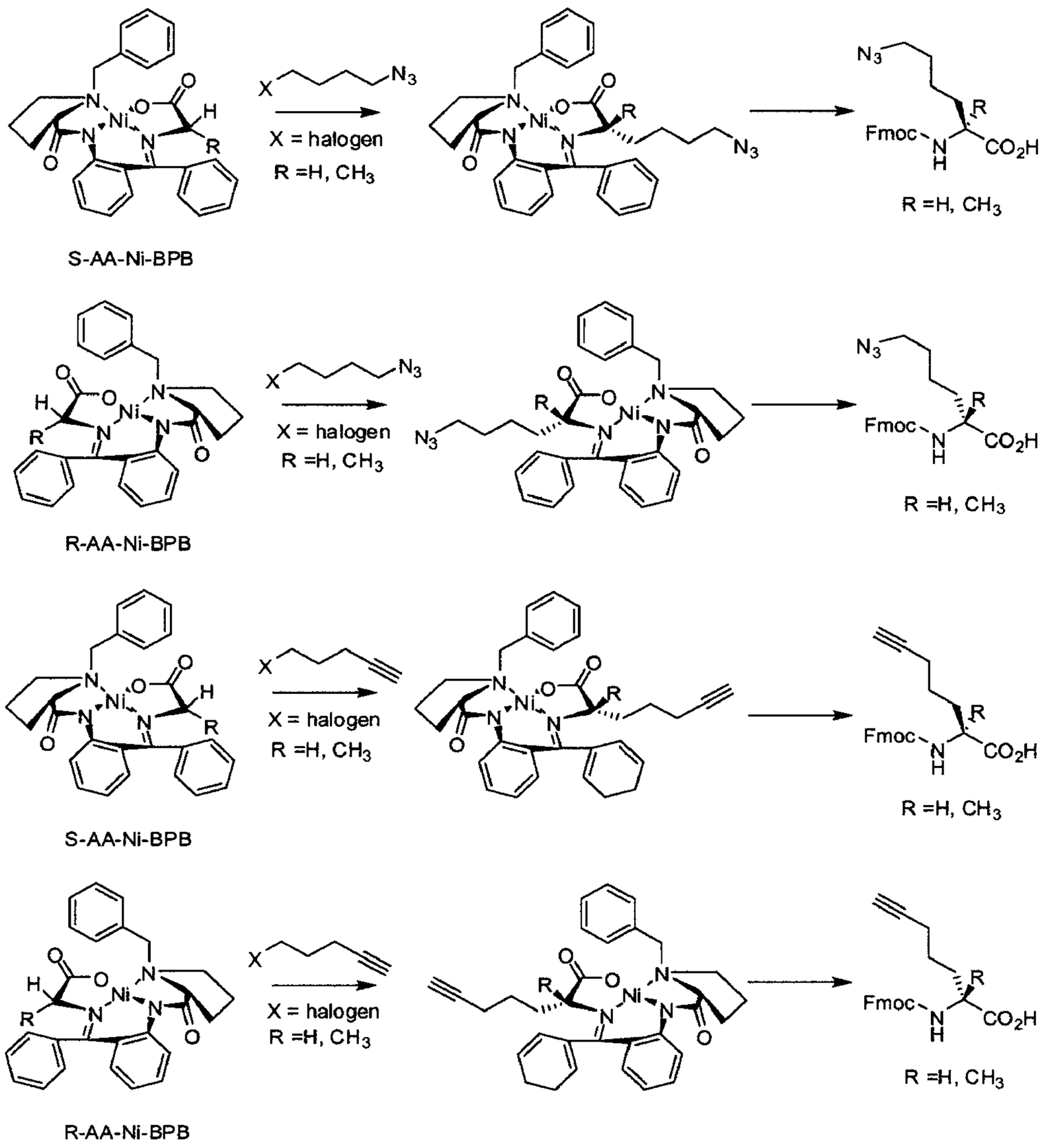
[00139] Longer peptidomimetic precursors are produced, for example, by conjoining individual synthetic peptides using native chemical ligation. Alternatively, the longer synthetic peptides are biosynthesized by well known recombinant DNA and protein expression techniques. Such techniques are provided in well-known standard manuals with detailed protocols. To construct a gene encoding a peptidomimetic precursor of this invention, the amino acid sequence is reverse translated to obtain a nucleic acid sequence encoding the amino acid sequence, preferably with codons that are optimum for the organism in which the gene is to be expressed. Next, a synthetic gene is made, typically by synthesizing oligonucleotides which encode the peptide and any regulatory elements, if necessary. The synthetic gene is inserted in a suitable cloning vector and transfected into a host cell. The peptide is then expressed under suitable conditions appropriate for the selected expression system and host. The peptide is purified and characterized by standard methods.

[00140] The peptidomimetic precursors are made, for example, in a high-throughput, combinatorial fashion using, for example, a high-throughput polychannel combinatorial synthesizer (*e.g.*, Thuramed TETRAS multichannel peptide synthesizer from CreoSalus, Louisville, KY or Model Apex 396 multichannel peptide synthesizer from AAPPTEC, Inc., Louisville, KY).

[00141] The following synthetic schemes are provided solely to illustrate the present invention and are not intended to limit the scope of the invention, as described herein. To simplify the drawings, the illustrative schemes depict azido amino acid analogs ε-azido-α-methyl-L-lysine and ε-azido-α -methyl-D-lysine, and alkyne amino acid analogs L-propargylglycine, (S)-2-amino-2-methyl-4-pentynoic acid, and (S)-2-amino-2-methyl-6-heptynoic acid. Thus, in the following synthetic schemes, each R_1, R_2, R_7 and R_8 is -H; each L_1 is $-(CH_2)_4-$; and each L_2 is

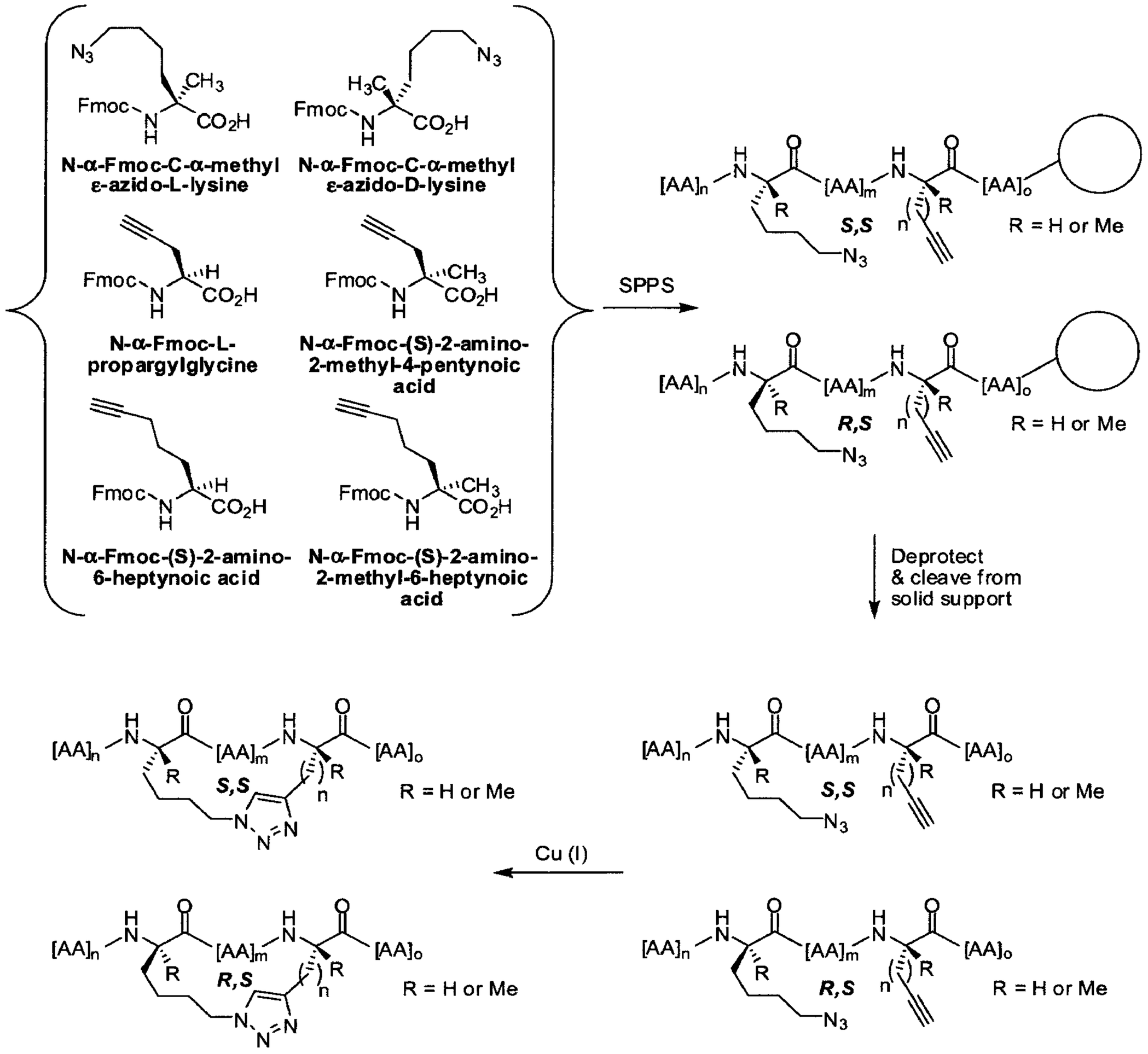
-(CH_2)-. However, as noted throughout the detailed description above, many other amino acid analogs can be employed in which R_1, R_2, R_7, R_8, L_1 and L_2 can be independently selected from the various structures disclosed herein.

[00142] Synthetic Scheme 1:



[00143] Synthetic Scheme 1 describes the preparation of several compounds of the invention. Ni(II) complexes of Schiff bases derived from the chiral auxiliary (S)-2-[N-(N'-benzylprolyl)amino]benzophenone (BPB) and amino acids such as glycine or alanine are prepared as described in Belokon *et al.* (1998), *Tetrahedron Asymm.* 9:4249-4252. The resulting complexes are subsequently reacted with alkylating reagents comprising an azido or alkynyl moiety to yield enantiomerically enriched compounds of the invention. If desired, the resulting compounds can be protected for use in peptide synthesis.

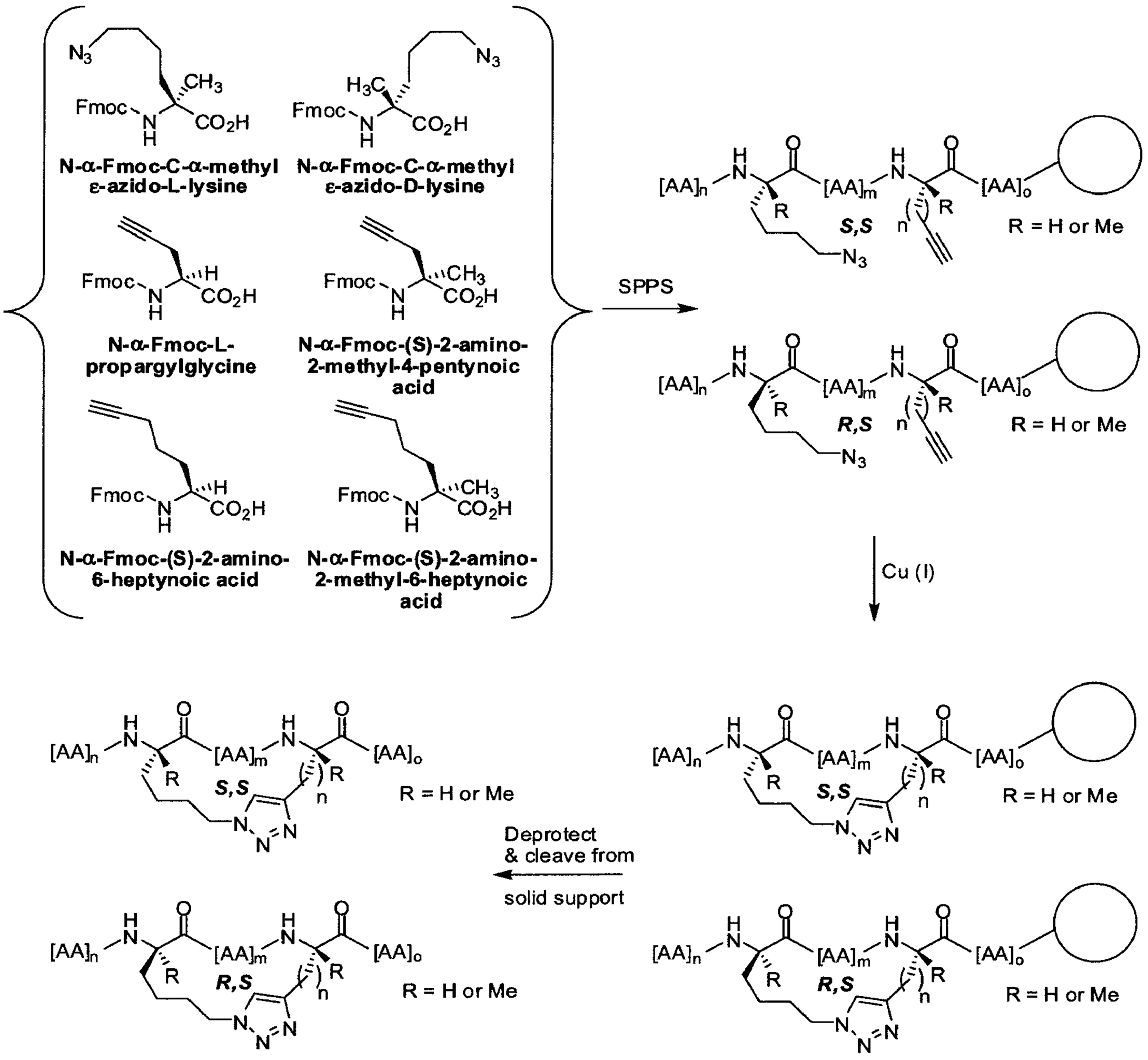
[00144] Synthetic Scheme 2:



[00145] In the general method for the synthesis of peptidomimetic macrocycles shown in Synthetic Scheme 2, the peptidomimetic precursor contains an azide moiety and an alkyne moiety and is synthesized by solution-phase or solid-phase peptide synthesis (SPPS) using the commercially available amino acid N-α-Fmoc-L-propargylglycine and the N-α-Fmoc-protected forms of the amino acids (S)-2-amino-2-methyl-4-pentynoic acid, (S)-2-amino-6-heptynoic acid, (S)-2-amino-2-methyl-6-heptynoic acid, N-methyl-ε-azido-L-lysine, and N-methyl-ε-azido-D-lysine. The peptidomimetic precursor is then deprotected and cleaved from the solid-phase resin by standard conditions (*e.g.*, strong acid such as 95% TFA). The peptidomimetic precursor is reacted as a crude mixture or is purified prior to reaction with a macrocyclization reagent such as a Cu(I) in organic or aqueous solutions (Rostovtsev *et al.* (2002), *Angew. Chem. Int. Ed.* 41:2596-2599; Tornoe *et al.* (2002), *J. Org. Chem.* 67:3057-3064; Deiters *et al.* (2003), *J. Am. Chem. Soc.* 125:11782-11783; 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 H_2O, THF, CH_3CN, DMF , DIPEA, tBuOH or a mixture thereof. In another

embodiment, the macrocyclization step is performed in DMF. In some embodiments, the macrocyclization step is performed in a buffered aqueous or partially aqueous solvent.

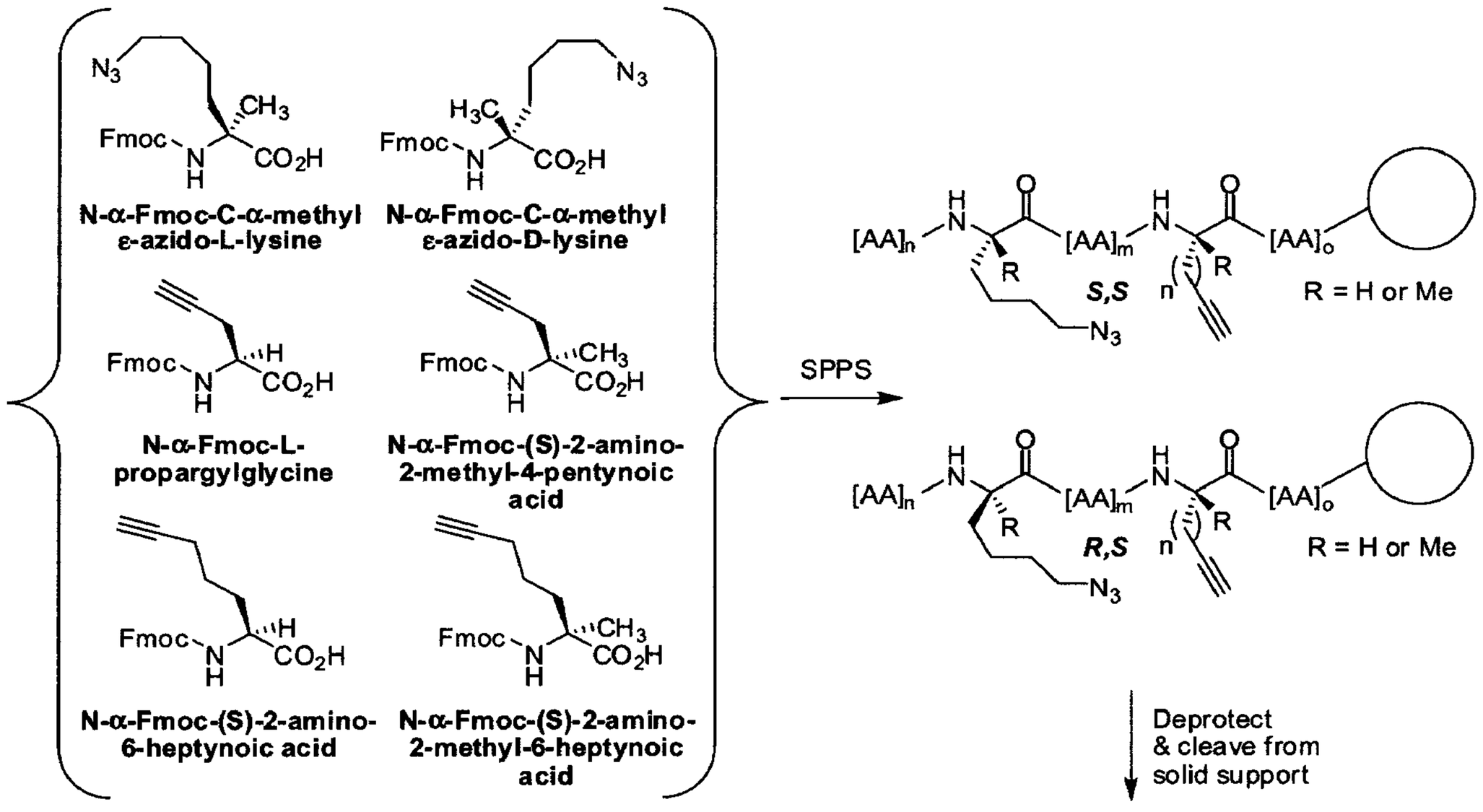
[00146] Synthetic Scheme 3:



[00147] In the general method for the synthesis of peptidomimetic macrocycles shown in Synthetic Scheme 3, the peptidomimetic precursor contains an azide moiety and an alkyne moiety and is synthesized by solid-phase peptide synthesis (SPPS) using the commercially available amino acid N-α-Fmoc-L-propargylglycine and the N-α-Fmoc-protected forms of the amino acids (S)-2-amino-2-methyl-4-pentynoic acid, (S)-2-amino-6-heptynoic acid, (S)-2-amino-2-methyl-6-heptynoic acid, N-methyl-ε-azido-L-lysine, and N-methyl-ε-azido-D-lysine. The peptidomimetic precursor is reacted with a macrocyclization reagent such as a Cu(I) reagent on the resin as a crude mixture (Rostovtsev *et al.* (2002), *Angew. Chem. Int. Ed.* 41:2596-2599; Tornoe *et al.* (2002), *J. Org. Chem.* 67:3057-3064; Deiters *et al.* (2003), *J. Am. Chem. Soc.* 125:11782-11783; 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

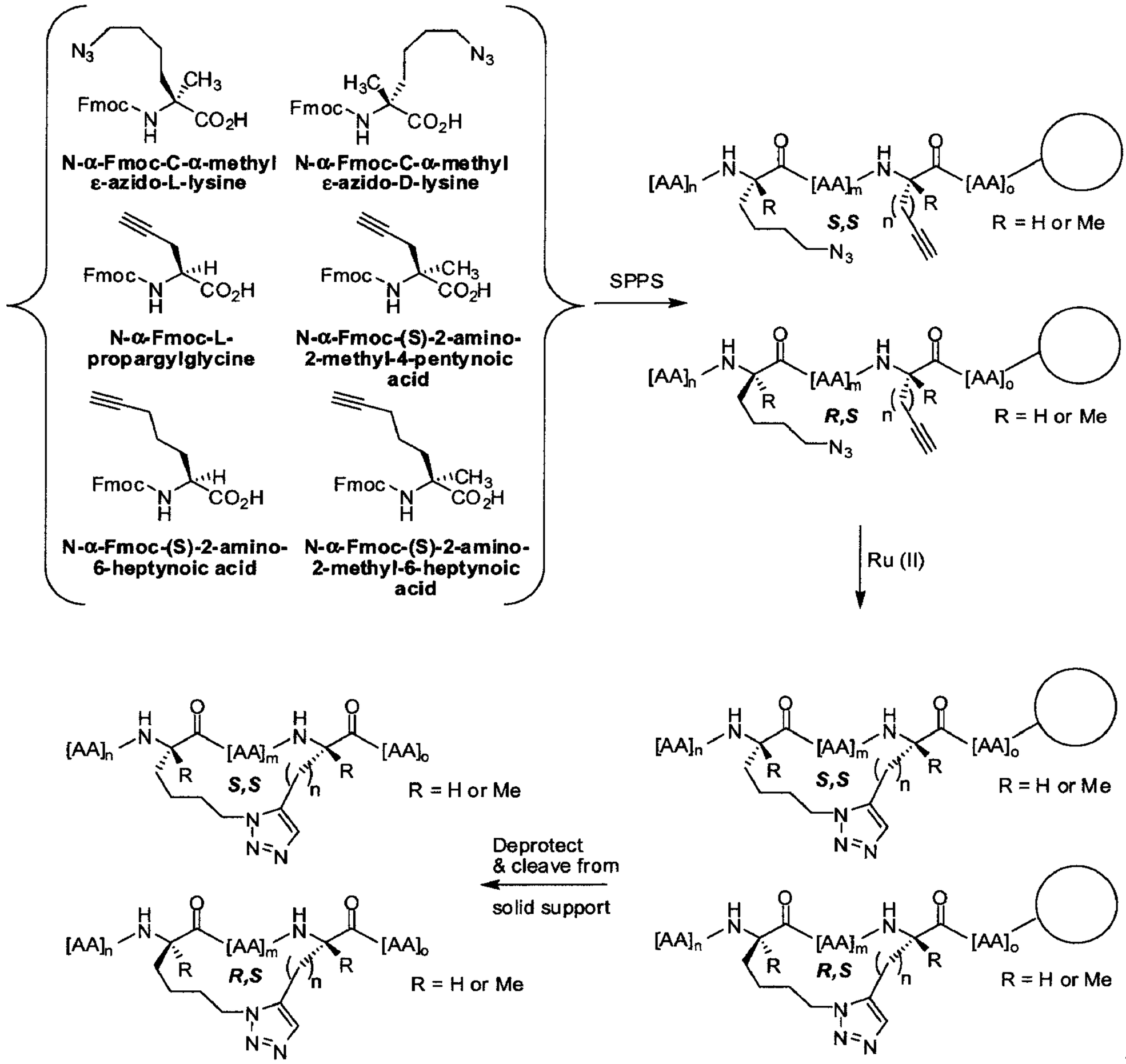
embodiments, the macrocyclization step is performed in a solvent chosen from the group consisting of CH_2Cl_2, $ClCH_2CH_2Cl$, DMF, THF, NMP, DIPEA, 2,6-lutidine, pyridine, DMSO, H_2O or a mixture thereof. In some embodiments, the macrocyclization step is performed in a buffered aqueous or partially aqueous solvent.

[00148] Synthetic Scheme 4:



[00149] In the general method for the synthesis of peptidomimetic macrocycles shown in Synthetic Scheme 4, the peptidomimetic precursor contains an azide moiety and an alkyne moiety and is synthesized by solution-phase or solid-phase peptide synthesis (SPPS) using the commercially available amino acid N-α-Fmoc-L-propargylglycine and the N-α-Fmoc-protected forms of the 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 $Cp^*RuCl(PPh_3)_2$ or $[Cp^*RuCl]_4$ (Rasmussen *et al.* (2007), *Org. Lett.* 9:5337-5339; Zhang *et al.* (2005), *J. Am. Chem. Soc.* 127:15998-15999). In some embodiments, the macrocyclization step is performed in a solvent chosen from the group consisting of DMF, CH_3CN and THF.

[00150] Synthetic Scheme 5:



[00151] In the general method for the synthesis of peptidomimetic macrocycles 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 $Cp^*RuCl(PPh_3)_2$ or $[Cp^*RuCl]_4$ (Rasmussen *et al.* (2007), *Org. Lett.* 9:5337-5339; Zhang *et al.* (2005), *J. Am. Chem. Soc.* 127:15998-15999). In some embodiments, the macrocyclization step is performed in a solvent chosen from the group consisting of CH_2Cl_2, $ClCH_2CH_2Cl$, CH_3CN, DMF, and THF.

[00152] Several exemplary peptidomimetic macrocycles are shown in Table 5. "Nle" represents norleucine and replaces a methionine residue. It is envisioned that similar linkers are used to synthesize peptidomimetic macrocycles based on the polypeptide sequences disclosed in Table 1 through Table 4.

TABLE 5

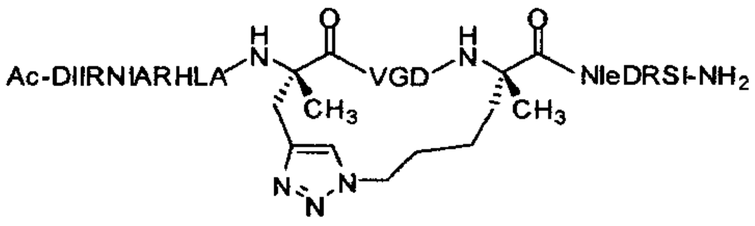
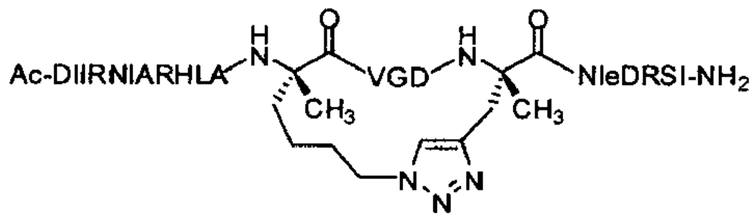
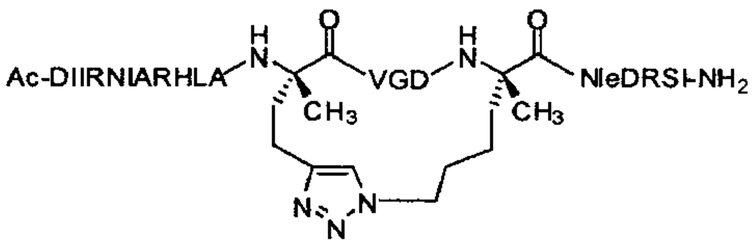
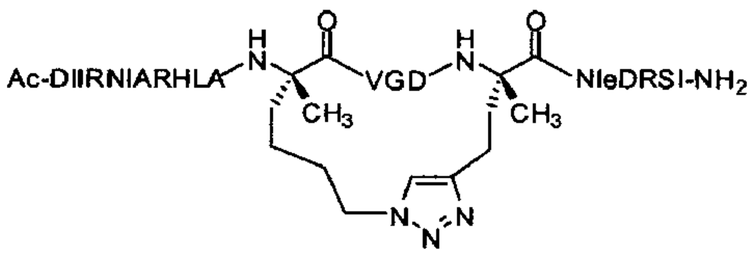
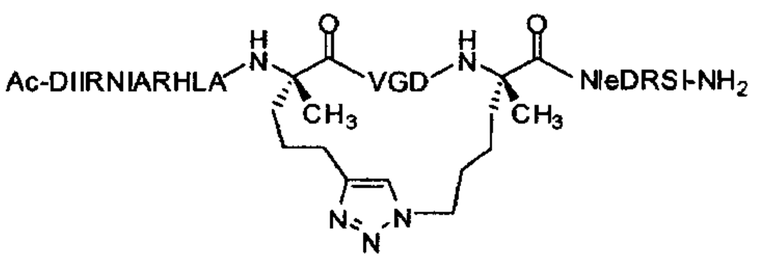
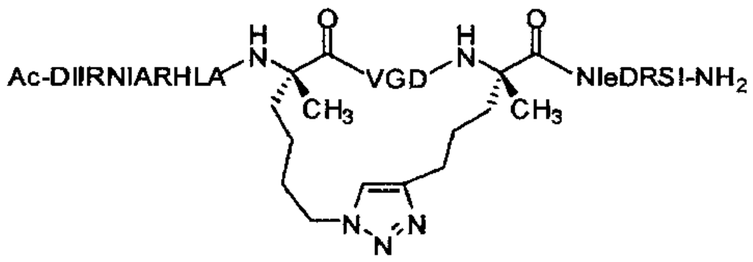
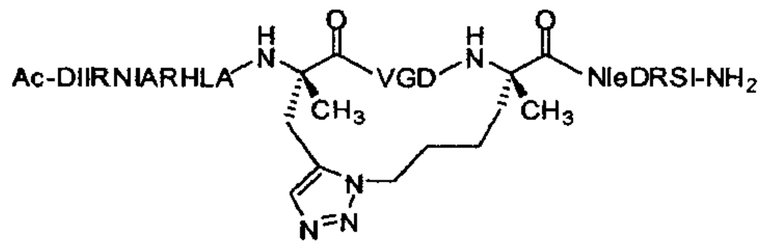
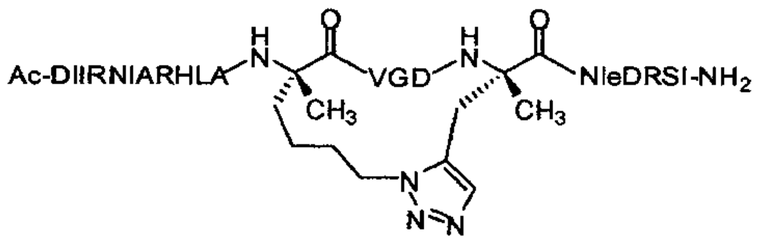
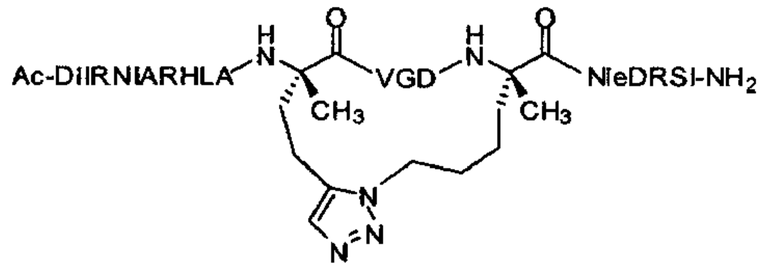
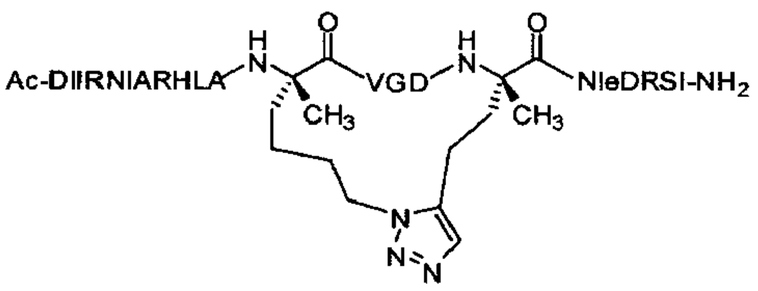
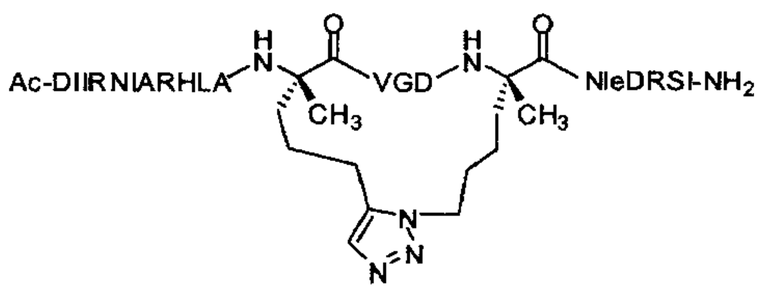
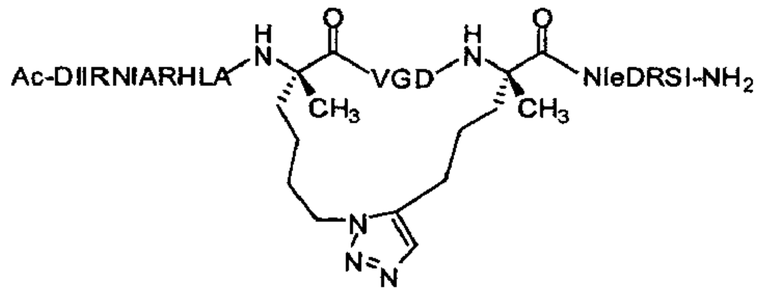
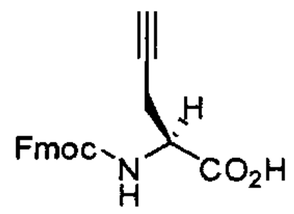
	MW = 2464
	MW = 2464
	MW = 2478
	MW = 2478
	MW = 2492
	MW = 2492
	MW = 2464
	MW = 2464
	MW = 2478
	MW = 2478
	MW = 2492
	MW = 2492

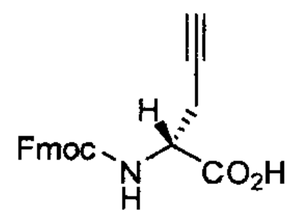
Table 5 shows exemplary peptidommimetic macrocycles of the invention. "Nle" represents norleucine.

[00153] The present invention contemplates the use of non-naturally-occurring amino acids and amino acid analogs in the synthesis of the peptidomimetic macrocycles described herein. Any amino acid or amino acid analog amenable to the synthetic methods employed for the synthesis of stable triazole containing peptidomimetic macrocycles can be used in the present invention. For example, L-propargylglycine is contemplated as a useful amino acid in the present invention. However, other alkyne-containing amino acids that contain a different amino acid side chain are also useful in the invention. For example, L-propargylglycine contains one methylene unit between the α-carbon of the amino acid and the alkyne of the amino acid side chain. The invention also contemplates the use of amino acids with multiple methylene units between the α-carbon and the alkyne. Also, the azido-analogs of amino acids L-lysine, D-lysine, alpha-methyl-L-lysine, and alpha-methyl-D-lysine are contemplated as useful amino acids in the present invention. However, other terminal azide amino acids that contain a different amino acid side chain are also useful in the invention. For example, the azido-analog of L-lysine contains four methylene units between the α-carbon of the amino acid and the terminal azide of the amino acid side chain. The invention also contemplates the use of amino acids with fewer than or greater than four methylene units between the α-carbon and the terminal azide. Table 6 shows some amino acids useful in the preparation of peptidomimetic macrocycles of the invention.

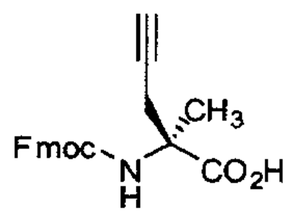
TABLE 6



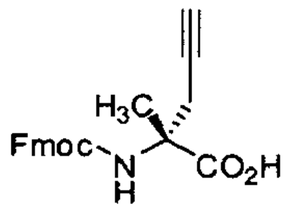
N-α-Fmoc-L-propargyl glycine



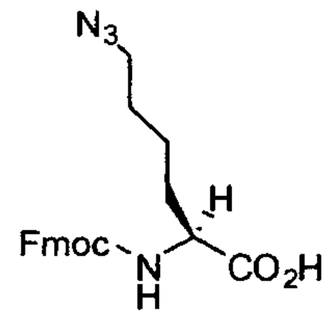
N-α-Fmoc-D-propargyl glycine



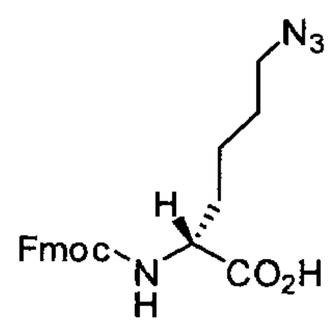
N-α-Fmoc-(S)-2-amino-2-methyl-4-pentynoic acid



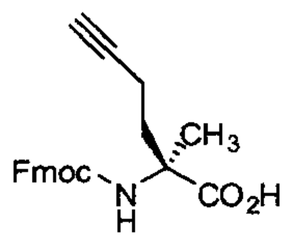
N-α-Fmoc-(R)-2-amino-2-methyl-4-pentynoic acid



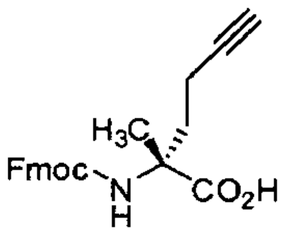
N-α-Fmoc-ε-azido-L-lysine



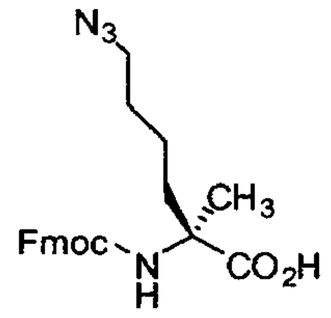
N-α-Fmoc-ε-azido-D-lysine



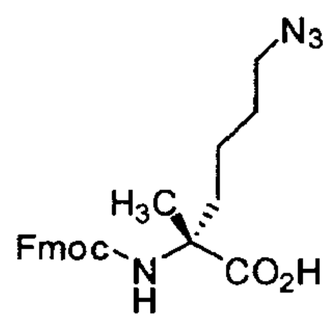
N-α-Fmoc-(S)-2-amino-2-methyl-5-hexynoic acid



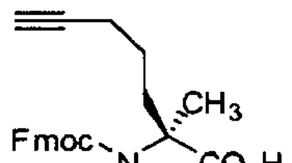
N-α-Fmoc-(R)-2-amino-2-methyl-5-hexynoic acid



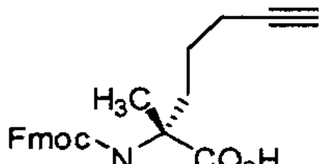
N-α-Fmoc-ε-azido-α-methyl-L-lysine



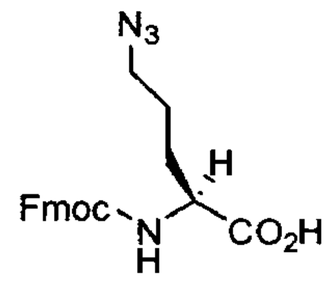
N-α-Fmoc-ε-azido-α-methyl-D-lysine



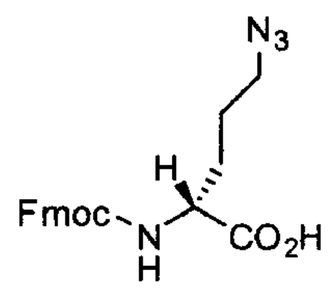
N-α-Fmoc-(S)-2-amino-2-methyl-6-heptynoic acid



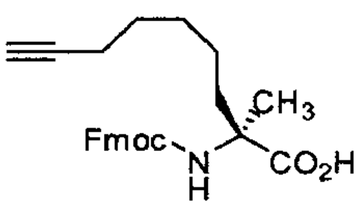
N-α-Fmoc-(R)-2-amino-2-methyl-6-heptynoic acid



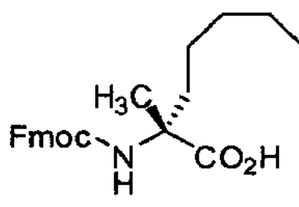
N-α-Fmoc-δ-azido-L-ornithine



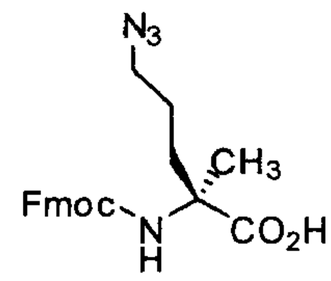
N-α-Fmoc-δ-azido-D-ornithine



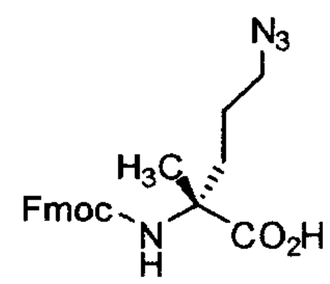
N-α-Fmoc-(S)-2-amino-2-methyl-7-octynoic acid



N-α-Fmoc-(R)-2-amino-2-methyl-7-octynoic acid



N-α-Fmoc-ε-azido-α-methyl-L-ornithine



N-α-Fmoc-ε-azido-α-methyl-D-ornithine

N-α-Fmoc-(S)-2-amino-2-methyl-8-nonynoic acid

N-α-Fmoc-(R)-2-amino-2-methyl-8-nonynoic acid

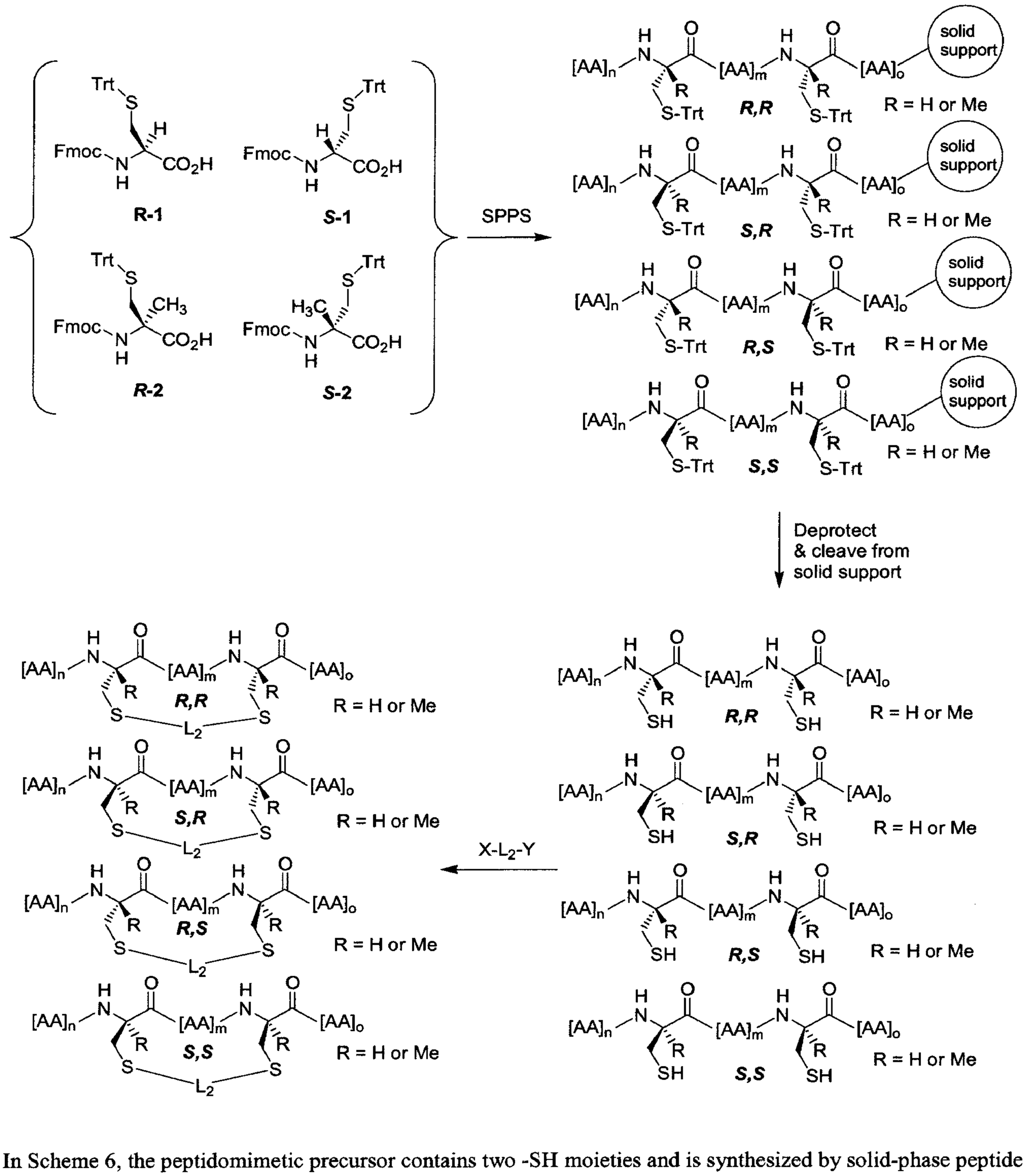
Table 6 shows exemplary amino acids useful in the preparation of peptidomimetic macrocycles of the invention.

[00154] 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-alpha-methyl-L-lysine, and ε-azido-alpha-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.

[00155] 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.

[00156] In other embodiments, peptidomimetic macrocycles of Formula III are synthesized. The following synthetic schemes describe the preparation of such compounds. To simplify the drawings, the illustrative schemes depict amino acid analogs derived from L-or D-cysteine, in which L_1 and L_3 are both -(CH_2)-. However, as noted throughout the detailed description above, many other amino acid analogs can be employed in which L_1 and L_3 can be independently selected from the various structures disclosed herein. The symbols "$[AA]_m$", "$[AA]_n$", "$[AA]_o$" 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.

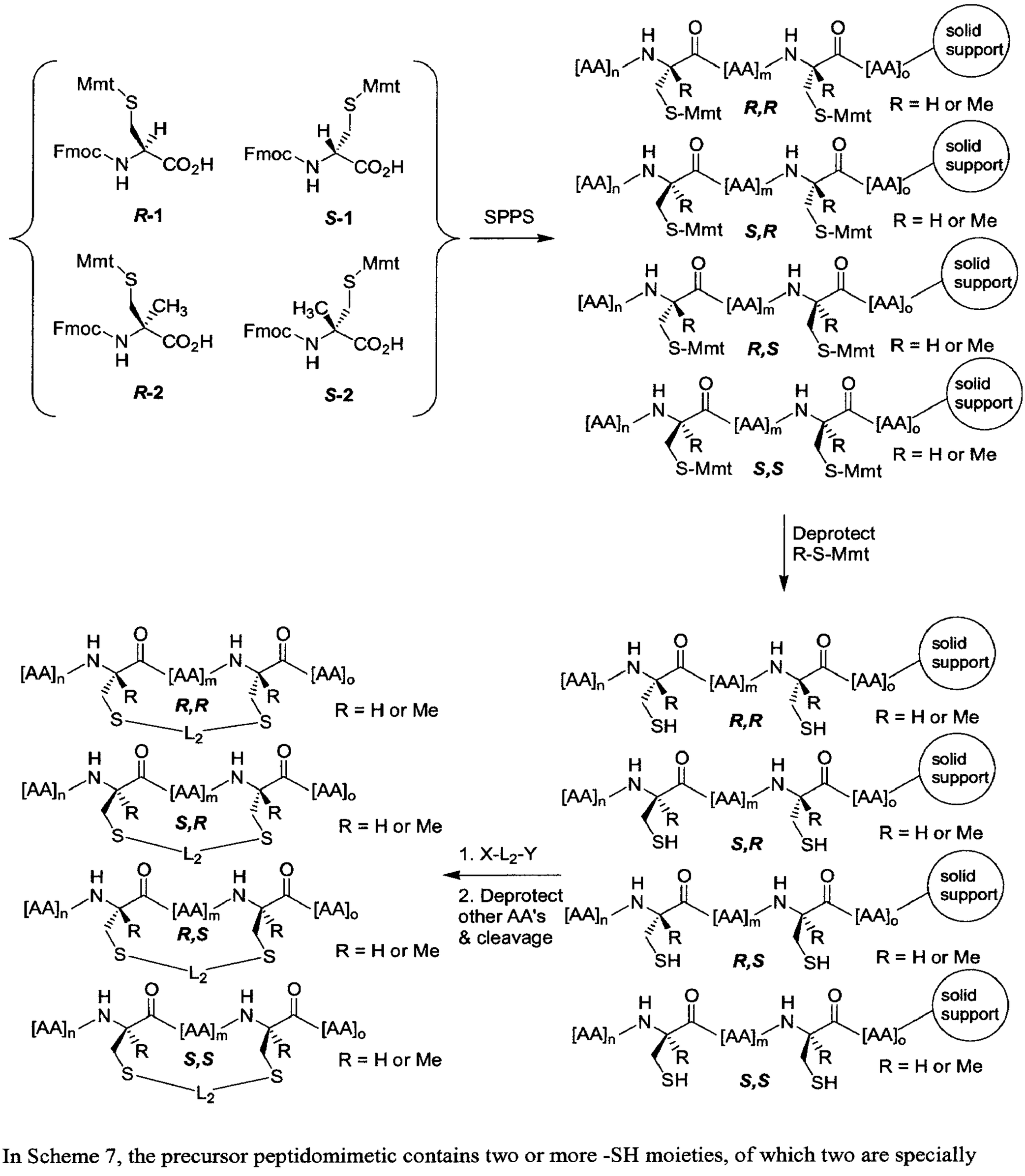
Synthetic Scheme 6:



[00157] In Scheme 6, the peptidomimetic precursor contains two -SH moieties and is synthesized by solid-phase peptide synthesis (SPPS) using commercially available N-α-Fmoc amino acids such as N-α-Fmoc-S-trityl-L-cysteine or N-α-Fmoc-S-trityl-D-cysteine. Alpha-methylated versions of D-cysteine or L-cysteine are generated by known methods (Seebach *et al.* (1996), *Angew. Chem. Int. Ed. Engl.* 35:2708-2748, and references therein) and then converted to the appropriately protected N-α-Fmoc-S-trityl monomers by known methods ("Bioorganic Chemistry: Peptides and Proteins", Oxford University Press, New York: 1998, the entire contents of which are incorporated herein by reference). The precursor peptidomimetic is then deprotected and cleaved from the solid-phase resin by standard conditions (*e.g.*, strong acid such as 95% TFA). The precursor peptidomimetic is reacted

as a crude mixture or is purified prior to reaction with X-L_2-Y in organic or aqueous solutions. In some embodiments the alkylation reaction is performed under dilute conditions (i.e. 0.15 mmol/L) to favor macrocyclization and to avoid polymerization. In some embodiments, the alkylation reaction is performed in organic solutions such as liquid NH_3 (Mosberg et al. (1985), J. Am.Chem. Soc. 107:2986-2987; Szewczuk et al. (1992), Int. J. Peptide Protein Res. 40 :233-242), NH_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 solvent used for the alkylation reaction is DMF or dichloroethane.

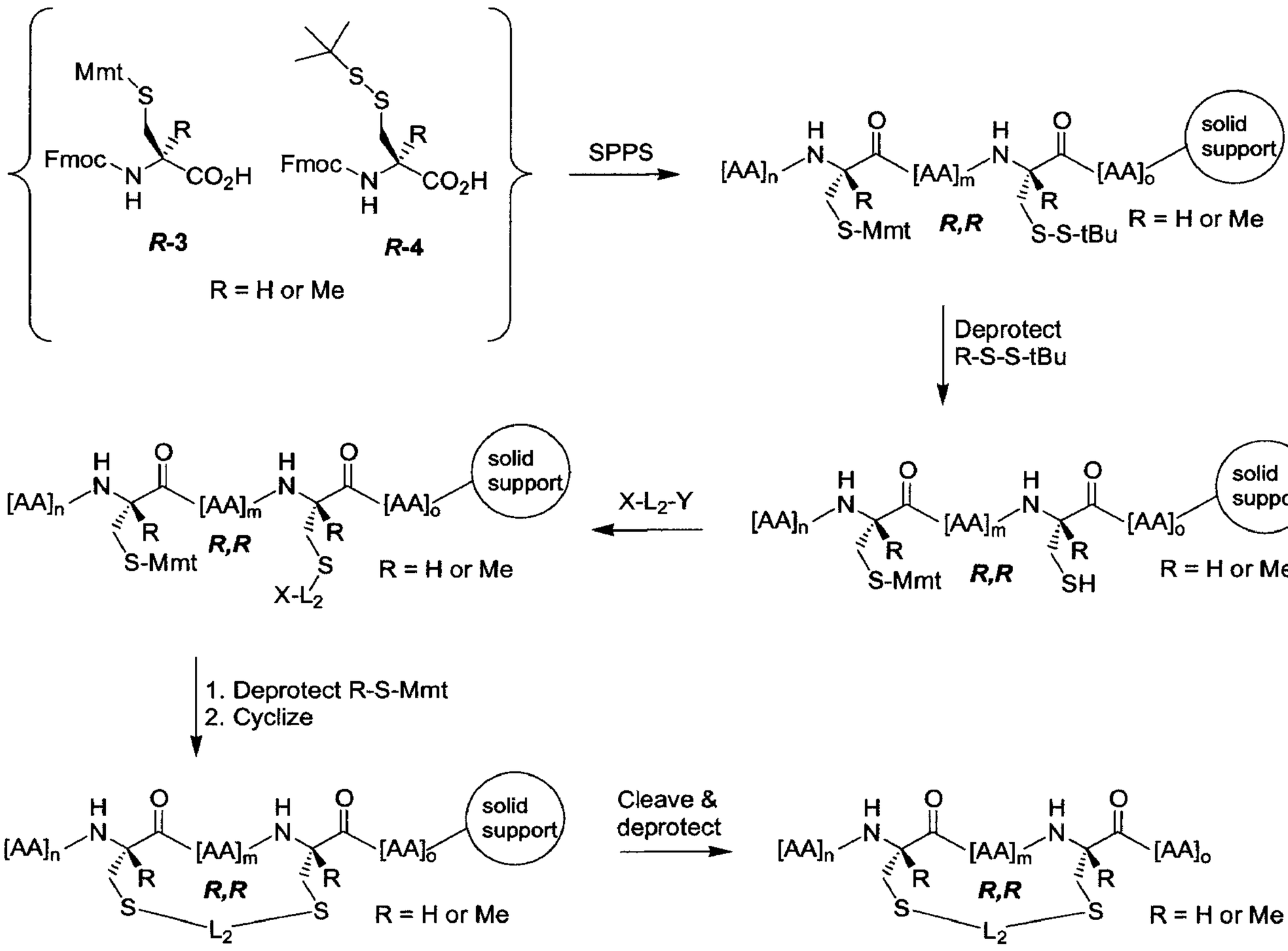
Synthetic Scheme 7:



[00158] 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-L_2-Y in an organic solution. For example, the reaction takes place in the presence of a hindered base such as diisopropylethylamine. In some embodiments, the alkylation reaction is performed in organic solutions such as liquid NH_3 (Mosberg *et al.* (1985), *J. Am.Chem. Soc.* 107:2986-2987; Szewczuk *et al.* (1992), *Int. J. Peptide Protein Res.* 40 :233-242), NH_3/MeOH or NH_3/DMF (Or *et al.* (1991), *J. Org. Chem.* 56:3146-3149). In other embodiments, the alkylation reaction is performed in DMF or dichloroethane. The peptidomimetic macrocycle is then deprotected and cleaved from the solid-phase resin by standard conditions (*e.g.*, strong acid such as 95% TFA).

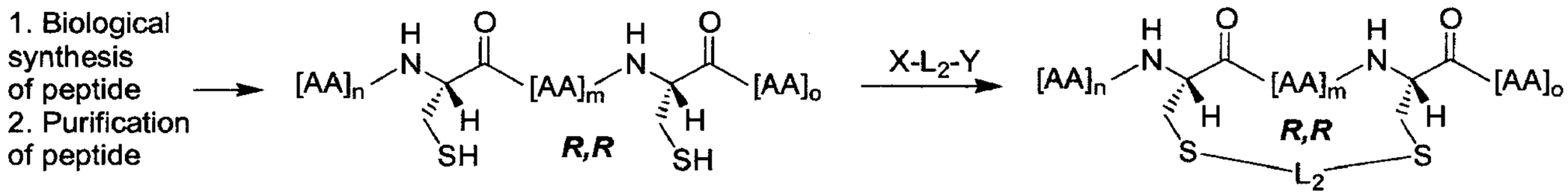
Synthetic Scheme 8:



[00159] 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-tButyl protecting group of the peptidomimetic precursor is selectively cleaved by known

conditions (*e.g.*, 20% 2-mercaptoethanol in DMF, reference: Galande *et al.* (2005), *J. Comb. Chem.* 7:174-177). The precursor peptidomimetic is then reacted on the resin with a molar excess 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:



[00160] 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.

[00161] Various embodiments for X and Y are envisioned which are suitable for reacting with thiol groups. In general, each X or Y is independently be selected from the general category shown in Table 5. For example, X and Y are halides such as –Cl, –Br or –I. Any of the macrocycle-forming linkers described herein may be used in any combination with any of the sequences shown in Tables 1-4 and also with any of the R– substituents indicated herein.

TABLE 5: Examples of Reactive Groups Capable of Reacting with Thiol Groups and Resulting Linkages

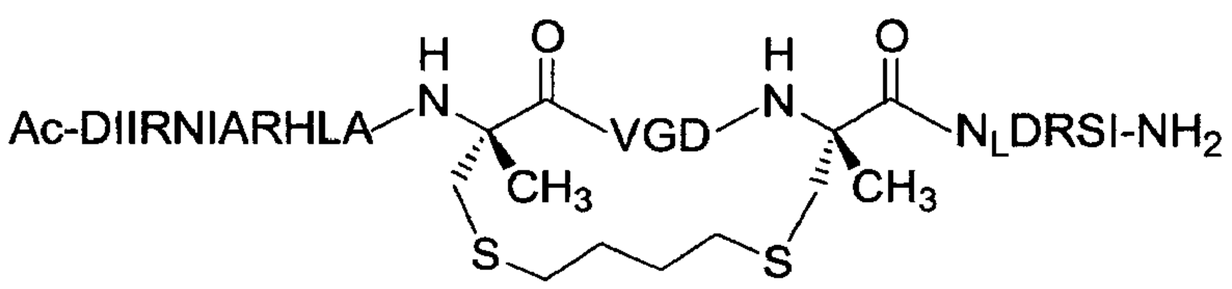
(1) X or Y	**(2) Resulting Covalent Linkage**
(3) acrylamide	(4) Thioether
(5) halide (e.g. alkyl or aryl halide)	(6) Thioether

TABLE **5: Examples of Reactive Groups Capable of Reacting with Thiol Groups and Resulting Linkages**

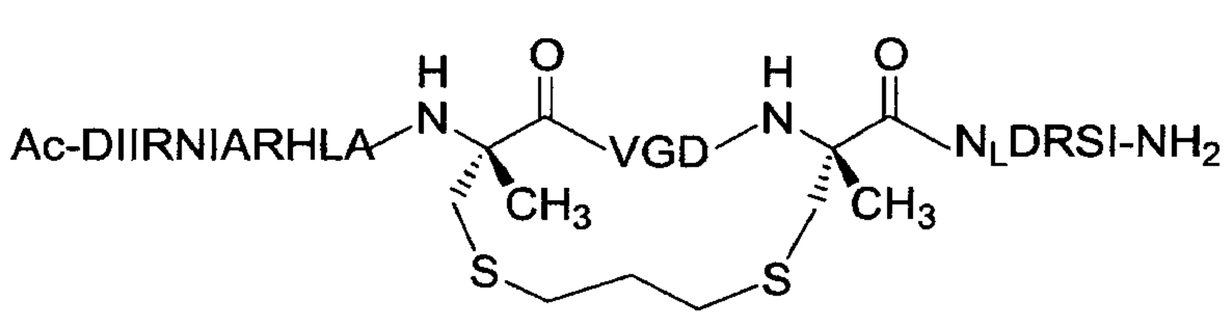
(1)	X or Y	(2)	Resulting Covalent Linkage
(7)	sulfonate	(8)	Thioether
(9)	aziridine	(10)	Thioether
(11)	epoxide	(12)	Thioether
(13)	haloacetamide	(14)	Thioether
(15)	maleimide	(16)	Thioether
(17)	sulfonate ester	(18)	Thioether

[00162] Table 6 shows exemplary macrocycles of the invention. "N_L" represents norleucine and replaces a methionine residue. It is envisioned that similar linkers are used to synthesize peptidomimetic macrocycles based on the polypeptide sequences disclosed in Table 1 through Table 4.

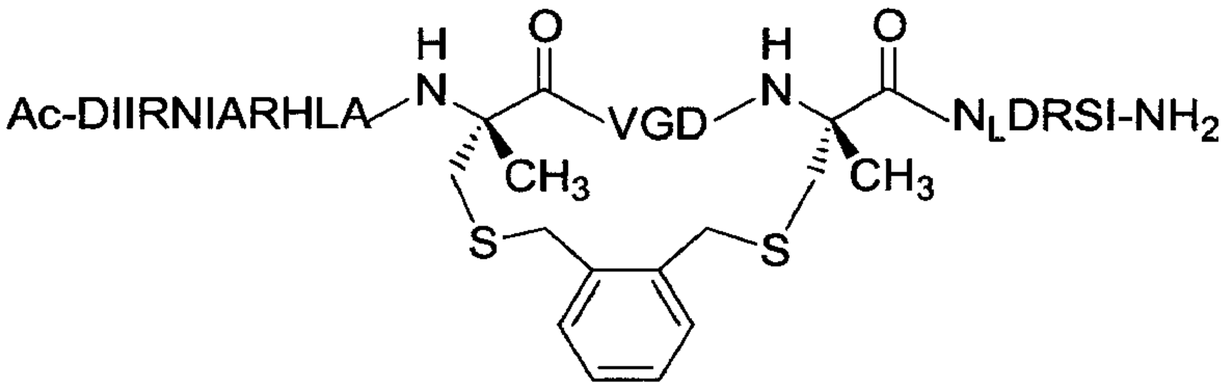
TABLE 6: Examples of Peptidomimetic Macrocycles of the Invention



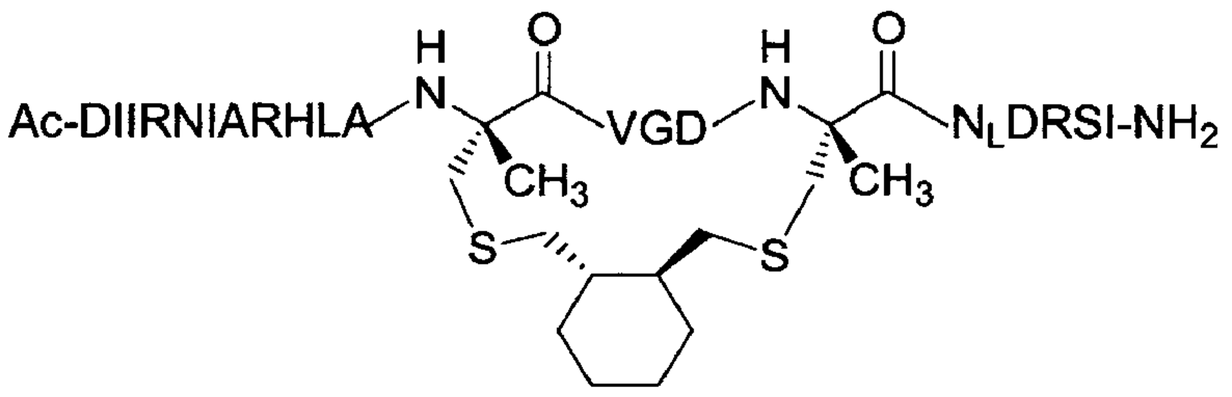
MW = 2477



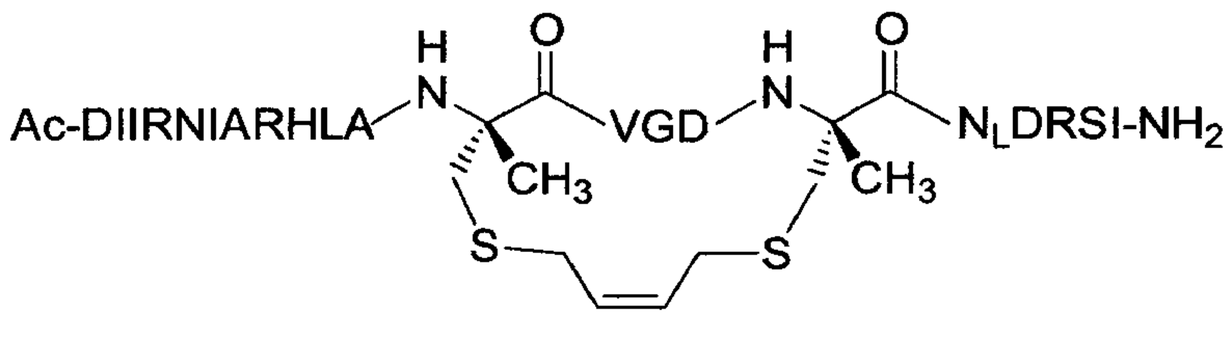
MW = 2463



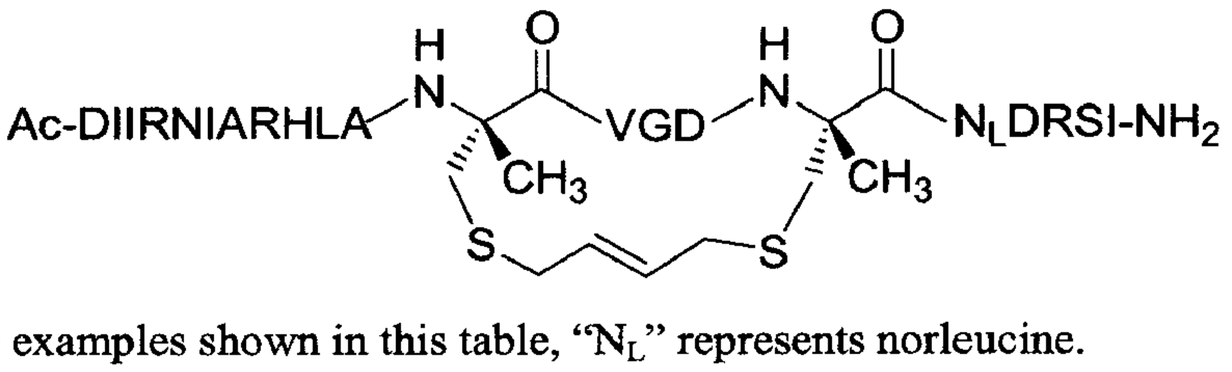
MW = 2525



MW = 2531



MW = 2475



MW = 2475

For the examples shown in this table, "N_L" represents norleucine.

[00163] 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 (III). 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.

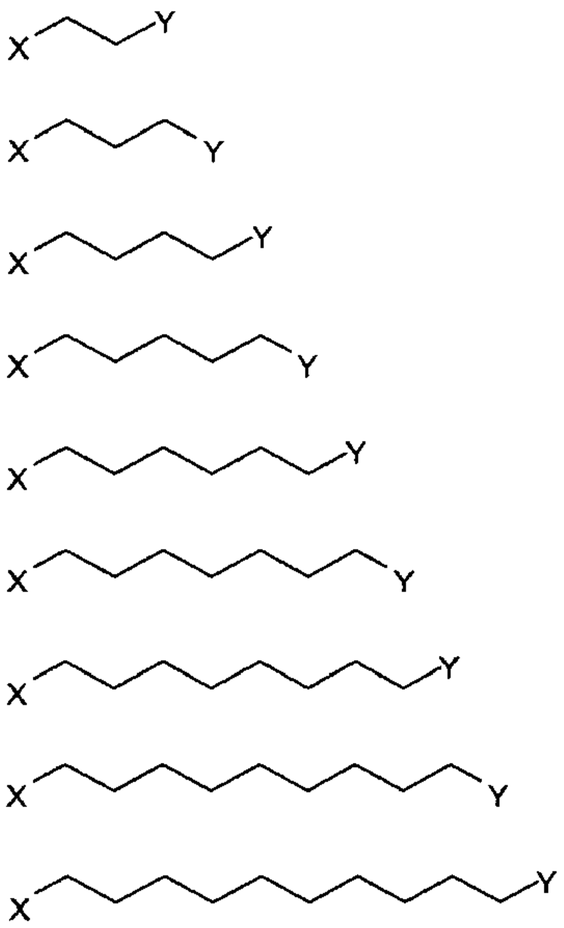
[00164] The invention includes macrocycles in which macrocycle-forming linkers are used to link two or more -SH moieties in the peptidomimetic precursors to form the peptidomimetic macrocycles of the invention. As described above, the macrocycle-forming linkers impart conformational rigidity, increased metabolic stability and/or increased cell penetrability. Furthermore, in some embodiments, the macrocycle-forming linkages stabilize the α-helical secondary structure of the peptidomimetic macrocyles. The macrocycle-forming linkers are of the formula X-L_2-Y, wherein both X and Y are the same or different moieties, as defined above. Both X and Y have the chemical characteristics that allow one macrocycle-forming linker –L_2- to bis alkylate the bis-sulfhydryl containing peptidomimetic precursor. As defined above, the linker –L_2- includes alkylene, alkenylene, alkynylene, heteroalkylene, cycloalkylene, heterocycloalkylene, cycloarylene, or heterocycloarylene, or –R_4–K–R_4–, all of which can be optionally substituted with an R_5 group, as defined above. Furthermore, one to three carbon atoms within the macrocycle-forming linkers –L_2-, other than the carbons attached to the –SH of the sulfhydryl containing amino acid, are optionally substituted with a heteroatom such as N, S or O.

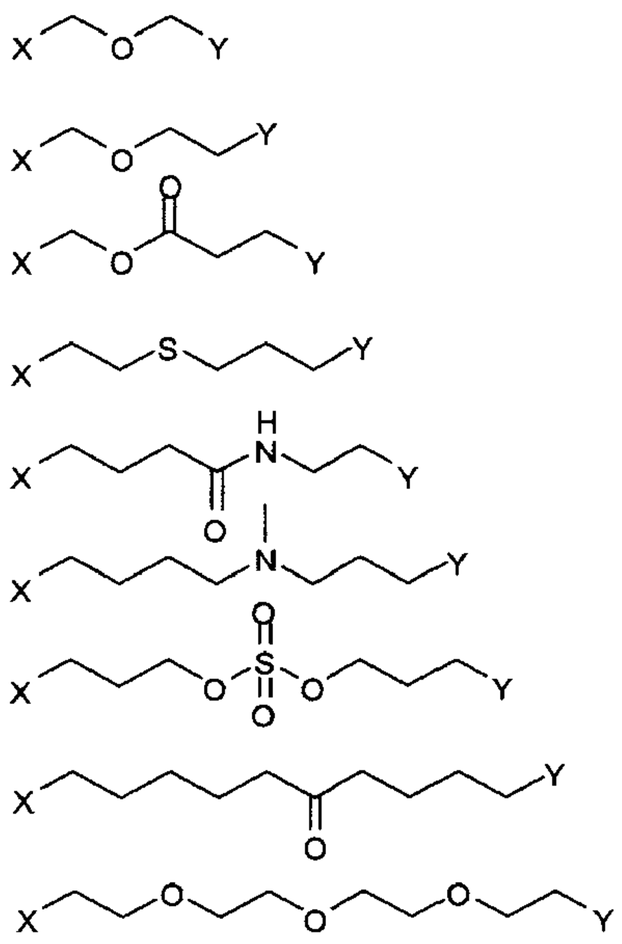
[00165] The L_2 component of the macrocycle-forming linker X-L_2-Y may be varied in length depending on, among other things, the distance between the positions of the two amino acid analogs used to form the peptidomimetic macrocycle. Furthermore, as the lengths of L_1 and/or L_3 components of the macrocycle-forming 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.

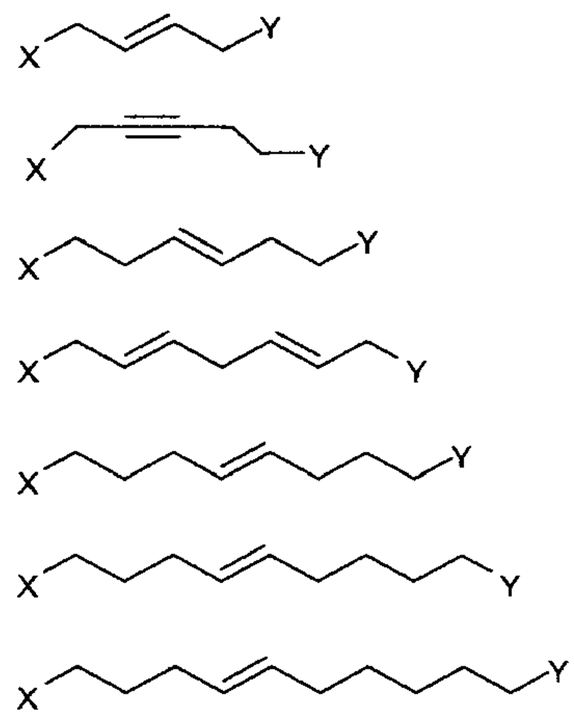
[00166] In some embodiments, L_2 is an alkylene group of the formula –$(CH_2)_n$–, where n is an integer between about 1 and about 15. For example, n is 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10. In other embodiments, L_2 is an alkenylene group. In still other embodiments, L_2 is an aryl group.

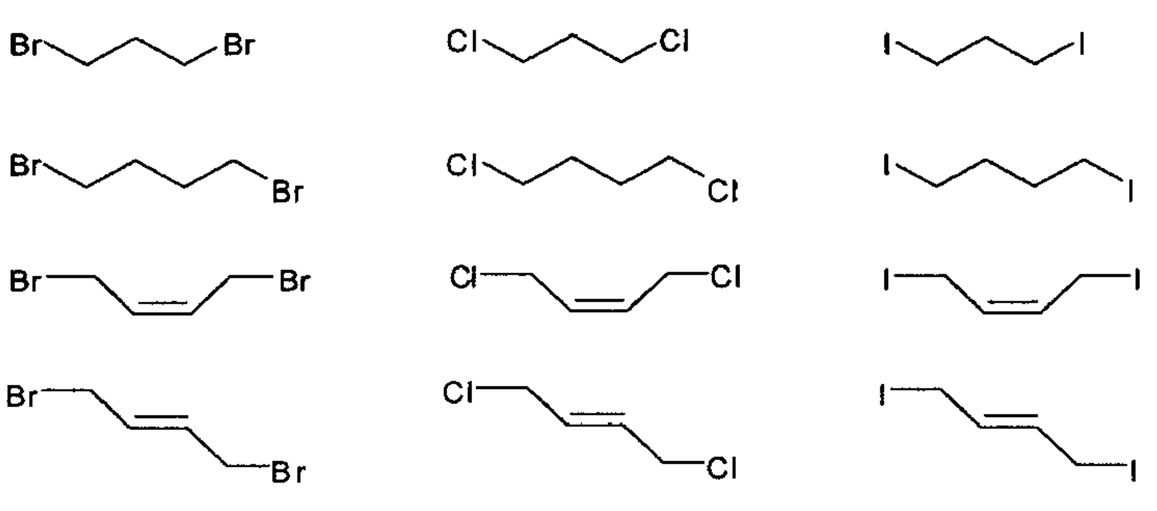
[00167] Table 7 shows additional embodiments of $X–L_2–Y$ groups.

TABLE 7. Exemplary $X–L_2–Y$ groups of the invention.



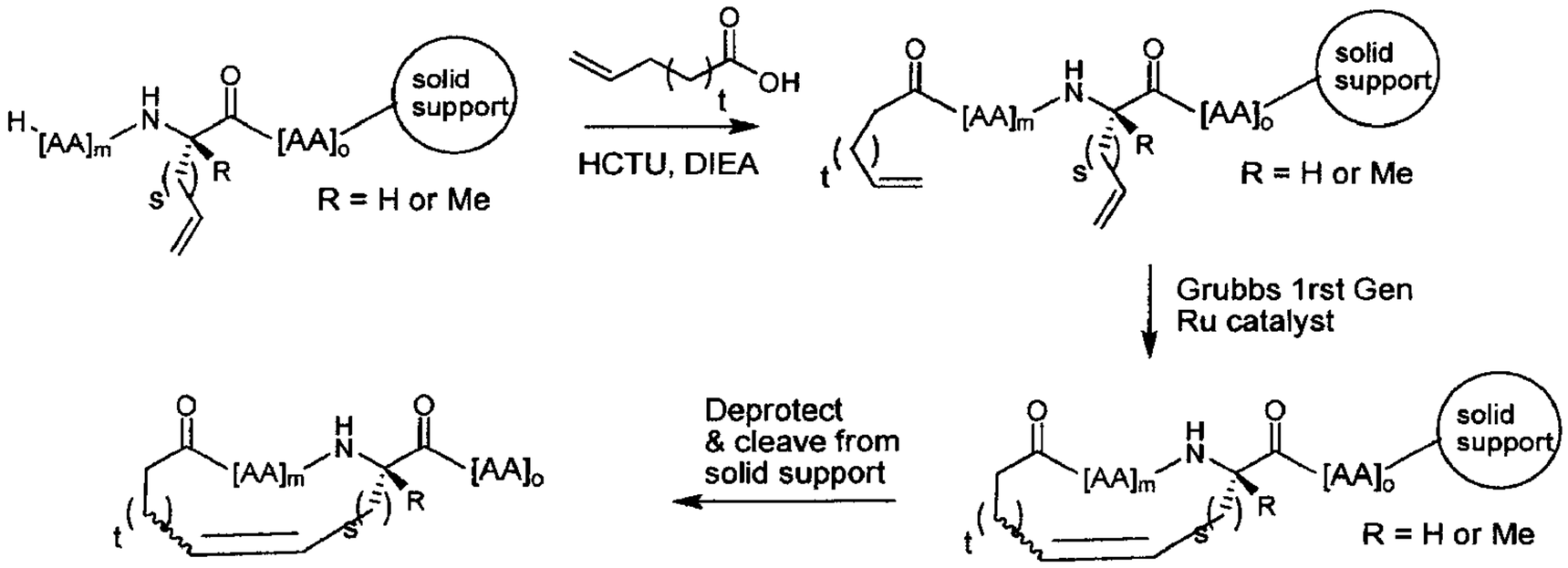






Each X and Y in this table, is, for example, independently Cl–, Br– or I–.

Scheme 10



[00168] In the general method for the synthesis of peptidomimetic macrocycles shown in Synthetic Scheme 10, the peptidomimetic precursor contains at least one olefin moiety and an alkyne moiety and is synthesized by solution-phase or solid-phase peptide synthesis (SPPS) using commericially available N-a-Fmoc-protected amino acids and the N-α-Fmoc-protected forms of the amino acid (S)-2-amino-2-methyl-6-heptenoic acid. The free N-terminus of the peptide is acylated with 6-heptenoic acid standard amide bond forming conditions. The terminal olefin of the acylating group is crosslinked to the terminal olefin of the internal amino acid sidechain using ruthenium catalyzed olefin metathesis as described in U.S. Patent 5,811,515. The peptidomimetic precursor is then deprotected and cleaved from the solid-phase resin by standard conditions (*e.g.*, strong acid such as 95% TFA).

[00169] Additional methods of forming peptidomimetic macrocycles which are envisioned as suitable to perform the present invention include those disclosed by Mustapa, M. Firouz Mohd et al., J. Org. Chem (2003), 68, pp. 8193-8198; Yang, 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 aminoacids are incorporated into the macrocycle precursor at the desired positions, which may 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.

Assays

[00170] The properties of the peptidomimetic macrocycles of the invention are assayed, for example, by using the methods described below.

Assay to Determine α-helicity.

[00171] 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, unmodified pro-apoptotic BH3 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 of the invention will possess an alpha-helicity of greater than 50%. To assay the helicity of peptidomimetic macrocyles of the invention, such as BH3 domain-based macrocycles, the compounds are dissolved in an aqueous solution (e.g. 50 mM potassium phosphate solution at pH 7, or distilled H_2O, to concentrations of 25-50 μM). Circular dichroism (CD) spectra are obtained on a spectropolarimeter (*e.g.*, Jasco J-710) 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. [Φ]222obs) by the reported value for a model helical decapeptide (Yang *et al.* (1986), *Methods Enzymol.* 130:208)).

Assay to Determine Melting Temperature (Tm).

[00172] A peptidomimetic macrocycle of the invention comprising a secondary structure such as an α-helix exhibits, for example, a higher melting temperature than a corresponding uncrosslinked polypeptide. Typically

peptidomimetic macrocycles of the invention exhibit Tm of > 60°C representing a highly stable structure in aqueous solutions. To assay the effect of macrocycle formation on meltine temperature, peptidomimetic macrocycles or unmodified peptides are dissolved in distilled H_2O (e.g. at a final concentration of 50 μM) and the Tm is determined by measuring the change in ellipticity over a temperature range (e.g. 4 to 95 °C) on a spectropolarimeter (*e.g.*, Jasco J-710) 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.

[00173] 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 may shield it from proteolytic cleavage. The peptidomimetic macrocycles of the present invention may 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=-1Xslope).

Ex Vivo Stability Assay.

[00174] 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 may be used. For example, a peptidomimetic macrocycle and/or a corresponding uncrosslinked polypeptide (2 mcg) are each incubated with fresh mouse, rat and/or human serum (e.g. 1-2 mL) at 37°C for 0, 1, 2, 4, 8, and 24 hours. Samples of differing macrocycle concentration may be prepared by serial dilution with serum. To determine the level of intact compound, the following procedure may be used: The samples are extracted 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 ± 2°C. The supernatants are then transferred to fresh 2 ml tubes and evaporated on Turbovap under N_2 < 10 psi, 37°C. The samples are reconstituted in 100μL of 50:50 acetonitrile:water and submitted to LC-MS/MS analysis. Equivalent or similar procedures for testing *ex vivo* stability are known and may be used to determine stability of macrocycles in serum.

In vitro Binding Assays.

[00175] To assess the binding and affinity of peptidomimetic macrocycles and peptidomimetic precursors to acceptor proteins, a fluorescence polarization assay (FPA) isused, 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).

[00176] 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 ismeasured, for example, by fluorescence polarization on a luminescence spectrophotometer (e.g. Perkin-Elmer LS50B). Kd values may be determined by nonlinear regression analysis using, for example, Graphpad Prism software (GraphPad Software, Inc., San Diego, CA). A peptidomimetic macrocycle of the invention shows, in some instances, similar or lower Kd than a corresponding uncrosslinked polypeptide.

[00177] Acceptor proteins for BH3-peptides such as BCL-2, BCL-X_L, BAX or MCL1 may, for example, be used in this assay. Acceptor proteins for p53 peptides such as MDM2 or MDMX may also be used in this assay.

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

[00178] To assess the binding and affinity of compounds that antagonize the interaction between a peptide (*e.g.* a BH3 peptide or a p53 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.

[00179] 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 ismeasured, for example, by fluorescence polarization on a luminescence spectrophotometer (e.g. Perkin-Elmer LS50B). Kd values may be determined by nonlinear regression analysis using, for example, Graphpad Prism software (GraphPad Software, Inc., San Diego, CA).

[00180] Any class of molecule, such as small organic molecules, peptides, oligonucleotides or proteins can be examined as putative antagonists in this assay. Acceptor proteins for BH3-peptides such as BCL2, BCL-XL, BAX or MCL1 can be used in this assay. Acceptor proteins for p53 peptides such as MDM2 or MDMX can be used in this assay.

Binding Assays in Intact Cells.

[00181] 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°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°C followed by further 2 hrs incubation at 4°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, including BCL2, MCL1, BCL-XL, A1, BAX, BAK, MDM2 or MDMX.

Cellular Penetrability Assays.

[00182] A peptidomimetic macrocycle is, for example, more cell permeable compared to a corresponding uncrosslinked polypeptide. In some embodiments, the peptidomimetic macrocycles are more cell permeable than a corresponding uncrosslinked polypeptides. Peptidomimetic macrocycles with optimized linkers possess, for example, cell penetrability that is at least two-fold greater than a corresponding uncrosslinked polypeptide, 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 polypeptides, intact cells are incubated with fluoresceinated peptidomimetic macrocycles or corresponding uncrosslinked polypeptides (10 µM) 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.

[00183] 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 µM) to identify those that kill at EC50<10 µM. 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.

[00184] 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 µL of fresh serum are then measured by LC-MS/MS as above.

In vivo Efficacy in Animal Models.

[00185] To determine the anti-oncogenic activity of peptidomimetic macrocycles of the invention *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 x

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 chemotherapeutics 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.

[00186] To determine the suitability of the peptidomimetic macrocycles of the invention for treatment of humans, clinical trials are performed. For example, patients diagnosed with cancer and in need of treatment are selected and separated in treatment and one or more control groups, wherein the treatment group is administered a peptidomimetic macrocycle of the invention, while the control groups receive a placebo or a known anti-cancer drug. The treatment safety and efficacy of the peptidomimetic macrocycles of the invention 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 macrocyle show improved long-term survival compared to a patient control group treated with a placebo.

Pharmaceutical Compositions and Routes of Administration

[00187] Methods of administration include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, oral, sublingual, intracerebral, intravaginal, transdermal, rectal, by inhalation, or topical by application to ears, nose, eyes, or skin.

[00188] The peptidomimetic macrocycles of the invention also include pharmaceutically acceptable derivatives or prodrugs thereof. A "pharmaceutically acceptable derivative" means any pharmaceutically acceptable salt, ester, salt of an ester, pro-drug or other derivative of a compound of this invention which, upon administration to a recipient, is capable of providing (directly or indirectly) a compound of this invention. Particularly favored pharmaceutically acceptable derivatives are those that increase the bioavailability of the compounds of the invention when administered to a mammal (*e.g.*, by increasing absorption into the blood of an orally administered compound) or which increases delivery of the active compound to a biological compartment (*e.g.*, the brain or lymphatic system) relative to the parent species. Some pharmaceutically acceptable derivatives include a chemical group which increases aqueous solubility or active transport across the gastrointestinal mucosa.

[00189] In some embodiments, the peptidomimetic macrocycles of the invention are modified by covalently or non-covalently joining appropriate functional groups to enhance selective biological properties. Such modifications

include those which increase biological penetration into a given biological compartment (*e.g.*, blood, lymphatic system, central nervous system), increase oral availability, increase solubility to allow administration by injection, alter metabolism, and alter rate of excretion.

[00190] Pharmaceutically acceptable salts of the compounds of this invention include those derived from pharmaceutically acceptable inorganic and organic acids and bases. Examples of suitable acid salts include acetate, adipate, benzoate, benzenesulfonate, butyrate, citrate, digluconate, dodecylsulfate, formate, fumarate, glycolate, hemisulfate, heptanoate, hexanoate, hydrochloride, hydrobromide, hydroiodide, lactate, maleate, malonate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, nitrate, palmoate, phosphate, picrate, pivalate, propionate, salicylate, succinate, sulfate, tartrate, tosylate and undecanoate. Salts derived from appropriate bases include alkali metal (*e.g.*, sodium), alkaline earth metal (*e.g.*, magnesium), ammonium and N-$(alkyl)_4^+$ salts.

[00191] For preparing pharmaceutical compositions from the compounds of the present invention, pharmaceutically acceptable carriers include either solid or liquid carriers. Solid form preparations include powders, tablets, pills, capsules, cachets, suppositories, and dispersible granules. A solid carrier can be one or more substances, which also acts as diluents, flavoring agents, binders, preservatives, tablet disintegrating agents, or an encapsulating material. Details on techniques for formulation and administration are well described in the scientific and patent literature, see, *e.g.*, the latest edition of Remington's Pharmaceutical Sciences, Maack Publishing Co, Easton PA.

[00192] 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.

[00193] 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.

[00194] Liquid form preparations include solutions, suspensions, and emulsions, for example, water or water/propylene glycol solutions. For parenteral injection, liquid preparations can be formulated in solution in aqueous polyethylene glycol solution. The term "parenteral" as used herein refers modes of administration including intravenous, intraarterial, intramuscular, intraperitoneal, intrasternal, and subcutaneous.

[00195] The pharmaceutical preparation is preferably in unit dosage form. In such form the preparation is subdivided into unit doses containing appropriate quantities of the active component. The unit dosage form can be a packaged preparation, the package containing discrete quantities of preparation, such as packeted tablets, capsules, and powders in vials or ampoules. Also, the unit dosage form can be a capsule, tablet, cachet, or lozenge itself, or it can be the appropriate number of any of these in packaged form.

[00196] When the compositions of this invention comprise a combination of a peptidomimetic macrocycle and one or more additional therapeutic or prophylactic agents, both the compound and the additional agent should be present at dosage levels of between about 1 to 100%, and more preferably between about 5 to 95% of the dosage normally administered in a monotherapy regimen. In some embodiments, the additional agents are administered separately, as part of a multiple dose regimen, from the compounds of this invention. Alternatively, those agents are part of a single dosage form, mixed together with the compounds of this invention in a single composition.

Methods of Use

[00197] In one aspect, the present invention provides 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 MDM2 system, labeled stabilized peptidomimetic macrocyles based on the p53 is used in an MDM2 binding assay along with small molecules that competitively bind to MDM2. Competitive binding studies allow for rapid *in vitro* evaluation and determination of drug candidates specific for the p53/MDM2 system. Likewise in the BH3/BCL-X_L anti-apoptotic system labeled peptidomimetic macrocycles based on BH3 can be used in a BCL-X_L binding assay along with small molecules that competitively bind to BCL-X_L. Competitive binding studies allow for rapid *in vitro* evaluation and determination of drug candidates specific for the BH3/BCL-X_L system. The invention further provides for the generation of antibodies against the peptidomimetic macrocycles. In some embodiments, these antibodies specifically bind both the peptidomimetic macrocycle and the p53 or BH3 peptidomimetic precursors upon which the peptidomimetic macrocycles are derived. Such antibodies, for example, disrupt the p53/MDM2 or BH3/BCL-XL systems, respectively.

[00198] In other aspects, the present invention provides for 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) BCL-2 family member expression or activity (*e.g.*, extrinsic or intrinsic apoptotic pathway abnormalities). It is believed that some BCL-2 type disorders are caused, at least in part, by an abnormal level of one or more BCL-2 family members (*e.g.*, over or under expression), or by the presence of one or more BCL-2 family members exhibiting abnormal activity. As such, the reduction in the level and/or activity of the BCL-2 family member or the enhancement of the level and/or activity of the BCL-2 family member, is used, for example, to ameliorate or reduce the adverse symptoms of the disorder.

[00199] In another aspect, the present invention provides methods for treating or preventing hyperproliferative disease by interfering with the interaction or binding between p53 and MDM2 in tumor cells. These methods comprise administering an effective amount of a compound of the invention to a warm blooded animal, including a human, or to tumor cells containing wild type p53. In some embodiments, the administration of the compounds of the present invention induce cell growth arrest or apoptosis. In other or further embodiments, the present invention is used to treat disease and/or tumor cells comprising elevated MDM2 levels. Elevated levels of MDM2 as used herein refers to MDM2 levels greater than those found in cells containing more than the normal copy number (2) of mdm2 or above about 10,000 molecules of MDM2 per cell as measured by ELISA and similar assays (Picksley *et al.* (1994), *Oncogene* 9, 2523 2529).

[00200] 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.

[00201] In some embodiments, the peptidomimetics macrocycles of the invention is used to treat, prevent, and/or diagnose cancers and neoplastic conditions. As used herein, the terms "cancer", "hyperproliferative" and "neoplastic" refer to cells having the capacity for autonomous growth, *i.e.*, an abnormal state or condition characterized by rapidly proliferating cell growth. Hyperproliferative and neoplastic disease states may be categorized as pathologic, *i.e.*, characterizing or constituting a disease state, or may be categorized as non-

pathologic, *i.e.*, a deviation from normal but not associated with a disease state. The term is meant to include all types of cancerous growths or oncogenic processes, metastatic tissues or malignantly transformed cells, tissues, or organs, irrespective of histopathologic type or stage of invasiveness. A metastatic tumor can arise from a multitude of primary tumor types, including but not limited to those of breast, lung, liver, colon and ovarian origin. "Pathologic hyperproliferative" cells occur in disease states characterized by malignant tumor growth. Examples of non-pathologic hyperproliferative cells include proliferation of cells associated with wound repair. Examples of cellular proliferative and/or differentiative disorders include cancer, *e.g.*, carcinoma, sarcoma, or metastatic disorders. In some embodiments, the peptidomimetics macrocycles are novel therapeutic agents for controlling breast cancer, ovarian cancer, colon cancer, lung cancer, metastasis of such cancers and the like.

[00202] 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.

[00203] Examples of proliferative disorders include hematopoietic neoplastic disorders. As used herein, the term "hematopoietic neoplastic disorders" includes diseases involving hyperplastic/neoplastic cells of hematopoietic origin, *e.g.*, arising from myeloid, lymphoid or erythroid lineages, or precursor cells thereof. Preferably, the diseases arise from poorly differentiated acute leukemias, *e.g.*, erythroblastic leukemia and acute megakaryoblastic leukemia. Additional exemplary myeloid disorders include, but are not limited to, acute promyeloid leukemia (APML), acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML) (reviewed in Vaickus (1991), *Crit Rev. Oncol./Hemotol.* 11:267-97); lymphoid malignancies include, but are not limited to acute lymphoblastic leukemia (ALL) which includes B-lineage ALL and T-lineage ALL, chronic lymphocytic leukemia (CLL), prolymphocytic leukemia (PLL), hairy cell leukemia (HLL) and Waldenstrom's macroglobulinemia (WM). Additional forms of malignant lymphomas include, but are not limited to non-Hodgkin lymphoma and variants thereof, peripheral T cell lymphomas, adult T cell leukemia/lymphoma (ATL), cutaneous T-cell lymphoma (CTCL), large granular lymphocytic leukemia (LGF), Hodgkin's disease and Reed-Stemberg disease.

[00204] 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.

[00205] 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.

[00206] 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.

[00207] 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.

[00208] Examples of cellular proliferative and/or differentiative disorders of the ovary include, but are not limited to, ovarian tumors such as, tumors of coelomic epithelium, serous tumors, mucinous tumors, endometrioid tumors, clear cell adenocarcinoma, cystadenofibroma, Brenner tumor, surface epithelial tumors; germ cell tumors such as mature (benign) teratomas, monodermal teratomas, immature malignant teratomas, dysgerminoma, endodermal sinus tumor, choriocarcinoma; sex cord-stomal tumors such as, granulosa-theca cell tumors, thecomafibromas, androblastomas, hill cell tumors, and gonadoblastoma; and metastatic tumors such as Krukenberg tumors.

[00209] In other or further embodiments, the peptidomimetics macrocycles described herein are used to treat, prevent or diagnose conditions characterized by overactive cell death or cellular death due to physiologic insult, etc. Some examples of conditions characterized by premature or unwanted cell death are or alternatively unwanted or excessive cellular proliferation include, but are not limited to hypocellular/hypoplastic, acellular/aplastic, or hypercellular/hyperplastic conditions. Some examples include hematologic disorders including but not limited to fanconi anemia, aplastic anemia, thalaessemia, congenital neutropenia, myelodysplasia

[00210] In other or further embodiments, the peptidomimetics macrocycles of the invention that act to decrease apoptosis are used to treat disorders associated with an undesirable level of cell death. Thus, in some embodiments, the anti-apoptotic peptidomimetics macrocycles of the invention are used to treat disorders such as those that lead to cell death associated with viral infection, *e.g.*, infection associated with infection with human immunodeficiency virus (HIV). A wide variety of neurological diseases are characterized by the gradual loss of specific sets of neurons, and the anti-apoptotic peptidomimetics macrocycles of the invention are used, in some embodiments, in the treatment of these disorders. Such disorders include Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS) retinitis pigmentosa, spinal muscular atrophy, and various forms of cerebellar degeneration. The cell loss in these diseases does not induce an inflammatory response, and apoptosis appears to be the mechanism of cell death. In addition, a number of hematologic diseases are associated with a decreased production of blood cells. These disorders include anemia associated with chronic disease, aplastic anemia, chronic neutropenia, and the myelodysplastic syndromes. Disorders of blood cell production, such as myelodysplastic syndrome and some forms of aplastic anemia, are associated with increased apoptotic cell death within the bone marrow. These disorders could result from the activation of genes that promote apoptosis, acquired deficiencies in stromal cells or hematopoietic survival factors, or the direct effects of toxins and

mediators of immune responses. Two common disorders associated with cell death are myocardial infarctions and stroke. In both disorders, cells within the central area of ischemia, which is produced in the event of acute loss of blood flow, appear to die rapidly as a result of necrosis. However, outside the central ischemic zone, cells die over a more protracted time period and morphologically appear to die by apoptosis. In other or further embodiments, the anti-apoptotic peptidomimetics macrocycles of the invention are used to treat all such disorders associated with undesirable cell death.

[00211] Some examples of immunologic disorders that are treated with the peptidomimetics macrocycles described herein include but are not limited to organ transplant rejection, arthritis, lupus, IBD, Crohn's disease, asthma, multiple sclerosis, diabetes, etc.

[00212] Some examples of neurologic disorders that are treated with the peptidomimetics macrocycles described herein include but are not limited to Alzheimer's Disease, Down's Syndrome, Dutch Type Hereditary Cerebral Hemorrhage Amyloidosis, Reactive Amyloidosis, Familial Amyloid Nephropathy with Urticaria and Deafness, Muckle-Wells Syndrome, Idiopathic Myeloma; Macroglobulinemia-Associated Myeloma, Familial Amyloid Polyneuropathy, Familial Amyloid Cardiomyopathy, Isolated Cardiac Amyloid, Systemic Senile Amyloidosis, Adult Onset Diabetes, Insulinoma, Isolated Atrial Amyloid, Medullary Carcinoma of the Thyroid, Familial Amyloidosis, Hereditary Cerebral Hemorrhage With Amyloidosis, Familial Amyloidotic Polyneuropathy, Scrapie, Creutzfeldt-Jacob Disease, Gerstmann Straussler-Scheinker Syndrome, Bovine Spongiform Encephalitis, a prion-mediated disease, and Huntington's Disease.

[00213] Some examples of endocrinologic disorders that are treated with the peptidomimetics macrocycles described herein include but are not limited to diabetes, hypothyroidism, hypopituitarism, hypoparathyroidism, hypogonadism, etc.

[00214] Examples of cardiovascular disorders (*e.g.*, inflammatory disorders) that are treated or prevented with the peptidomimetics macrocycles of the invention include, but are not limited to, atherosclerosis, myocardial infarction, stroke, thrombosis, aneurism, heart failure, ischemic heart disease, angina pectoris, sudden cardiac death, hypertensive heart disease; non-coronary vessel disease, such as arteriolosclerosis, small vessel disease, nephropathy, hypertriglyceridemia, hypercholesterolemia, hyperlipidemia, xanthomatosis, asthma, hypertension, emphysema and chronic pulmonary disease; or a cardiovascular condition associated with interventional procedures ("procedural vascular trauma"), such as restenosis following angioplasty, placement of a shunt, stent, synthetic or natural excision grafts, indwelling catheter, valve or other implantable devices. Preferred cardiovascular disorders include atherosclerosis, myocardial infarction, aneurism, and stroke.

EXAMPLES

[00215] The following section provides illustrative examples of the present invention.

[00216] Example 1. Synthesis of Peptidomimetic Macrocycles of the Invention.

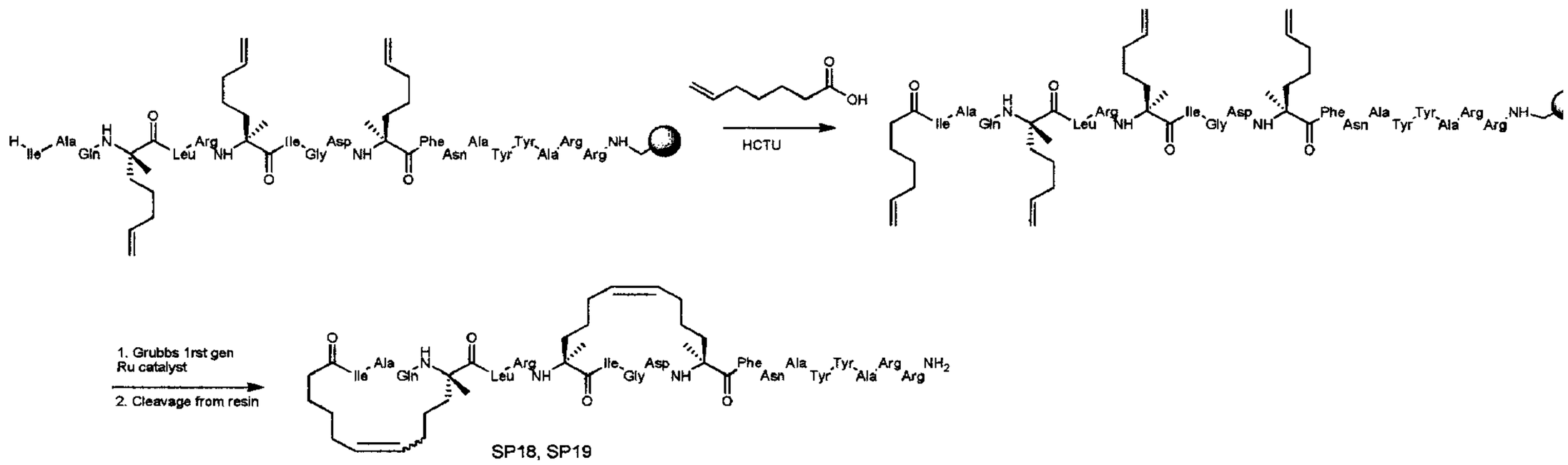
α-helical BID, BIM and p53 peptidomimetic macrocycles were synthesized, purified and analyzed as previously described (Walensky et al (2004) Science 305:1466-70; Walensky et al (2006) Mol Cell 24:199-210; Bernal et al (2007) J. Am Chem Soc. 9129, 2456-2457) and as indicated below. The macrocycles used in this study are shown in Figure 1. The corresponding uncrosslinked polypeptides represent the natural counterparts of the peptidomimetic macrocycles of the invention.

[00217] Alpha,alpha-disubstituted non-natural amino acids containing olefinic side chains were synthesized according to Williams et al. (1991) J. Am. Chem. Soc. 113:9276; Schafmeister et al. (2000) J. Am. Chem Soc. 122:5891 and Verdine et al PCT WO 2008/121767. Peptidomimetic macrocycles were designed by replacing two or more naturally occurring amino acids (see Figure 1) with the corresponding synthetic amino acids. Substitutions were made at the i and i+4 or i and i+7 positions. Macrocycles were generated by solid phase peptide synthesis followed by olefin metathesis-based crosslinking of the synthetic amino acids via their olefin-containing side chains.

[00218] In the sequences shown, the following abbreviations are used: "Nle" represents norleucine, "Aib" represents 2-aminoisobutyric acid, "Ac" represents acetyl, and "Pr" represents propionyl. Amino acids represented as "$" are alpha-Me S5-pentenyl-alanine olefin amino acids connected by an all-carbon i to i+4 crosslinker comprising one double bond. Amino acids represented as "$r5" are alpha-Me R5-pentenyl-alanine olefin amino acids connected by an all-carbon i to i+4 crosslinker comprising one double bond. Amino acids represented as "$s8" are alpha-Me S8-octenyl-alanine olefin amino acids connected by an all-carbon i to i+7 crosslinker comprising one double bond. Amino acids represented as "$r8" are alpha-Me R8-octenyl-alanine olefin amino acids connected by an all-carbon i to i+7 crosslinker comprising one double bond. Amino acids represented as "St" connect two all-carbon crosslinkers (S-5/R-5 bis-pentenyl amino acids). Amino acids represented as "Hep" are olefin-crosslinked N-terminal heptenoic acids. The crosslinkers are linear all-carbon crosslinker comprising eight or eleven carbon atoms between the alpha carbons of each amino acid.

[00219] The non-natural amino acids (R and S enantiomers of the 5-carbon olefinic amino acid and the S enantiomer of the 8-carbon olefinic amino acid) were characterized by nuclear magnetic resonance (NMR) spectroscopy (Varian Mercury 400) and mass spectrometry (Micromass LCT). Peptide synthesis was 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 were employed. Non-natural amino acids (4 equiv) were coupled with a 1:1:2 molar ratio of HATU (Applied Biosystems)/HOBt/DIEA. Olefin metathesis was performed in the solid phase using 10 mM Grubbs catalyst (Blackewell et al. 1994 supra) (Strem Chemicals) dissolved in degassed dichloromethane and reacted for 2 hours at room temperature. Isolation of metathesized compounds was achieved by trifluoroacetic acid-mediated deprotection and cleavage, ether precipitation to yield the crude product, and 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).

Example 2. Synthesis of N-terminal cross-linked SP-18 & SP19 macrocycles



SP18, SP19

The peptides were elongated on a Thuramed Tetras automated multichannel peptide synthesizer starting with a 4-(2'4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucylaminomethyl linked polystyrene resin (Rink AM resin). The amino acids (5 eq) were coupled using standard solid phase protocols based on fluorenylmethoxycarbonyl (Fmoc) protection and 2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) as the coupling agent (5 eq). Double coupling was used during the automated process for all of the amino acids except for the α-methylated Fmoc-protected olefinic amino acids which were single coupled with longer reaction times. After the final amino acid was added to the peptide, the Fmoc group was removed and the free amine was acylated using acetic anhydride in 10% DIEA. The linear peptide was assembled as above on resin (0.5 mmol based on initial resin loading) incorporating the desired Fmoc-protected olefinic amino acid. After the coupling of the last amino acid, the N-terminus was acylated with 6-heptenoic acid (5 eq) using the method outlined above. The resin was washed with DCM. The resin was dried under reduced pressure and taken up in an anhydrous DCM solution of Grubbs I catalyst (20 mL, 4 mg/mL, 0.02 mmol). After 18 h, the reaction was filtered and the resin was washed with DCM. The olefin metathesis step was repeated until the starting material was fully consumed. The cyclized peptide was simultaneously cleaved from the resin and the protecting groups on the sidechains removed by treating the resin with a solution (15 mL) of trifluoroacetic acid (TFA) (93.5%), water (2.5%), triisopropylsilane (TIPS), (2.5%), and ethanedithiol (EDT) (2.5%). Chilled diethylether (200 mL) was added after 4 h. The mixture was centrifuged and the supernatant decanted. The pellet was suspended in 1:1 acetonitrile/water (50 mL) and lyophilized. The crude peptide was purified using C_{18} reversed-phase HPLC with acetonitrile and water (with 0.1% TFA) as the mobile phase. The fractions containing the desired peptide were pooled. The fractions were lyophilized twice in 50:50 acetonitrile : HCl (aq) (60 mN, then 10 mN) and once in 50:50 acetonitrile: water to give SP18 or SP19 as a colorless solid (SP18: 16 mg, SP19: 32 mg).

Example 3. Sample and Standard Curve Preparation:

[00220] For *in vivo* plasma stability studies 50 μL of 10mM of each macrocycle in DMSO was combined with 9950 μL rat plasma (1:200 v/v) and mixed by vortexing (4 minutes). This stock was serially diluted in rat plasma to yield 9 standards (20 – 20,000, or 100 – 50,000 ng/mL range). High concentration (early time point) test samples were diluted 10:1 or 5:1 in blank plasma. All samples, including plasma blank, were combined 1:1 v/v with internal standard peptide in plasma.

Example 4. Pharmacokinetic Analysis

[00221] The IV dose formulation is prepared by dissolving peptidomimetic macrocycles of the invention in 5 % DMSO/ D5W to achieve a 10 mg/Kg/dose. Canulated Crl:CD®(SD) male rats (7-8 weeks old, Charles River Laboratories) are used in these studies. Intravenous doses are administered via the femoral cannula and the animals are dosed at 10 mL/kg per single injection. Blood for pharmacokinetic analysis is collected at 10 time points (0.0833, 0.25, 0.5,1, 2, 4, 6, 8, 12 and 24 hrs post-dose). Animals are terminated (without necropsy) following their final sample collection.

[00222] The whole blood samples are centrifuged (~1500 x g) for 10 min at ~4 °C. Plasma is prepared and transferred within 30 min of blood collection/centrifugation to fresh tubes that are frozen and stored in the dark at ~-70 °C until they are prepared for LC-MS/MS analysis.

[00223] Sample extraction is achieved, for example, by adding 10 µL of 50% formic acid to 100 µL plasma (samples or stds), following by vortexing for 10 seconds. 500 µL acetonitrile is added to the followed by vortexing for 2 minutes and centrifuged at 14,000rpm for 10 minutes at ~4°C. Supernatants are transferred to clean tubes and evaporated on turbovap <10 psi at 37°C. Prior to LC-MS/MS analysis samples are reconstituted with 100µL of 50:50 acetonitrile:water.

[00224] The peak plasma concentration (C_{max}), the time required to achieve the peak plasma concentration (t_{max}), the plasma terminal half-life ($t_{1/2}$), the area under the plasma concentration time curve (AUC), the clearance and volume of distribution are calculated from the plasma concentration data. All pharmacokinetic calculations are done using WinNonlin version 4.1 (Pharsight Corp) by non-compartmental analysis. Results of this analysis for peptidomimetic macrocycles of the invention are shown in Figure 2.

[00225] The following LC-MS/MS method is used. In brief, the LC-MS/MS instruments used was an API 365 (Applied Biosystems). The analytical column was a Phenomenex Synergi (4µ, Polar-RP, 50mm x 2 mm) and mobile phases A (0.1 % formic acid in water) and B (0.1 % formic acid in methanol) are pumped at a flow rate of 0.4 ml/min to achieve the following gradient:

Time (min)	% B
0	15
0.5	15
1.5	95
4.5	95
4.6	15
8.0	Stop

MRM: 814.0 to 374.2 (positive ionization)

Example 5. Determination Of Apparent Affinity To Serum Proteins (K_d*).

[00226] The measurement of apparent Kd values for serum protein by EC_{50} shift analysis provides a simple and rapid means of quantifying the propensity of experimental compounds to bind serum albumin and other serum proteins. A linear relationship exists between the apparent EC'_{50} in the presence of serum protein (EC_{50}) and the amount of serum protein added to an *in vitro* assay. This relationship is defined by the binding affinity of the compound for serum proteins, expressed as K_d*. This term is an experimentally determined, apparent

dissociation constant that may result from the cumulative effects of multiple, experimentally indistinguishable, binding events. The form of this relationship is presented here in Eq. (1), and its derivation can be found in Copeland et al, Biorg. Med Chem Lett. 2004, 14:2309-2312.

(1) $$EC'_{50} = EC_{50} + P\left(\frac{n}{1+\frac{K_d^*}{EC_{50}}}\right)$$

[00227] A significant proportion of serum protein binding can be ascribed to drug interactions with serum albumin, due to the very high concentration of this protein in serum (35–50 g/L or 530–758 μM). To calculate the Kd value for these compounds we have assumed that the shift in EC_{50} upon protein addition can be ascribed fully to the serum albumin present in the added serum, where P is 700 μM for 100% serum, P is 70 μM for 10% serum, etc. We further made the simplifying assumption that all of the compounds bind serum albumin with a 1:1 stoichiometry, so that the term n in Eq. (1) is fixed at unity. With these parameters in place we calculated the K_d* value for each cross-linked polypeptide from the changes in EC_{50} values with increasing serum (and serum protein) concentrations by nonlinear regression analysis of Eq. 1 using Mathematica 4.1 (Wolfram Research, Inc., www.wolfram.com). EC'_{50} values in whole blood are estimated by setting P in Eq. 1 to 700 μM [serum albumin].

[00228] The free fraction in blood is estimated per the following equation, as derived by Trainor, Expert Opin. Drug Disc., 2007, 2(1):51-64, where the total serum albumin concentration (for example, $[HSA]_{total}$) is set at 700 μM. The formula below may be used with any type of serum albumin, including rat serum albumin.

(2) $$FreeFraction = \frac{K_d^*}{K_d^* + [HSA]_{total}}$$

Example 6. Determination Of α-Helicity

Two vials (1.0 mg) of each sample were dissolved in 50% acetonitrile/50% water for a final concentration of 1.0 mg/ml. 100 μL or approximately 0.1 mg of each sample was aliquoted into each vial. Three 30 μL samples were taken for amino acid analysis. All samples were lyophilized overnight then stored at -20°C. Samples were diluted to several different concentrations (1.0 mg/ml, 0.5 mg/ml, 0.1 mg/ml, and 0.05 mg/ml) and put into varying path length cells (1.0 mm, 2.0 mm, 5.0 mm, and 10.0 mm). All samples were visually inspected for debris and scans were taken of each sample at 5°C to determine ideal path length and concentration for solubility. The samples were soluble at 0.05 mg/ml in 20mM phosphoric acid pH 2.0 buffer. All scans and temperature melts were performed in this buffer condition (benign buffer – 20mM phosphoric acid pH 2.0) in a 10.0 mm CD cell. All samples were run on a Jasco J-815 spectropolarimeter using the Spectra Manager software package. Samples were dissolved in 2.0 mL benign buffer (denoted 0%TFE) or buffer containing 5%, 10%, 15%, 20% or 50% Trifluoroethanol (TFE) from Sigma-Aldrich (catalog T63002). CD scans were run from 250-190 nm at 5°C. Data was collected every 0.2 nm. Appropriate buffer blanks were run before each CD scan and the buffer was subtracted from each run. Temperature melts were run from 5°C - 80°C with reverse melts from 80°C – 5°C immediately following. Data was collected every 0.2 degrees. Amino acid analysis was done using the AccQ Tag System (Waters, Milford, MA) on an Agilent 1100 HPLC. Briefly, the peptide aliquots were

hydrolyzed by adding 200uL of 6M HCl to each aliquot and heating the samples at 110 deg C for 24hr. The samples were then vacuum dried and the resulting residue was resuspended in 200uL of 200mM HCl. Using the reagents provided in the AccQ Tag Chemistry Kit, each free amino acid in 20uL of the hydrolysate was derivatized with a quinoline moiety. HPLC was used to separate the individual amino acids for each hydrolysate sample using a custom gradient and a custom column, and the abundance of each was measured by UV at 254nm. A sodium acetate buffer, pH 5.05 and a 60/40 (v/v) acetonitrile/water were the running buffers. By comparing the area of each peak to a set of standards with a known amount of each amino acid, the absolute amounts of each amino acid present in each hydrolysate sample were determined. Using the sequence of each peptide, the concentration of the peptide was determined using either the amount of alanine or leucine in the sample. All data was imported and saved in Excel files where percent helix, molar ellipticity, or concentrations by AAA were calculated. Percent helicity in aqueous solution was determined by dividing the molar ellipticity (222 nm) in 0% TFE for each crosslinked peptide by the molar ellipticity (222 nm) in 50% TFE for the parent peptide, with the assumption that the parent peptide is 100% helical in 50% TFE.

[00229] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

CLAIMS

WHAT IS CLAIMED IS:

1. A method of increasing the *in vivo* half-life of a helical polypeptide by installing one or more cross-links, wherein the *in vivo* half-life of said polypeptide is increased at least 50-fold relative to a corresponding polypeptide lacking said cross-links.
2. The method of claim 1, wherein the *in vivo* half-life of said helical polypeptide is increased on average at least 50-fold.
3. The method of claim 1, wherein the *in vivo* half-life of said helical polypeptide is increased at least 100-fold.
4. The method of claim 1, wherein the *in vivo* half-life of said helical polypeptide is increased at least 150-fold.
5. The method of claim 1, wherein the *in vivo* half-life of said helical polypeptide is increased at least 200-fold.
6. The method of claim 1, wherein at least one of the crosslinks connects two α-carbon atoms.
7. The method of claim 1, wherein one α-carbon atom to which one crosslink is attached is substituted with a substituent of formula R–.
8. The method of claim 7, wherein two α-carbon atoms to which one crosslink is attached are substituted with independent substituents of formula R–.
9. The method of claim 7, wherein R– is alkyl.
10. The method of claim 7, wherein R– is methyl.
11. The method of claim 1, wherein one cross-link is installed in said helical polypeptide.
12. The method of claim 1, wherein two cross-links are installed in said helical polypeptide.
13. The method of claim 1, wherein one crosslink is formed of consecutive carbon-carbon bonds.
14. The method of claim 1, wherein one crosslink contains at least 8 consecutive bonds.
15. The method of claim 1, wherein one crosslink contains 9 consecutive bonds.
16. The method of claim 1, wherein one crosslink contains 12 consecutive bonds.
17. The method of claim 1, wherein one crosslink comprises at least 7 carbon atoms.
18. The method of claim 1, wherein one crosslink comprises at least 10 carbon atoms.
19. The method of claim 1, wherein the crosslinked polypeptide comprises an α-helical domain of a BCL-2 family member.
20. The method of claim 1, wherein the crosslinked polypeptide comprises a BH3 domain.
21. The method of claim 1, wherein the crosslinked polypeptide comprises at least 60% of a sequence in Table 1, 2, 3 or 4.
22. The method of claim 1, wherein the crosslinked polypeptide comprises at least 80% of a sequence in Table 1, 2, 3 or 4.
23. The method of claim 1, wherein the crosslinked polypeptide penetrates cell membranes by an energy-dependent process and binds to an intracellular target.
24. The method of claim 1, wherein the cross-linked polypeptide is selected such that the apparent serum binding affinity (Kd*) of the crosslinked polypeptide is 1 micromolar or greater.
25. The method of claim 24, wherein the crosslinked polypeptide has a Kd* of 1 to 700 micromolar.
26. The method of claim 24, wherein the crosslinked polypeptide has a Kd* of 1 to 70 micromolar.
27. The method of claim 24, wherein the crosslinked polypeptide has a Kd* of 1 to 10 micromolar.
28. The method of claim 24, wherein the crosslinked polypeptide has a Kd* of 3, 10, 70 micromolar or greater.
29. The method of claim 1, wherein the crosslinked polypeptide is selected such that it possesses an estimated free fraction in human blood of 0.1 – 50%.
30. The method of claim 1, wherein the crosslinked polypeptide is selected such that it possesses an estimated free fraction in human blood of 0.15 – 10%.

31. A helical polypeptide comprising one or more cross-links, wherein the cross-linked helical polypeptide has an *in vivo* half-life at least 50-fold greater than that of a corresponding polypeptide lacking said cross-links.
32. The polypeptide of claim 31, wherein the cross-linked helical polypeptide has an *in vivo* half-life at least 100-fold greater than that of a corresponding polypeptide lacking said cross-links.
33. The polypeptide of claim 31, wherein the cross-linked helical polypeptide has an *in vivo* half-life at least 150-fold greater than that of a corresponding polypeptide lacking said cross-links.
34. The polypeptide of claim 31, wherein the cross-linked helical polypeptide has an *in vivo* half-life at least 200-fold greater than that of a corresponding polypeptide lacking said cross-links.
35. The polypeptide of claim 31, wherein at least one of the crosslinks connects two α-carbon atoms.
36. The polypeptide of claim 31, wherein one α-carbon atom to which one crosslink is attached is substituted with a substituent of formula R–.
37. The polypeptide of claim 36, wherein two α-carbon atoms to which one crosslink is attached are substituted with independent substituents of formula R–.
38. The polypeptide of claim 36, wherein R– is alkyl.
39. The polypeptide of claim 36, wherein R– is methyl.
40. The polypeptide of claim 31, wherein one cross-link is installed in said helical polypeptide.
41. The polypeptide of claim 31, wherein two cross-links are installed in said helical polypeptide.
42. The polypeptide of claim 31, wherein one crosslink is formed of consecutive carbon-carbon bonds.
43. The polypeptide of claim 31, wherein one crosslink contains at least 8 consecutive bonds.
44. The polypeptide of claim 31, wherein one crosslink contains 9 consecutive bonds.
45. The polypeptide of claim 31, wherein one crosslink contains 12 consecutive bonds.
46. The polypeptide of claim 31, wherein one crosslink comprises at least 7 carbon atoms.
47. The polypeptide of claim 31, wherein one crosslink comprises at least 10 carbon atoms.
48. The polypeptide of claim 31, wherein the crosslinked polypeptide comprises an α-helical domain of a BCL-2 family member.
49. The polypeptide of claim 31, wherein the crosslinked polypeptide comprises a BH3 domain.
50. The polypeptide of claim 31, wherein the crosslinked polypeptide comprises at least 60% of a sequence in Table 1, 2, 3 or 4.
51. The polypeptide of claim 31, wherein the crosslinked polypeptide comprises at least 80% of a sequence in Table 1, 2, 3 or 4.
52. The polypeptide of claim 31, wherein the polypeptide penetrates cell membranes by an energy-dependent process and binds to an intracellular target.
53. The polypeptide of claim 31, wherein the polypeptide is substituted with one or more of a halogen, alkyl group, a fluorescent moiety, affinity label, targeting moiety, or a radioisotope.
54. The polypeptide of claim 31, wherein the polypeptide provides a therapeutic effect.
55. The polypeptide of claim 31, wherein the polypeptide is in a pharmaceutically acceptable salt form.
56. The polypeptide of claim 31, wherein the apparent serum binding affinity (Kd*) of the crosslinked polypeptide is 1 micromolar or greater.
57. The polypeptide of claim 56, wherein the crosslinked polypeptide has a Kd* of 1 to 700 micromolar.
58. The polypeptide of claim 56, wherein the crosslinked polypeptide has a Kd* of 1 to 70 micromolar.
59. The polypeptide of claim 56, wherein the crosslinked polypeptide has a Kd* of 1 to 10 micromolar.
60. The polypeptide of claim 56, wherein the crosslinked polypeptide has a Kd* of 3, 10, 70 micromolar or greater.
61. The polypeptide of claim 31, wherein the crosslinked polypeptide possesses an estimated free fraction in human blood of 0.1 – 50%.

62. The polypeptide of claim 31, wherein the crosslinked polypeptide possesses an estimated free fraction in human blood of 0.15 – 10%.
63. A pharmaceutical composition comprising a polypeptide of any of the foregoing claims, in combination or association with a pharmaceutically acceptable diluent or carrier.

64. The method of claim 1, wherein the cross-linked polypeptide is selected such that the % helicity of the crosslinked polypeptide is 25% or greater at room temperature under aqueous conditions.
65. The method of claim 1, wherein the cross-linked polypeptide is selected such that the % helicity of the crosslinked polypeptide is 50% or greater at room temperature under aqueous conditions.
66. The method of claim 1, wherein the cross-linked polypeptide is selected such that the % helicity of the crosslinked polypeptide is 75% or greater at room temperature under aqueous conditions.
67. The method of claim 1, wherein the *in vivo* half-life of said polypeptide is determined after intravenous administration.
68. The polypeptide of claim 31, wherein the percent helicity at room temperature under aqueous conditions of the crosslinked polypeptide is at least 2-fold greater than that of a corresponding polypeptide lacking said cross-links.
69. The polypeptide of claim 31, wherein the percent helicity at room temperature under aqueous conditions of the crosslinked polypeptide is at least 5-fold greater than that of a corresponding polypeptide lacking said cross-links.
70. The polypeptide of claim 31, wherein the percent helicity at room temperature under aqueous conditions of the crosslinked polypeptide is at least 10-fold greater than that of a corresponding polypeptide lacking said cross-links.
71. The polypeptide of claim 31, wherein the *in vivo* half-life of said polypeptide is determined after intravenous administration.

Figure 1.

Stapled Peptides	Protein	Sequence	Calculated m/z (M+H)	Calculated m/z (M+3H)	Found m/z (M+3H)
SP1	BID	Ac-DIIRNIARHLAVGDNleDRSI-NH2	2438.40	813.47	813.7
SP11	BID	Ac-DIIRNIARHLAQVGDSNleDRSI-NH2	2403.32	801.78	801.87
SP7	BID	Ac-DIIRNIARHLA$VAibD$AARSI-NH2	2380.39	794.14	794.48
SP5	BID	Pr-RNIARHLA$VAibD$NleDRSI-NH2	2139.25	713.76	713.79
SP8	BID	Pr-RNIARHLA$VAibD$FARSI-NH2	2129.25	710.42	710.3
SP10	BID	Pr-RNIARHLAVGDNleAibRSI-NH2	2081.25	694.42	694.42
SP12	BID	HepIARLAVGD$NleDRSI-NH2	1869.13	935.07	935.35 (M+2H)
SP13	BID	Ac-DIIRNIA$r5HLAStVGD$NleDRSI-NH2	2447.41	816.48	816.7
SP14	BID	Ac-DIIRNIA$HLAStVGD$r5NleDRSI-NH2	2447.41	816.48	816.7
SP2	BIM	Ac-IWIAQELRIGDFNAYYARR-NH2	2646.43	882.82	883.15
SP9	BIM	Ac-IWIAQALRIGDFNAYYARR-NH2	2588.43	863.48	863.85
SP3	BIM	Ac-IWIAQQLRIGDFNAYYARR-NH2	2645.45	882.49	882.62
SP15	BIM	Ac-IWIAQALRRIGDEFNAYYARR-NH2	2623.40	875.14	874.94
SP16	BIM	Ac-IIAQLRIGDFNAYYARR-NH2	2581.48	861.16	861.64
SP18	BIM	HepIAQLRIGD$FNAYYARR-NH2	2397.36	799.79	800.38
SP19	BIM	HepIAQLRIGD$FNAYYARR-NH2	2397.36	799.79	800.38
SP20	BIM	Ac-IWIA$r5ALRStIGD$FNAYYARR-NH2	2625.48	875.83	876.39
SP21	BIM	Ac-IWIA$ALRStIGDEFN$s8YYARR-NH2	2725.53	909.18	909.79
SP22	p53	Ac-QSQQTFSNLWRLLPQN-NH2	2001.0304	1001.02	1001.18 (M+2H)
SP23	p53	Ac-QSQQTF$r8NLWRLL$QN-NH2	2109.1607	1055.08	1054.98 (M+2H)
SP17	p53	Ac-QS$QTFStNLWRLL$s8QN-NH2	2146.22	1073.61	1073.9 (M+2H)

WO 2010/060112 PCT/US2009/065824
1/27
CA 02743177 2011-05-10

Figure 2.

Stapled Peptides	T1/2 (hr)	C0 (ng/mL)	AUCall (hr*ng/mL)	Cl_obs (mL/hr/kg)	MRTinf_obs (hr)
SP1	*4.3*	*153985*	*268275*	*44.2*	*2.2*
*SP11	*1.3*	*1080*	*1136*	*2732*	*1.6*
SP7	*3.9*	*158078*	*402899*	*23.9*	*3.1*
SP5	*2.4*	*270900*	*127629*	*91.1*	*0.6*
SP8	*19.9*	*147963*	*73751*	*119.8*	*2.3*
SP10	*21.8*	*51651*	*35591*	*216.3*	*3.0*
*SP12	*5.9*	*6400*	*69035*	*30.2*	*9.2*
*SP13	*9.5*	*6100*	*82965*	*26.4*	*13.2*
*SP14	*6.7*	*7200*	*74344*	*43.6*	*8.7*
SP2	*5.7*	*70325*	*170685*	*81.8*	*3.1*
SP9	*7.3*	*158857*	*124448*	*49.8*	*4.5*
SP3	*11.1*	*186101*	*156183*	*66.0*	*4.3*
*SP15	*0.4*	*48050*	*8430*	*448.0*	*0.2*
*SP16	*4.5*	*22900*	*125889*	*24.0*	*4.4*
*SP18	*2.2*	*41400*	*99799*	*28.7*	*2.7*
*SP19	*3.2*	*14500*	*80708*	*31.7*	*4.0*
*SP20	*10.8*	*1950*	*15000*	*181.0*	*14.1*
*SP21	*5.7*	*1450*	*7000*	*418.6*	*6.6*
*SP22	*<0.1*	*15100*	*75*	*42000.0*	*<0.1*
*SP23	*1.8*	*36000*	*48000*	*106.0*	*1.8*
*SP17	*3.3*	*72800*	*102958*	*59.7*	*3.0*

2 mg/mL iv bolus in Sprague-Dawley rats (10 mg/kg)
*** 0.6 mg/mL iv bolus in Sprague-Dawley rats (3 mg/kg)**

Figure 3a

A) IV plasma half-life of SP1 in Sprague-Dawley rat; 2 mg/mL iv bolus

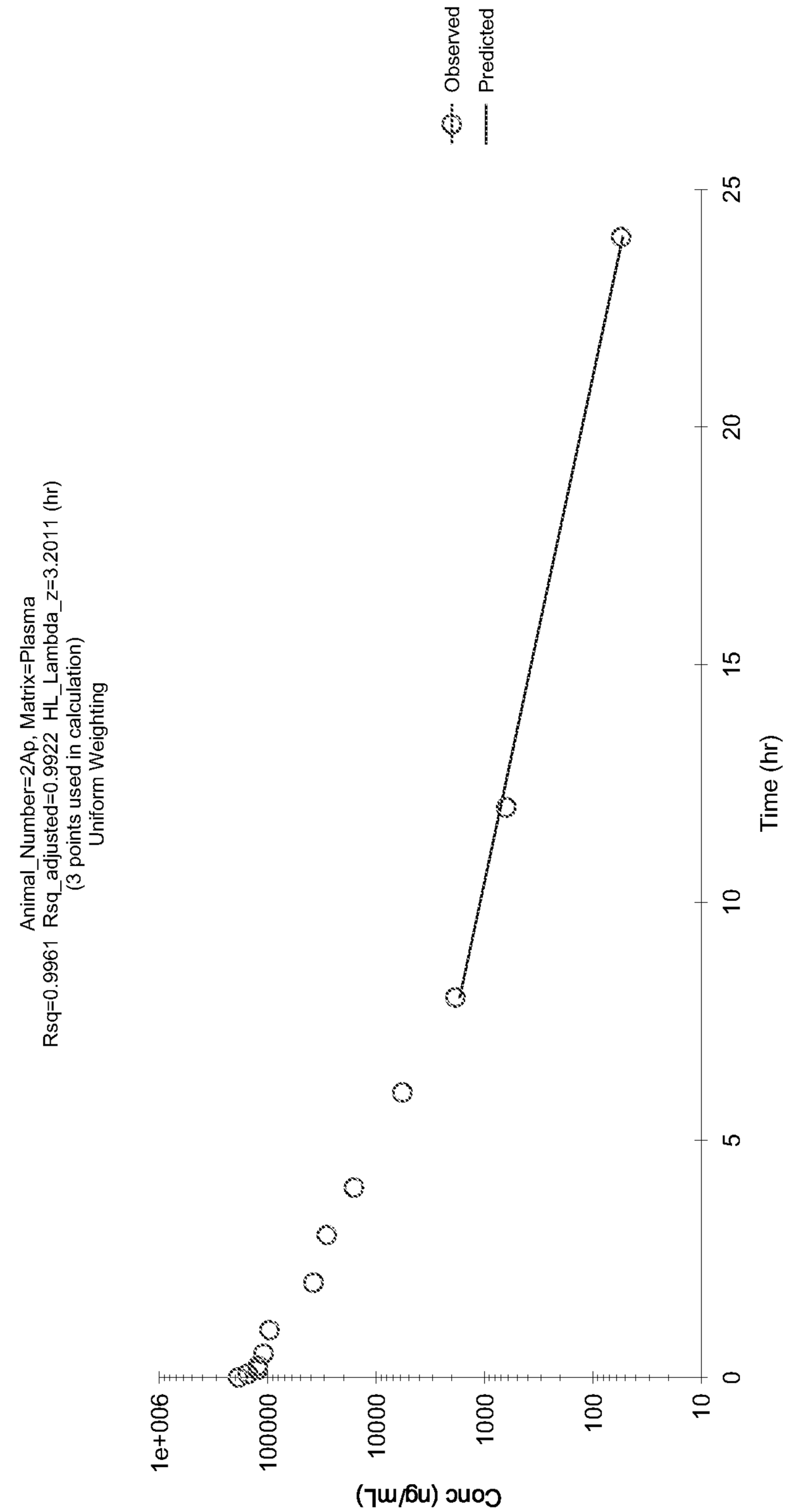


Figure 3b

B) IV plasma half-life of SP2 in Sprague-Dawley rat; 2 mg/mL iv bolus

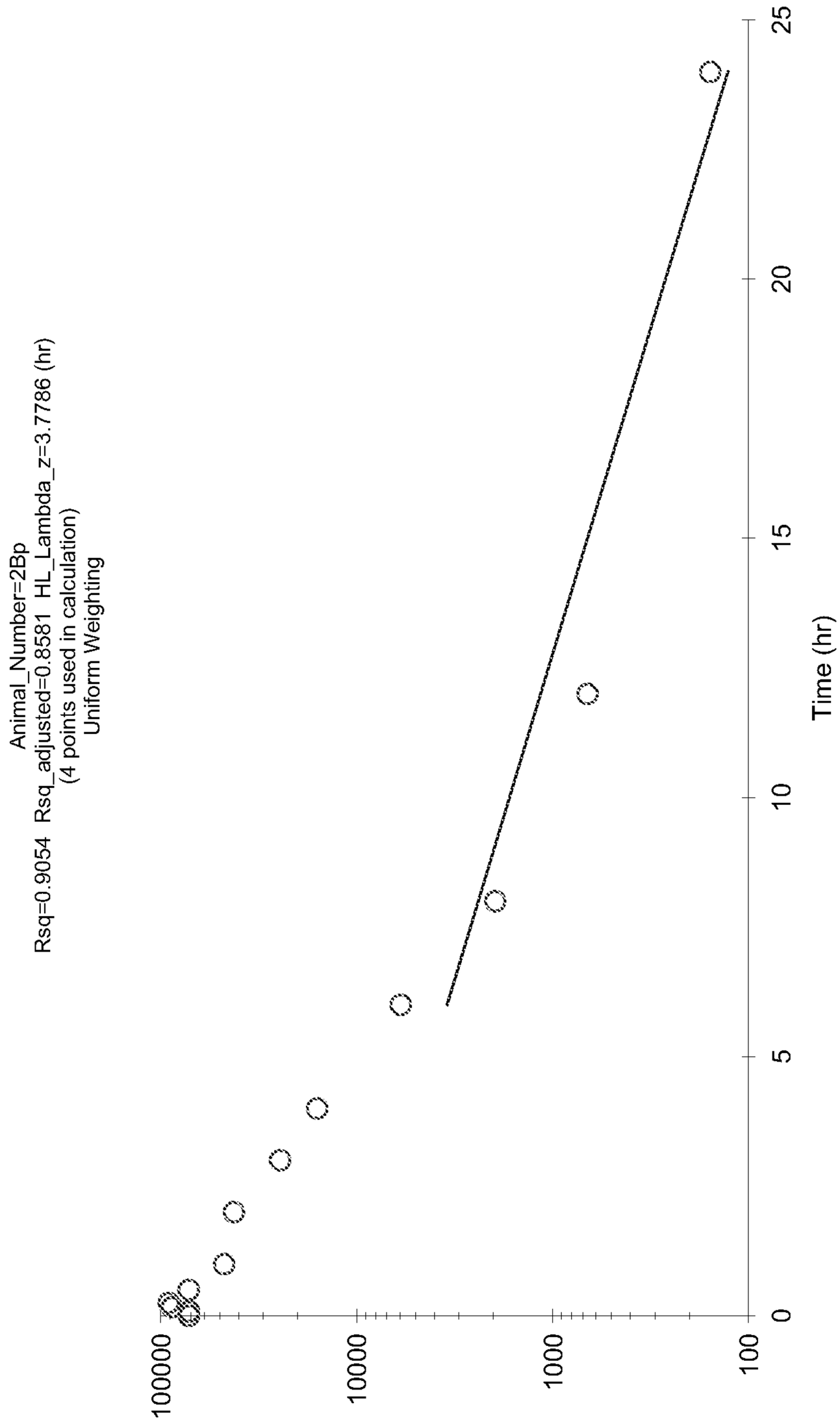


Figure 3c

C) IV plasma half-life of SP3 in Sprague-Dawley rat; 2 mg/mL iv bolus

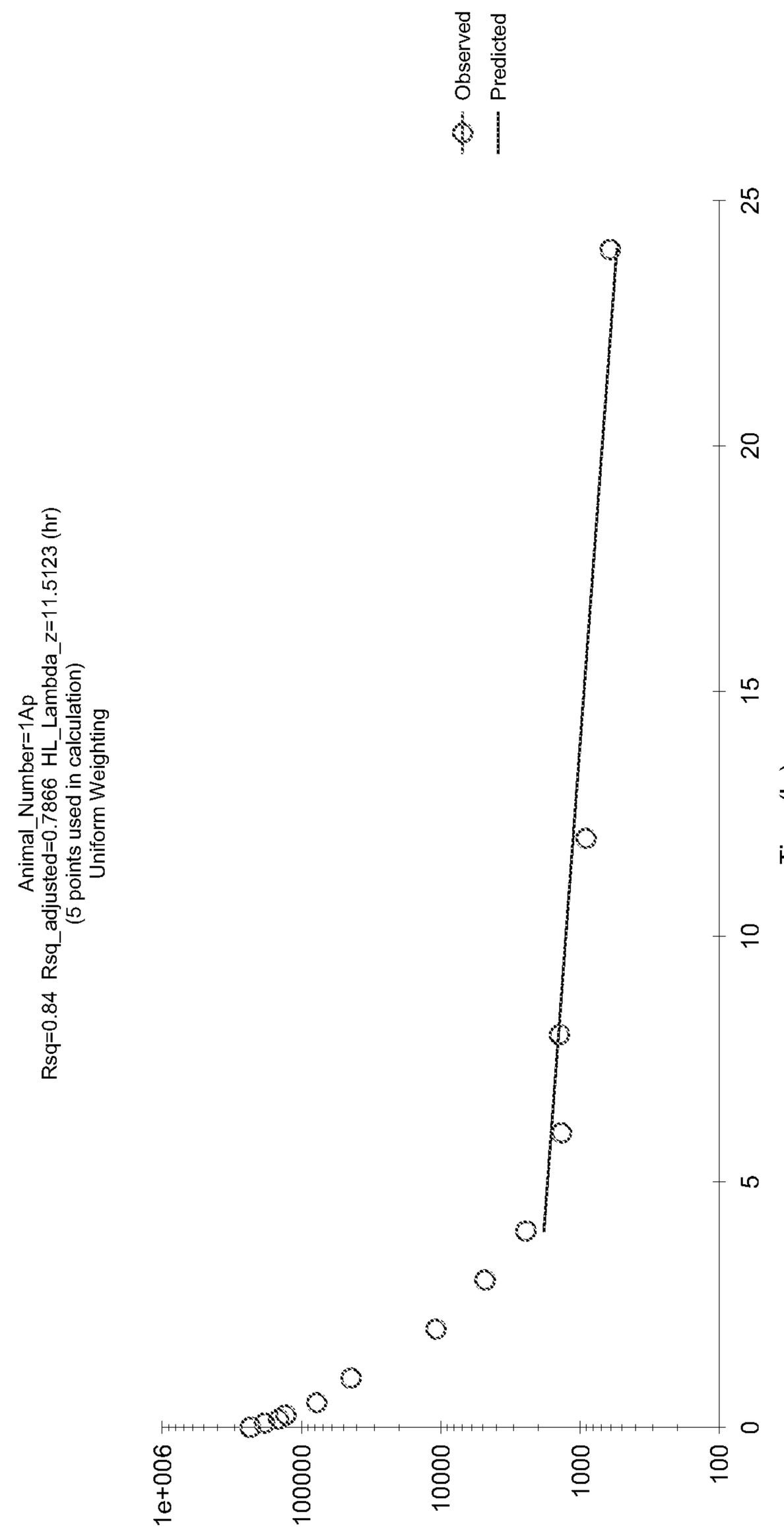


Figure 3d

D) IV plasma half-life of SP5 in Sprague-Dawley rat; 2 mg/mL iv bolus

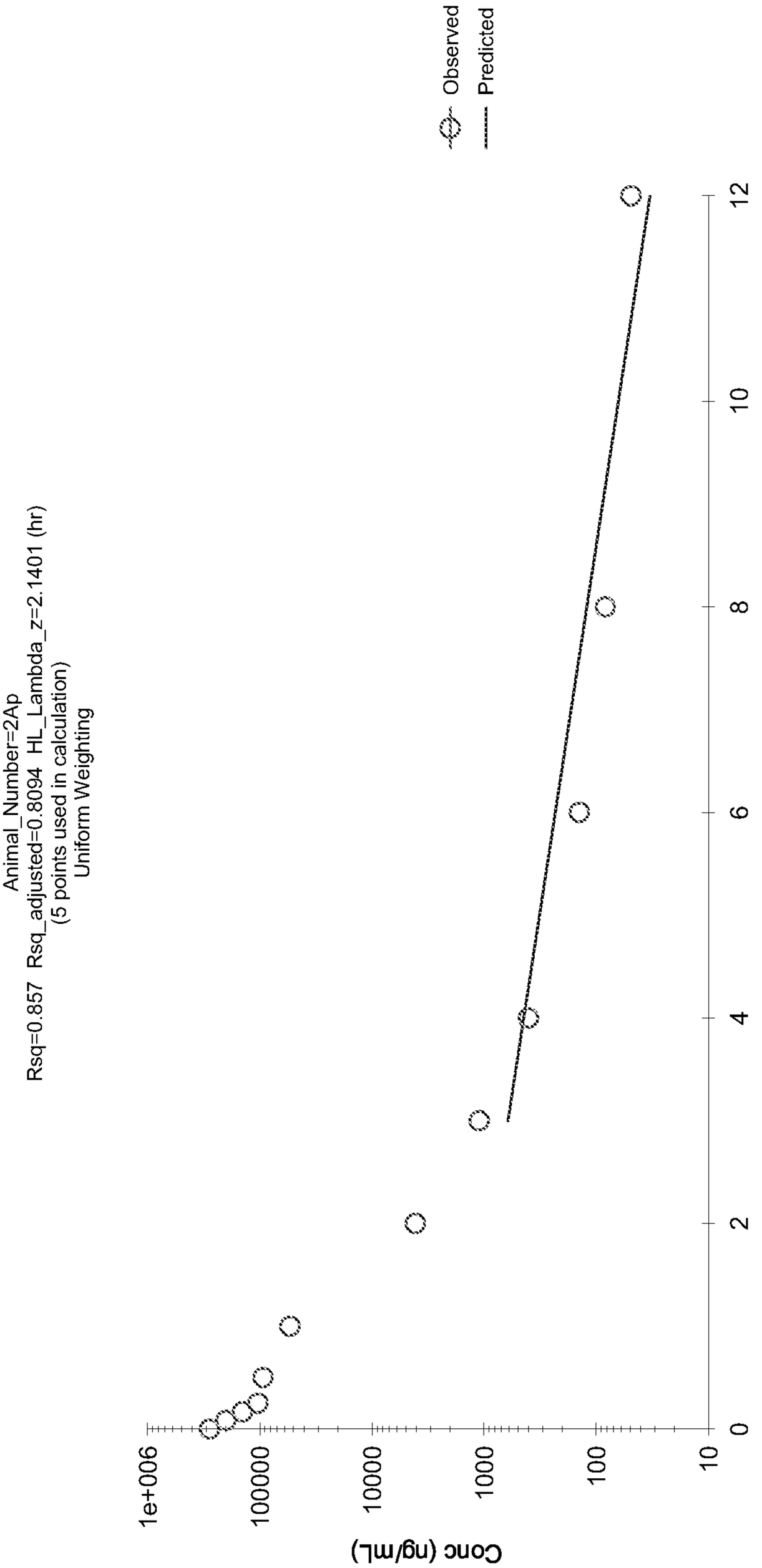


Figure 3e

E) IV plasma half-life of SP7 in Sprague-Dawley rat; 2 mg/mL iv bolus

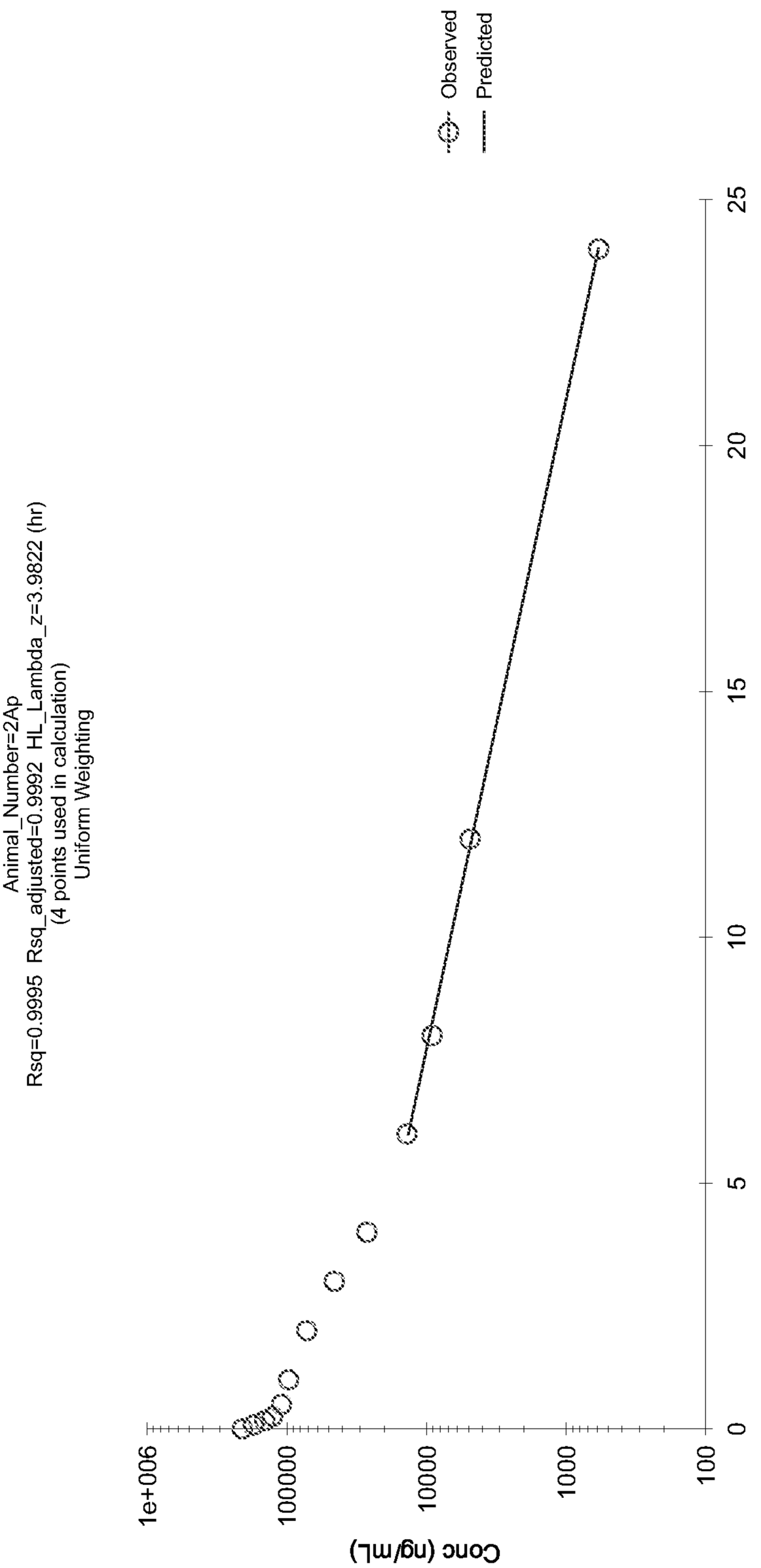


Figure 3f

F) IV plasma half-life of SP8 in Sprague-Dawley rat; 2 mg/mL iv bolus

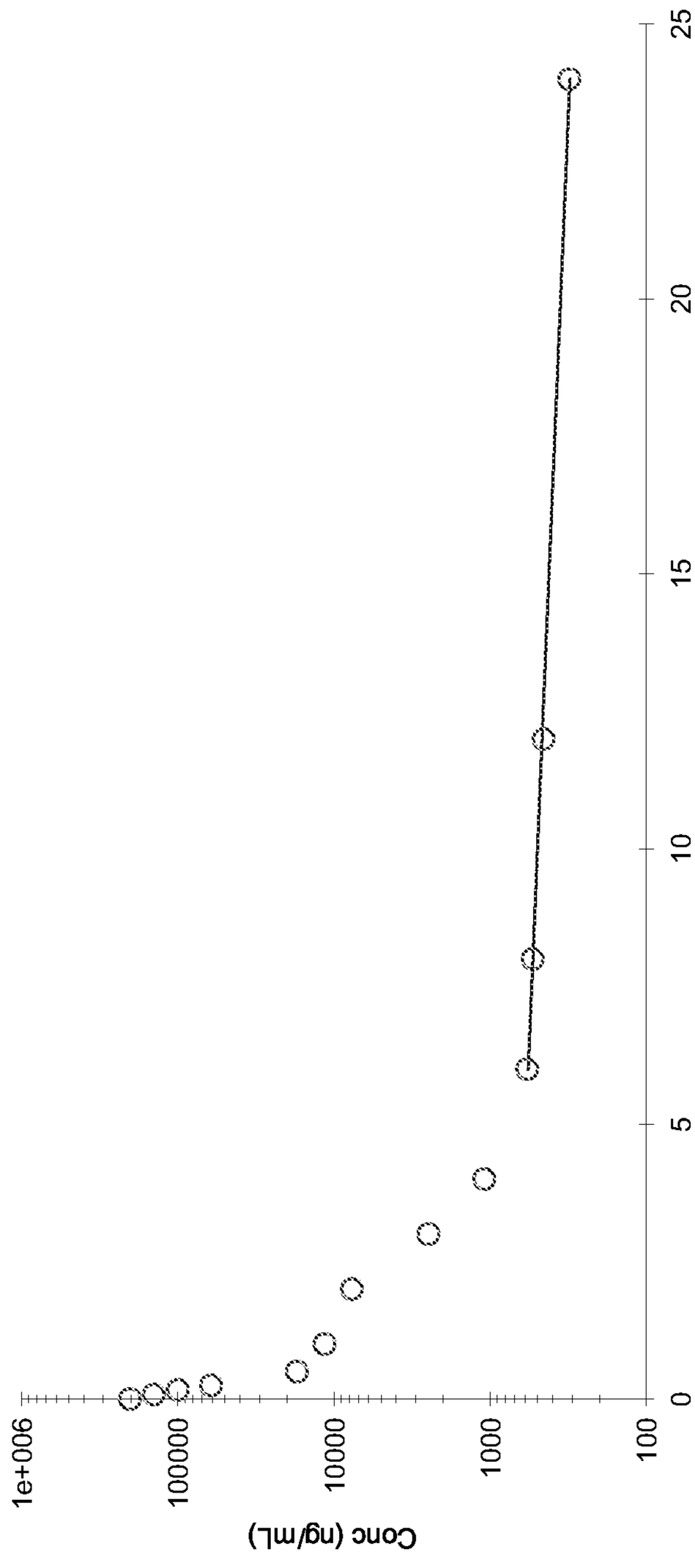


Figure 3g

G) IV plasma half-life of SP9 in Sprague-Dawley rat; 2 mg/mL iv bolus

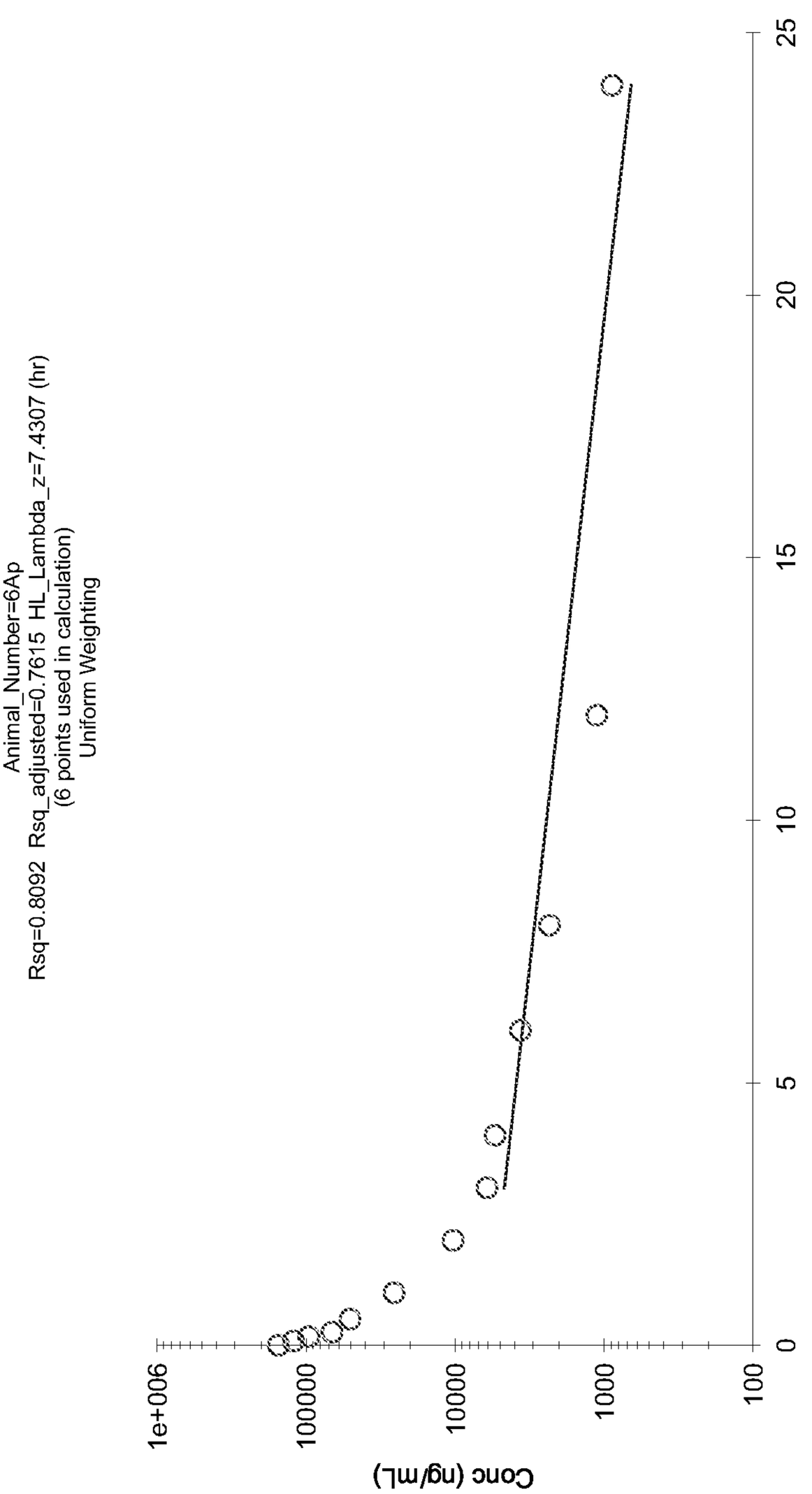


Figure 3h

H) IV plasma half-life of SP10 in Sprague-Dawley rat; 2 mg/mL iv bolus

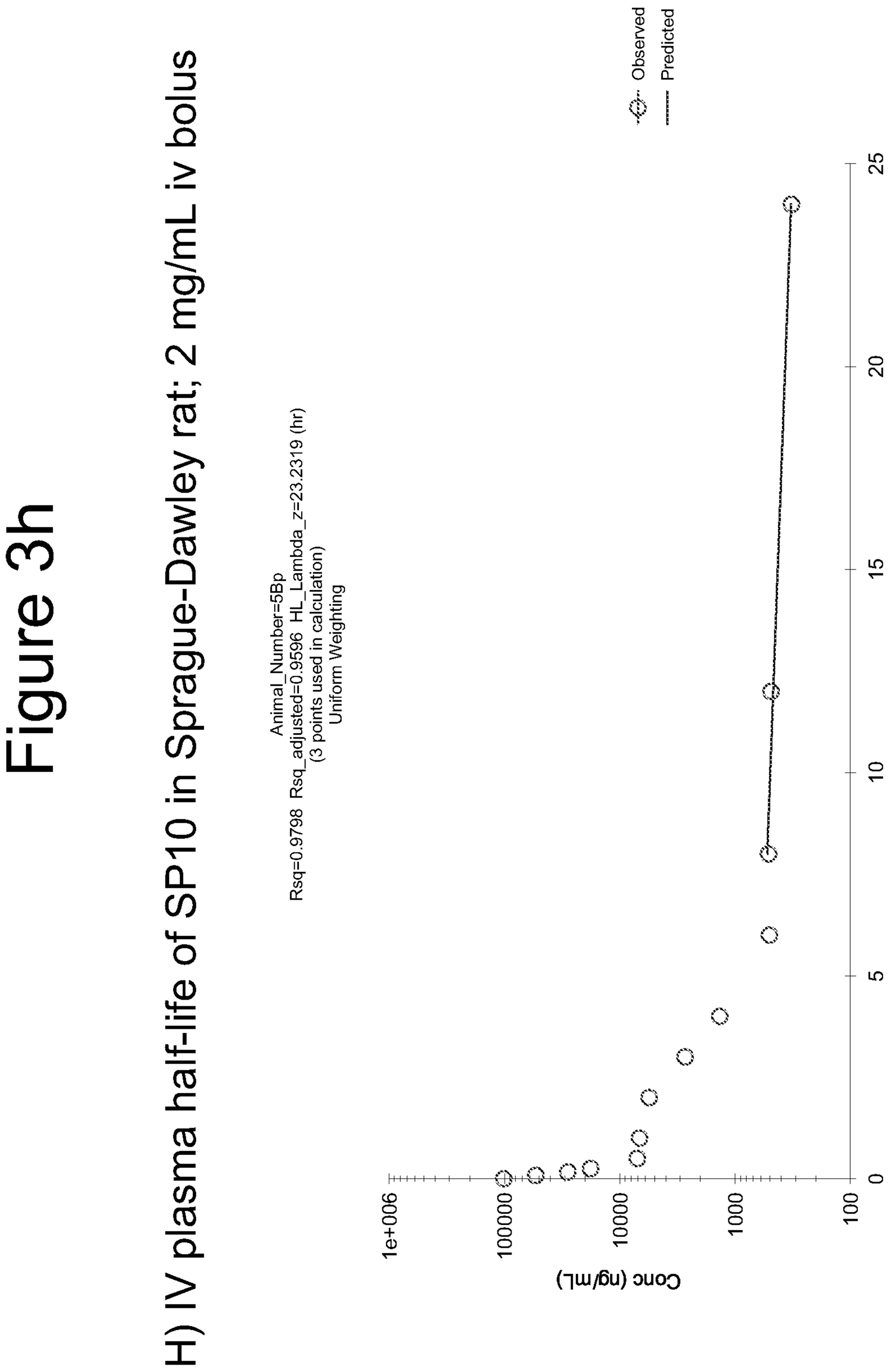


Figure 3i

Fig. 3i) IV plasma half-life of SP11 in Sprague-Dawley rat; 0.6 mg/mL iv bolus

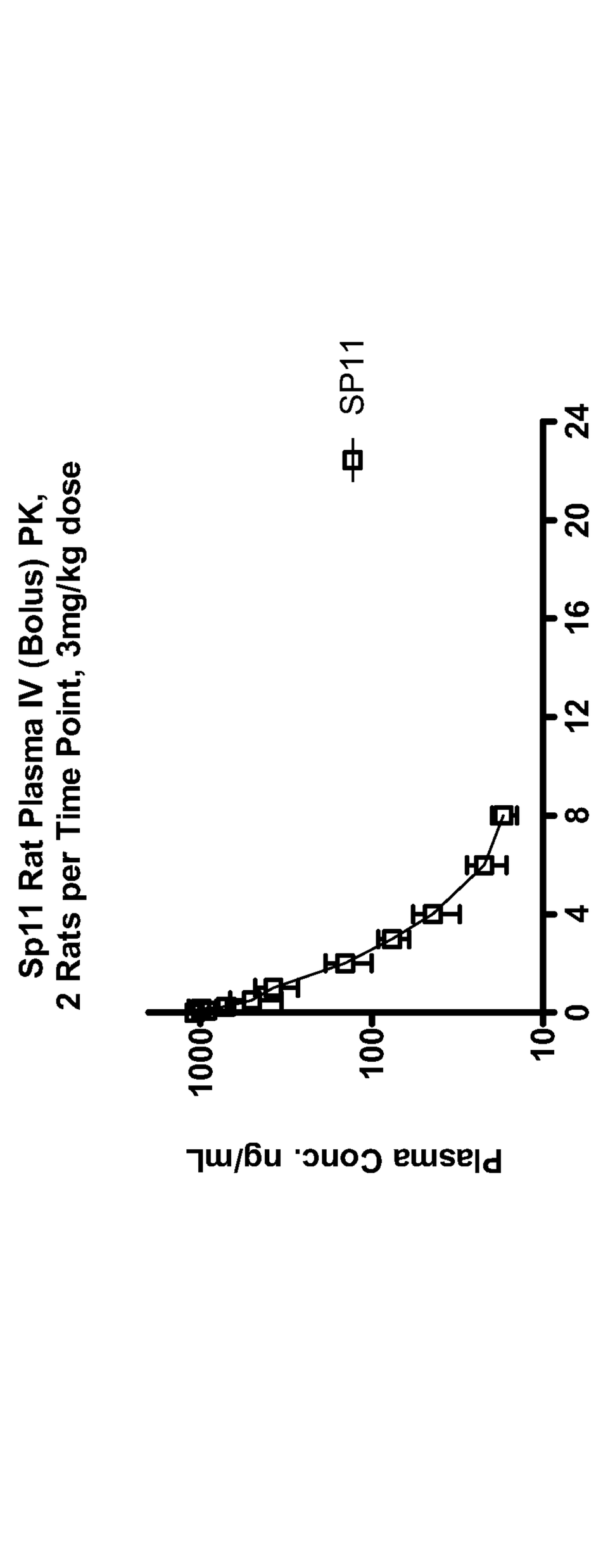


Figure 3j

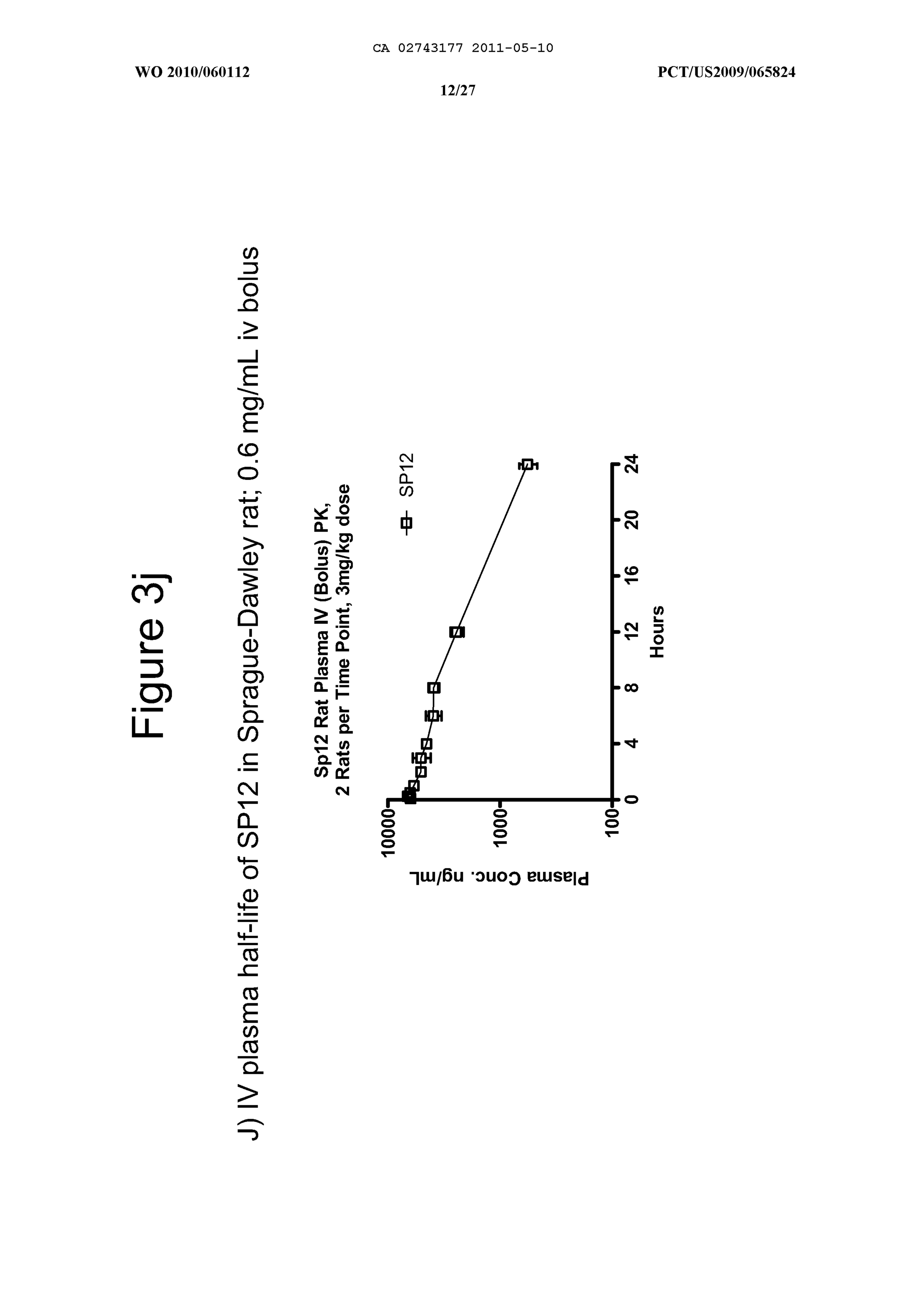

J) IV plasma half-life of SP12 in Sprague-Dawley rat; 0.6 mg/mL iv bolus
Sp12 Rat Plasma IV (Bolus) PK,
2 Rats per Time Point, 3mg/kg dose
SP12
Plasma Conc. ng/mL
10000
1000
100
0
4
8
12
16
20
24
Hours

Figure 3k

K) IV plasma half-life of SP13 in Sprague-Dawley rat; 0.6 mg/mL in 5% Dextrose

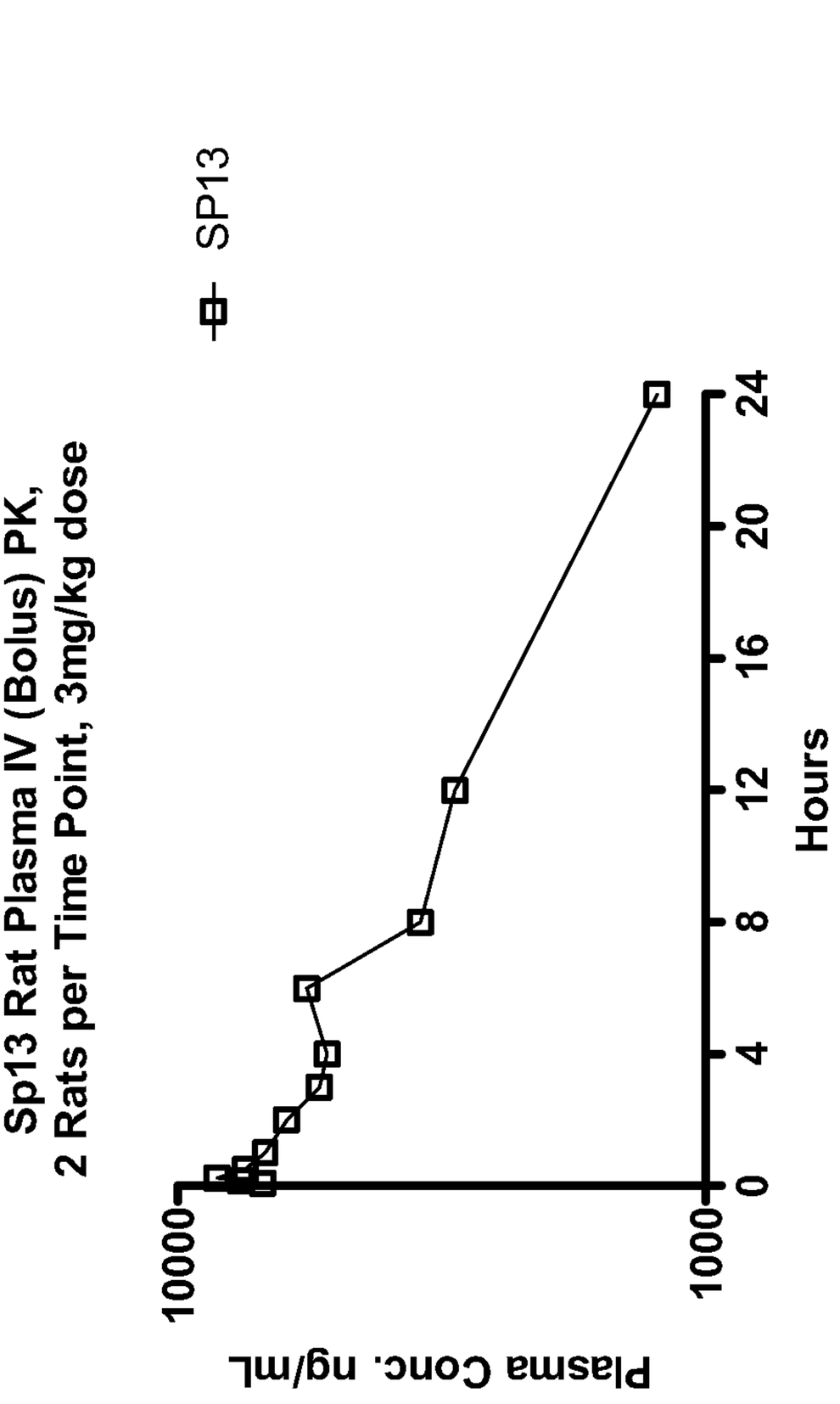
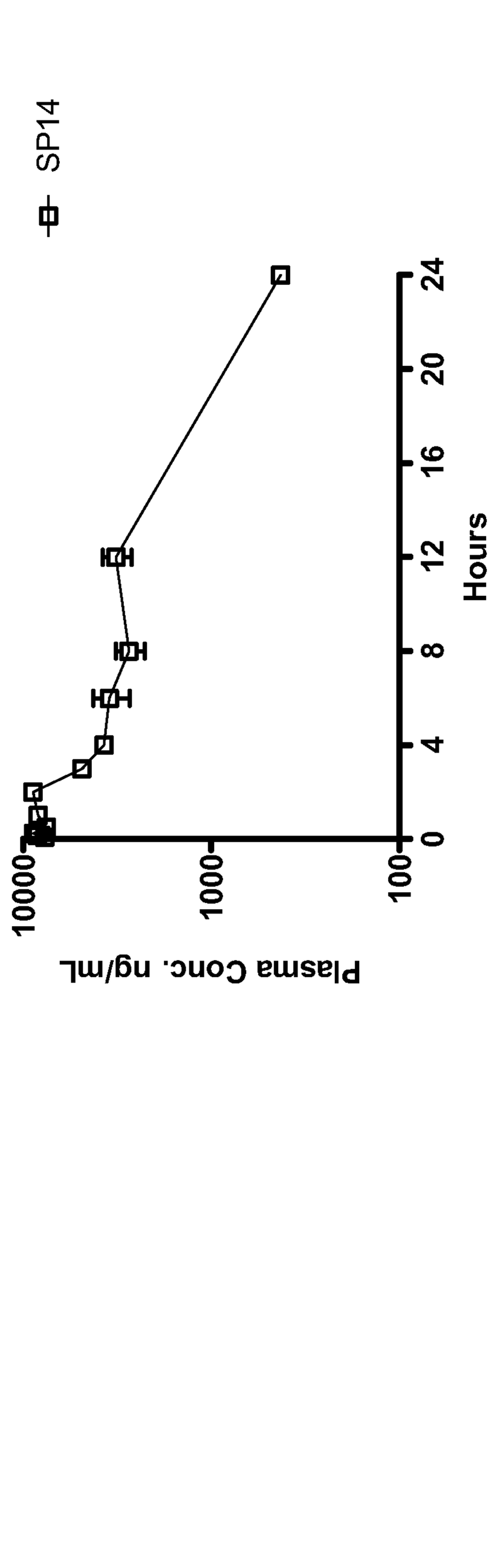


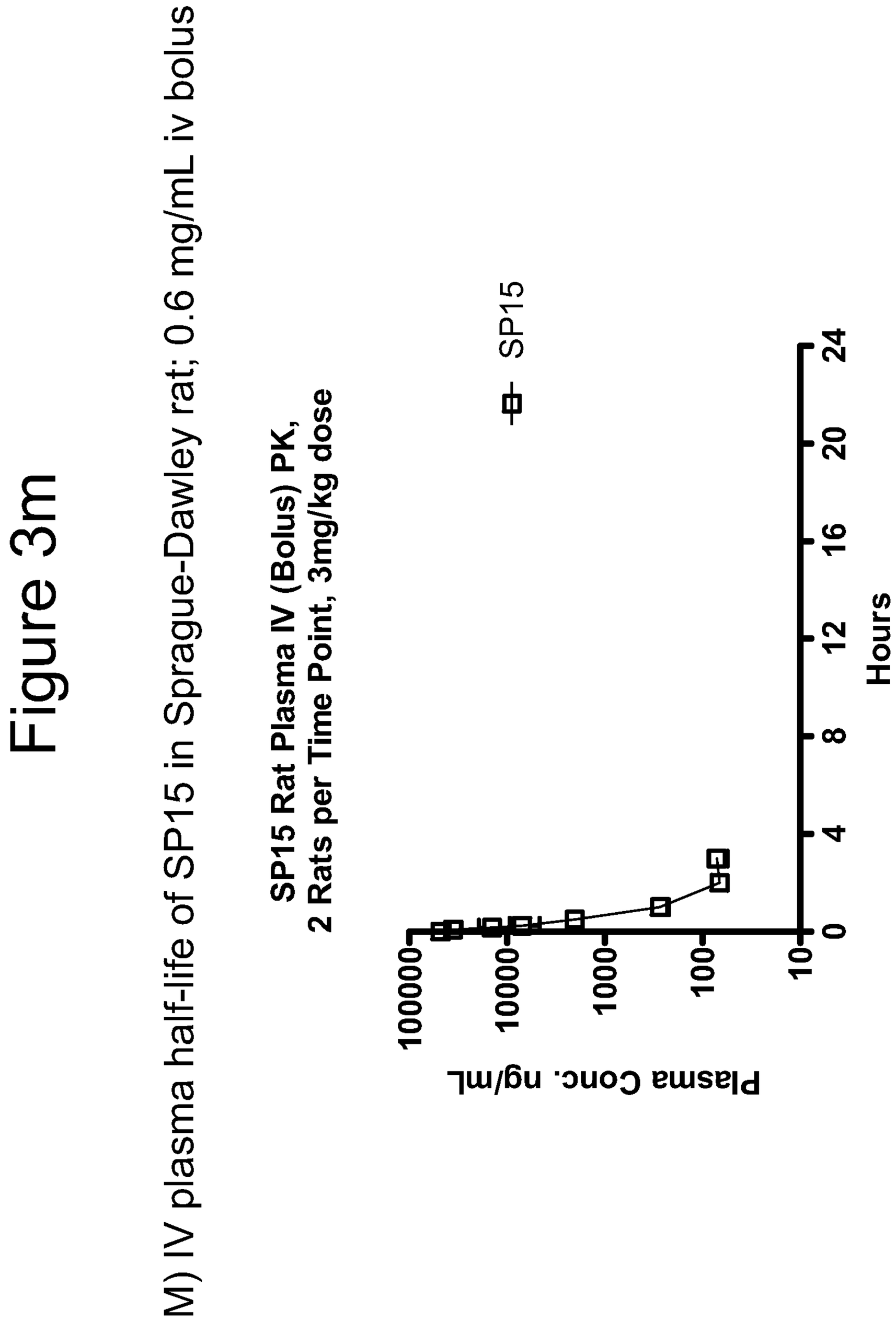
Figure 3L

half-life of SP14 in Sprague-Dawley rat; 0.6 mg/mL i.v bolus



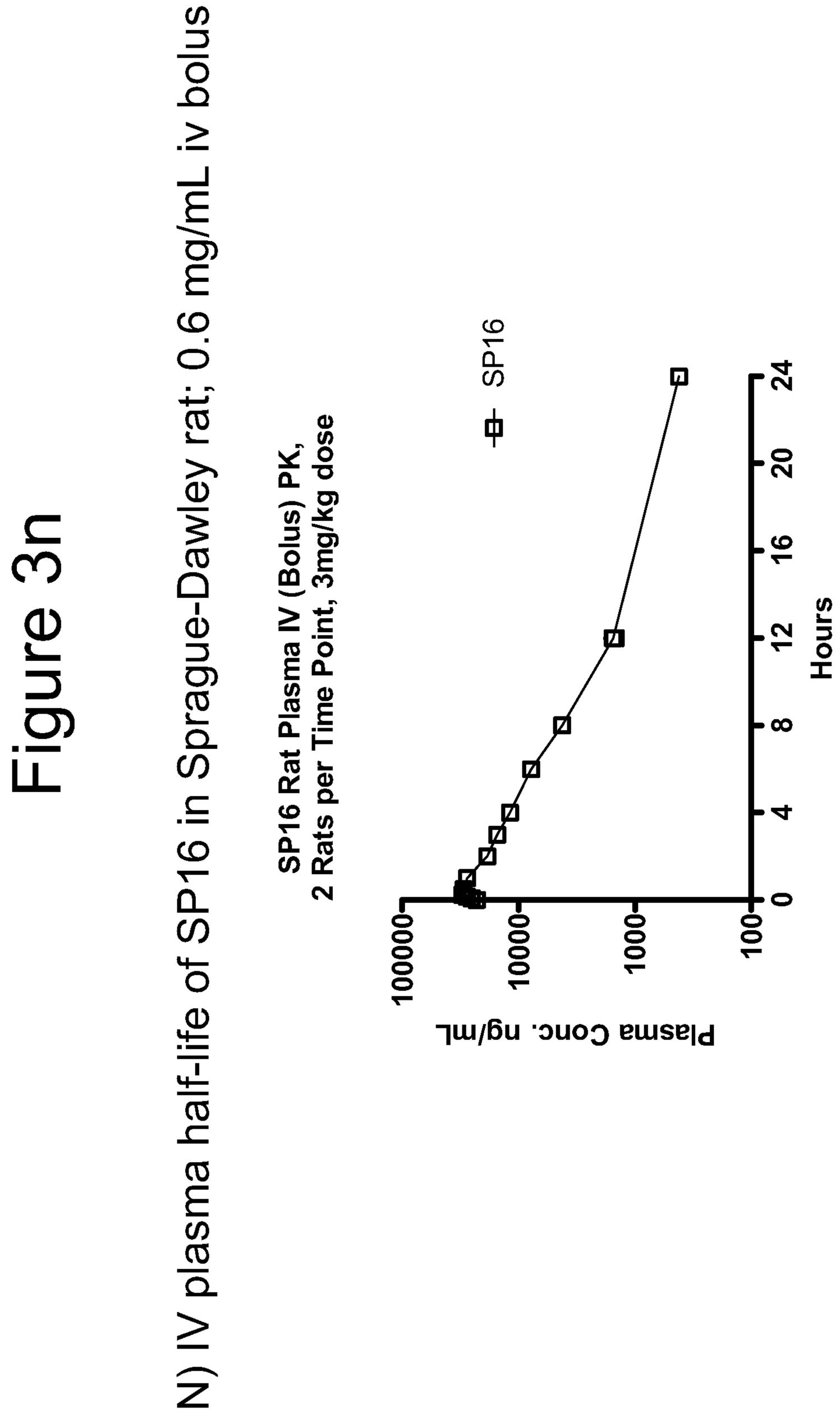
L) IV plasma

Figure 3m



M) IV plasma half-life of SP15 in Sprague-Dawley rat; 0.6 mg/mL in

Figure 3n



N) IV plasma half-life of SP16 in Sprague-Dawley rat; 0.6 mg/mL i.v bolus

Figure 30

O) IV plasma half-life of SP17 in Sprague-Dawley rat; 0.6 mg/mL in

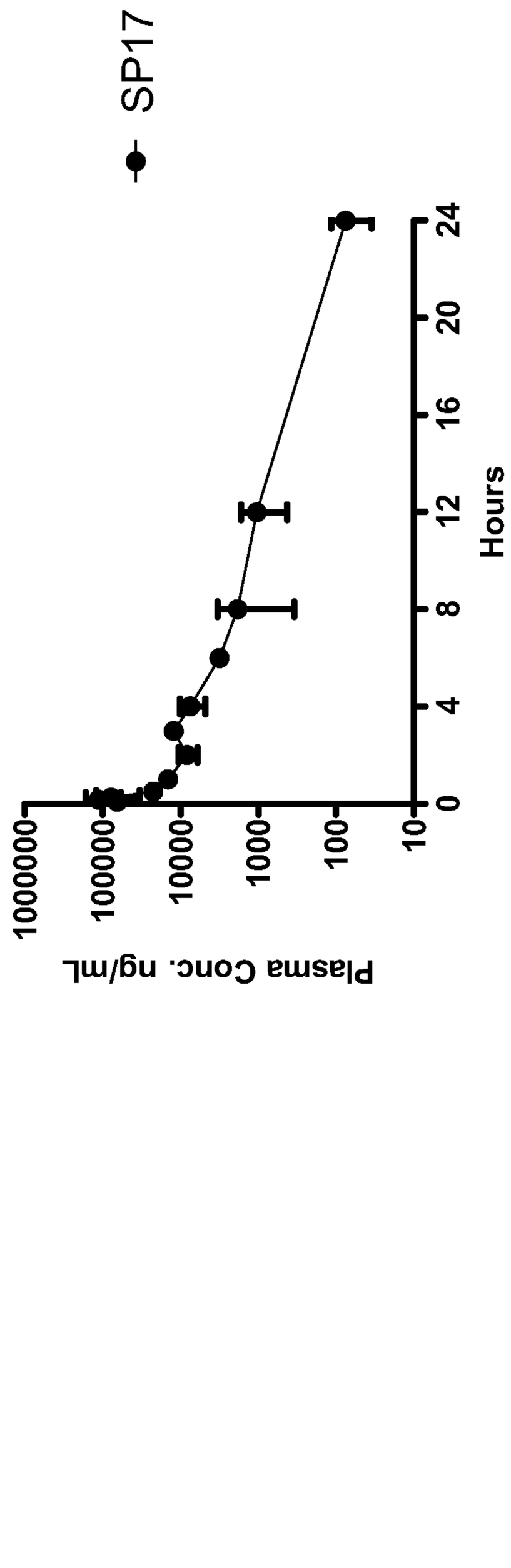
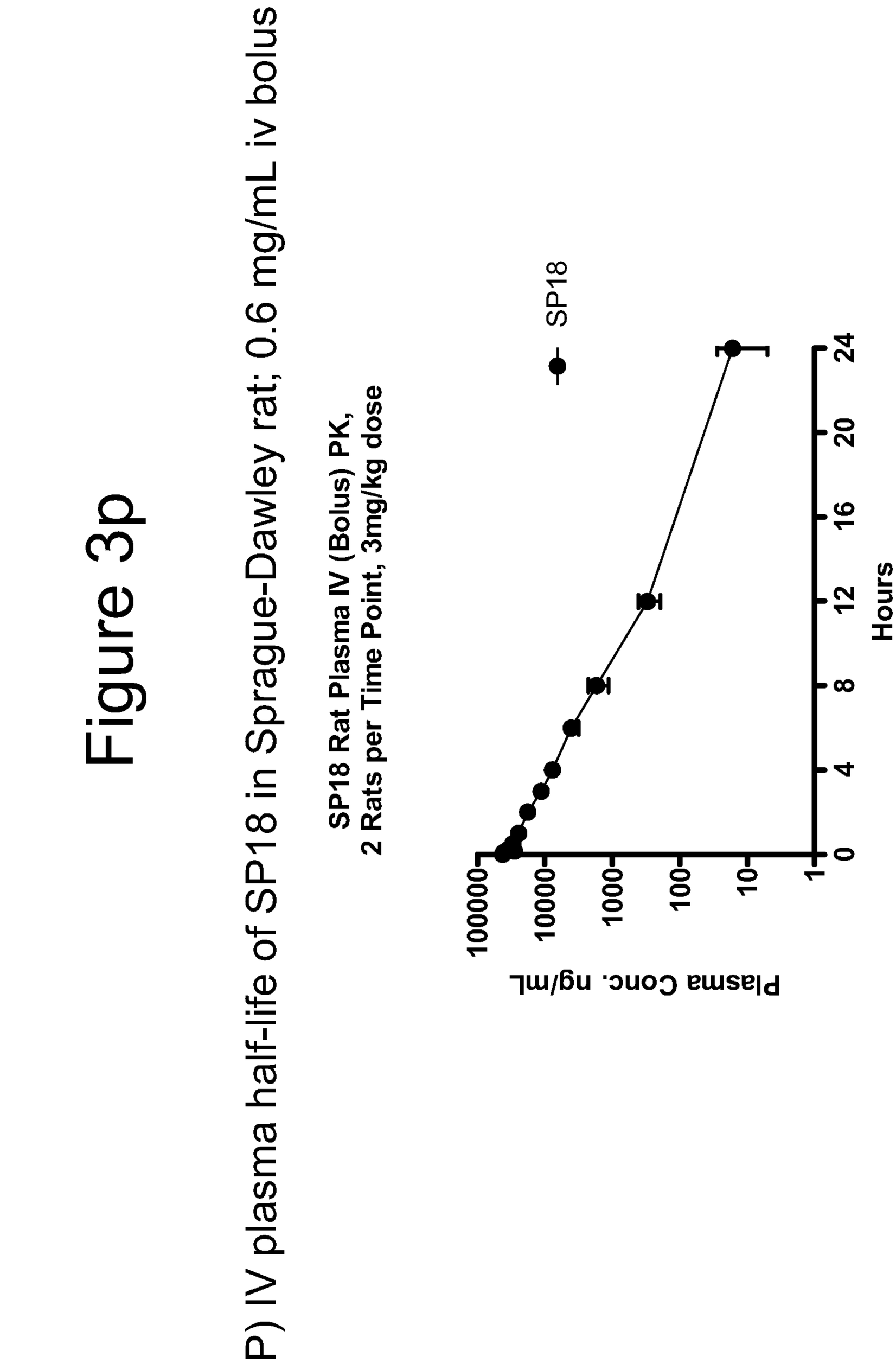


Figure 3p



P) IV plasma half-life of SP18 in Sprague-Dawley rat; 0.6 mg/mL in

Figure 3q

Q) IV plasma half-life of SP19 in Sprague-Dawley rat; 0.6 mg/mL iv bolus

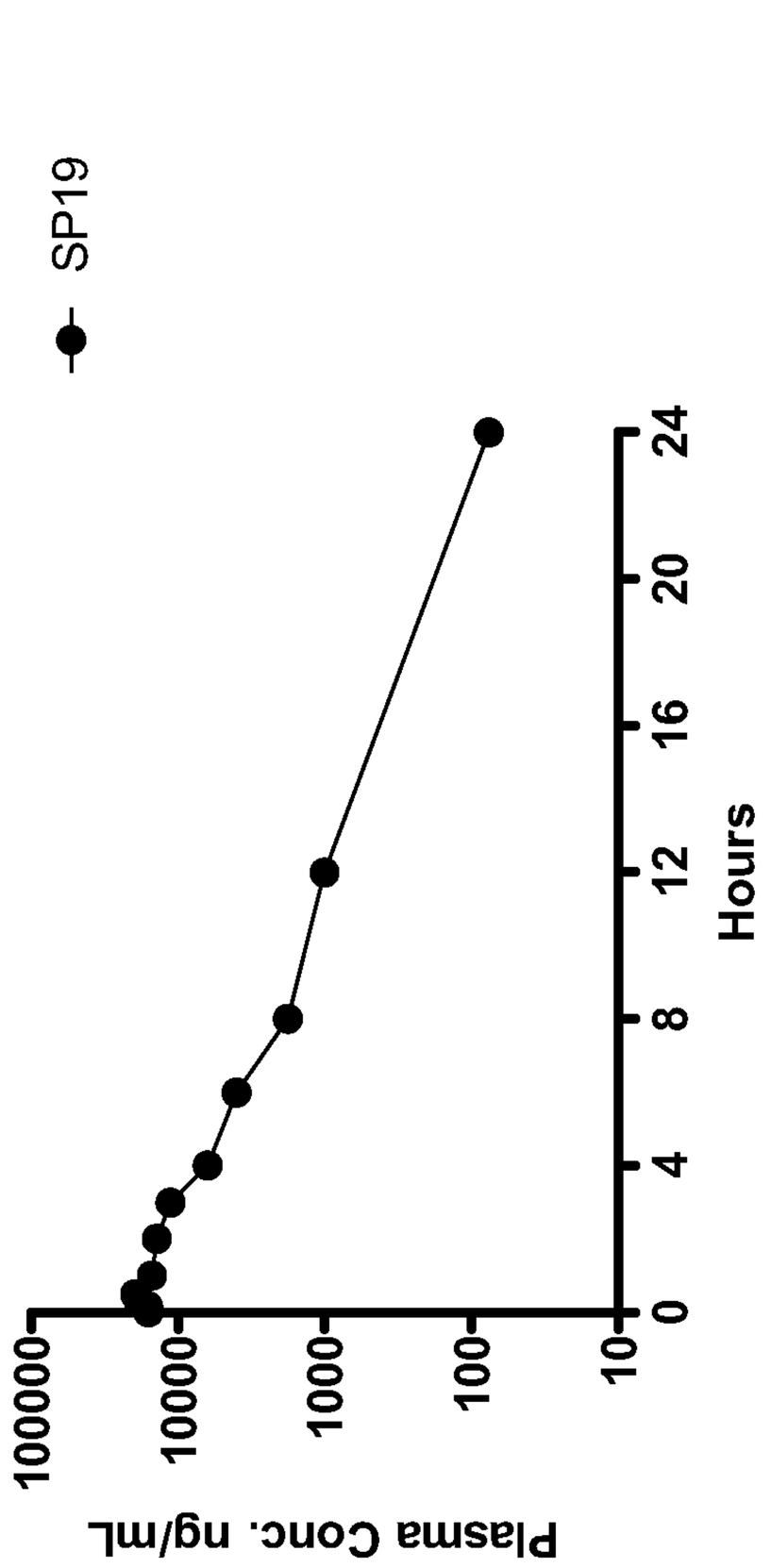


Figure 3r

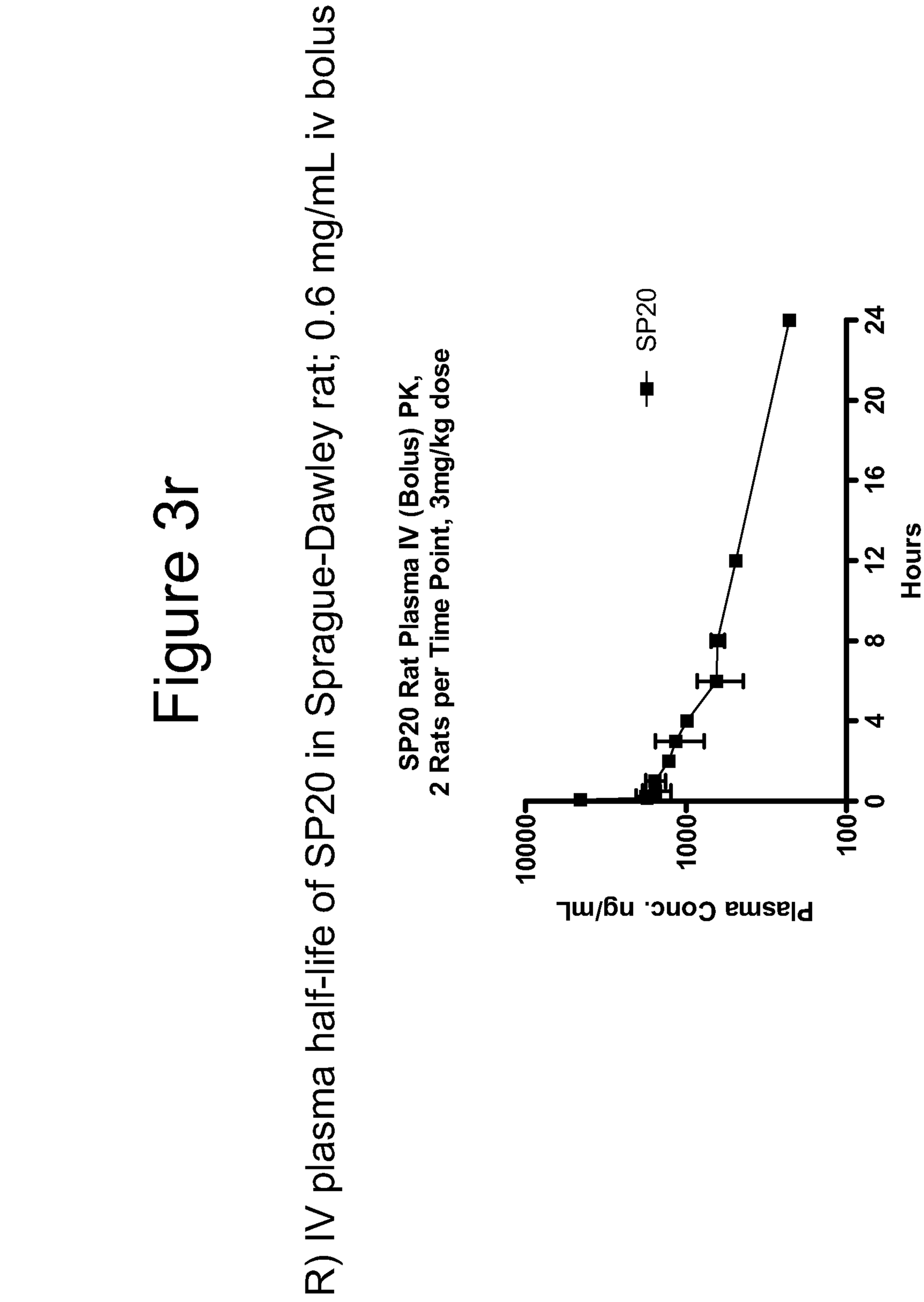

R) IV plasma half-life of SP20 in Sprague-Dawley rat; 0.6 mg/mL iv bolus
SP20 Rat Plasma IV (Bolus) PK,
2 Rats per Time Point, 3mg/kg dose
SP20
Plasma Conc. ng/mL
10000
1000
100
0
4
8
12
16
20
24
Hours

Figure 3s

half-life of SP21 in Sprague-Dawley rat; 0.6 mg/mL in

S) IV plasma bolus

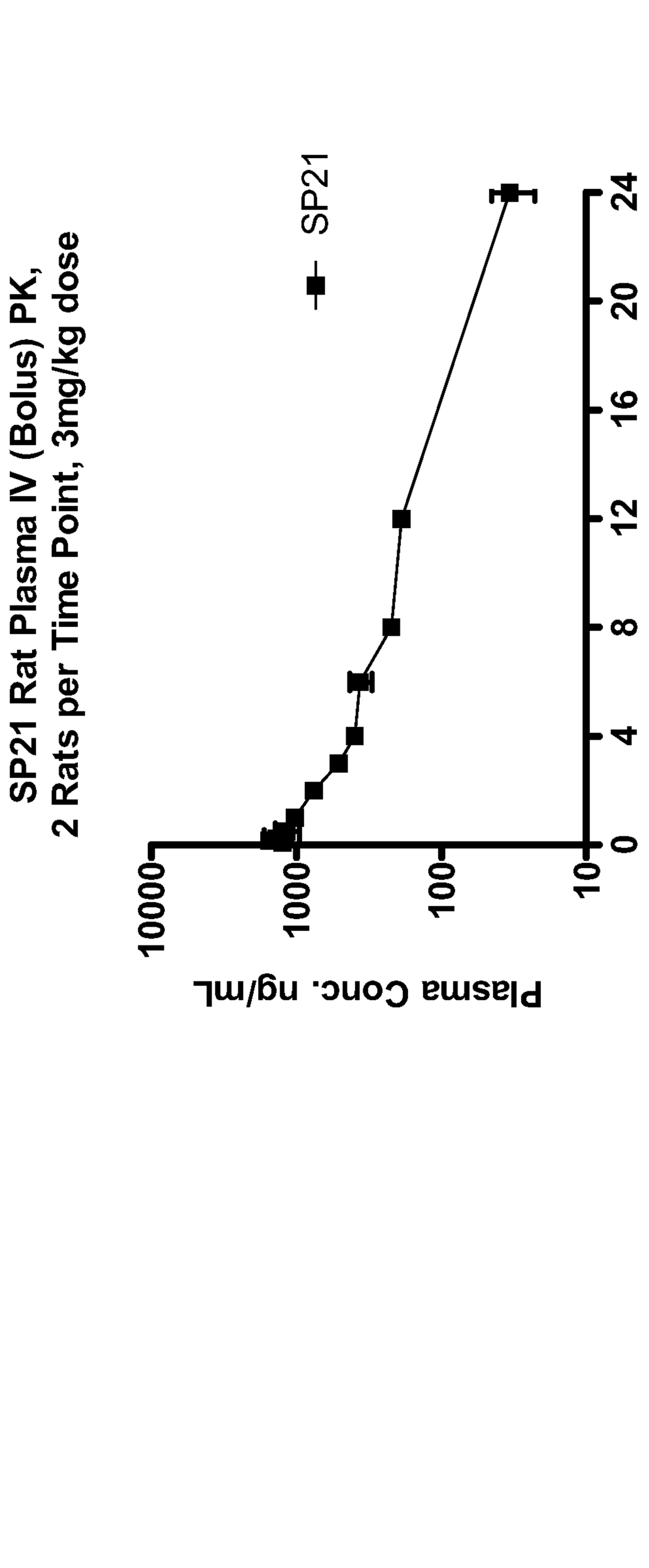


Figure 3t

T) IV plasma half-life of SP22 in Sprague-Dawley rat; 0.6 mg/mL i.v bolus

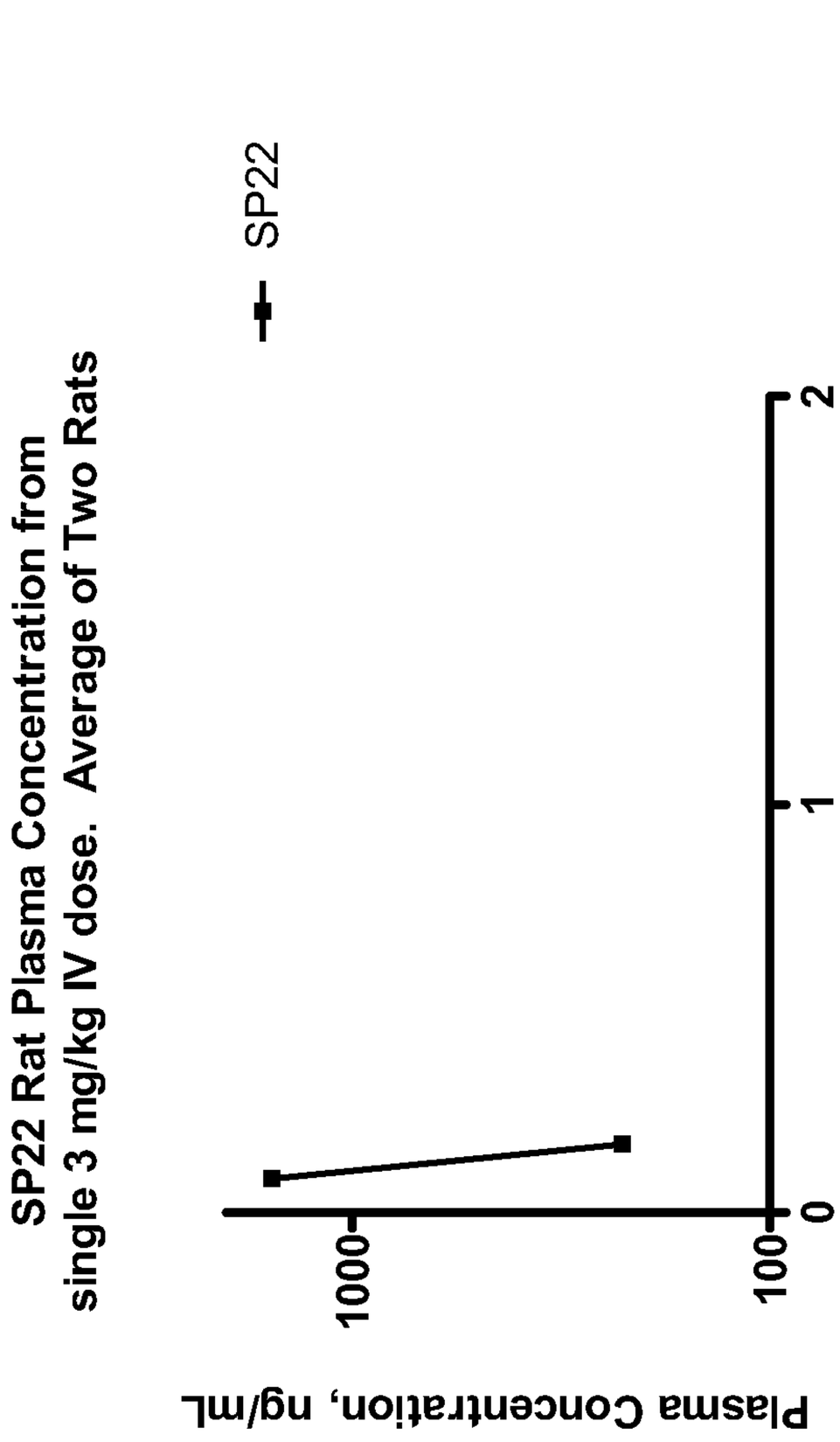


Figure 3U

half-life of SP23 in Sprague-Dawley rat; 0.6 mg/mL i

U) IV plasma

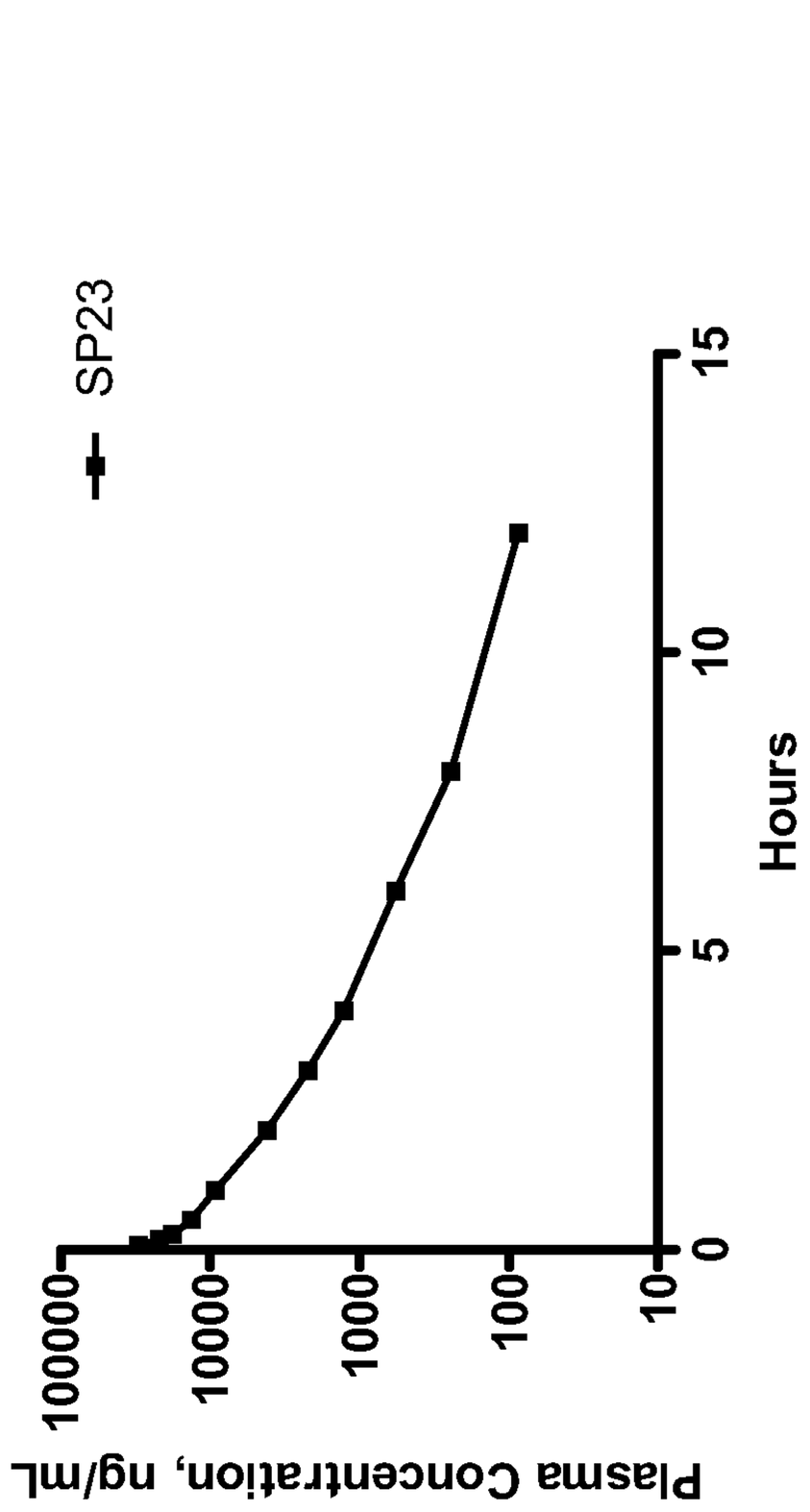


Figure 4.

Stapled Peptides	BH3	No serum EC50, μM	2% serum EC50, μM	10% serum EC50, μM	Serum Kd*	Free Fraction est. in blood, μM
SP1	BID	1.2	35.5	>100	<0.1	<0.1%
SP7	BID	0.9	17.0	72.1	<0.1	<0.1%
SP5	BID	1.6	7.6	22.4	3.7	0.61%
SP8	BID	2.2	5.3	14.6	10.1	1.66%
SP10	BID	2.4	4.8	9.7	20.1	3.25%
SP2	BIM	1.3	20.0	>100	<0.1	<0.1%
SP9	BIM	1.3	4.3	17.0	4.5	0.75%
SP3	BIM	1.2	2.7	12.2	6.5	1.07%

Figure 5

PK PARAMETERS	SP2 1 mg/kg IV monkey	SP2 3 mg/kg IV monkey	SP2 10 mg/kg IV monkey	SP2 10 mg/kg IV rat
T ½ (hr)	2.2	2	1.9	5.7
MRTinf (hr)	1.8	1.9	2.2	3.1
Cmax (ng/ml)	19049	51540	180010	70325
AUC all (hr*ng/ml)	27275	110669	427932	170685

Figure 6

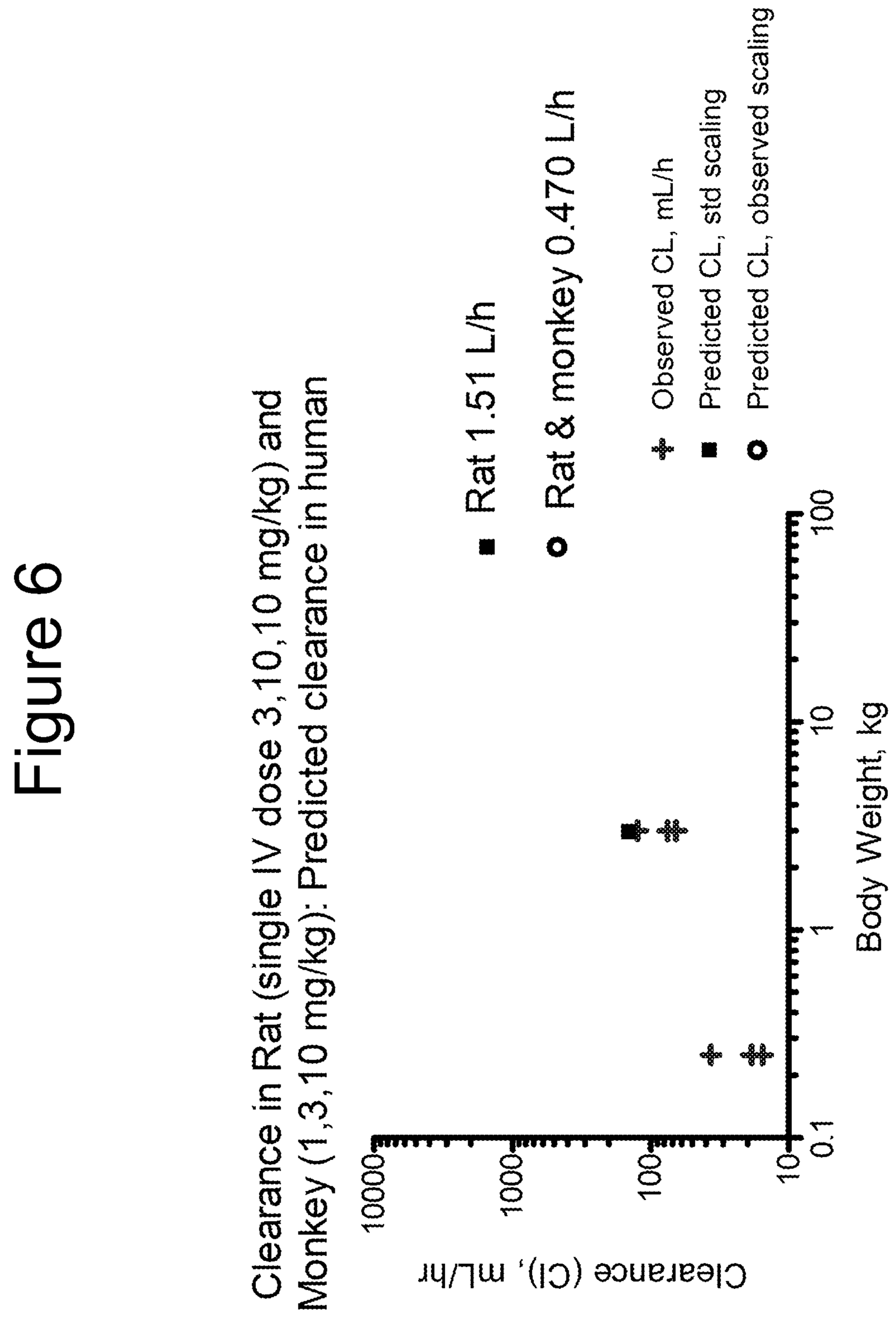

Clearance in Rat (single IV dose 3,10 mg/kg) and Monkey (1,3,10 mg/kg): Predicted clearance in human
Clearance (Cl), mL/hr
10000
1000
100
10
0.1
1
10
100
Body Weight, kg
Observed CL, 470 L/h
Predicted CL, 1.51 L/h
Predicted CL, Rat & monkey 0.mL/h std scaling
Observed scaling

Figure 7

Peptide	Protein	Molar Elliptcity Benign (222 in 0%TFE)	Molar Ellipticity 50%TFE (222 in 50%TFE)	Molar Ellipticity TFE - Molar Ellipticity Benign	% Helix benign compared to 50%TFE parent
SP1 (Parent)	BID	-8154	-28690	-20536	28.4
SP13	BID	-5446	-15935	-10489	19.0
SP14	BID	-5844	-16995	-11151	20.4
SP11	BID	-1889	-13268	-11379	6.6
SP9 (parent)	BIM	-14911	-16684	-1773	89.4
SP15	BIM	-2000	-14293	-12293	12.0
SP18	BIM	-6064	-7267	-1202	36.3
SP19	BIM	-6237	-7642	-1404	37.4
SP16	BIM	-14410	-15149	-738	86.4
SP20	BIM	-19244	-21042	-1798	115.3
SP21	BIM	-11173	-29433	-18261	67.0
SP23 (parent)	p53	-17994	-23530	-5536	76.5
SP17	p54	-12294	-13998	-1704.145013	52.2
SP22	p55	-646	-4030	-3384	2.7

WO 2010/060112 PCT/US2009/065824
27/27
CA 02743177 2011-05-10