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(54) Titre : CONTROLE DE LA FUSION DE MEMBRANE HOTE VIRALE AVEC DES HELICES ARTIFICIELLES A BASE DE SUCCEDANE DE LIAISON HYDROGENE

(54) Title: CONTROL OF VIRAL-HOST MEMBRANE FUSION WITH HYDROGEN BOND SURROGATE-BASED ARTIFICIAL HELICES

(57) Abrégé/Abstract:
The present invention relates to a peptide having one or more stable, internally-constrained HBS α- helices, where the peptide mimics at least a portion of a class I C-peptide helix or at least a portion of a class I N-peptide helix of a viral (e.g., HIV-I) coiled-coil assembly. Methods of inhibiting viral infectivity of a subject by administering these peptides are also disclosed.

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(54) Title: CONTROL OF VIRAL-HOST MEMBRANE FUSION WITH HYDROGEN BOND SURROGATE-BASED ARTIFICIAL HELICES

(57) Abstract: The present invention relates to a peptide having one or more stable, internally-constrained HBS α- helices, where the peptide mimics at least a portion of a class I C-peptide helix or at least a portion of a class I N-peptide helix of a viral (e.g., HIV-I) coiled-coil assembly. Methods of inhibiting viral infectivity of a subject by administering these peptides are also disclosed.

CONTROL OF VIRAL–HOST MEMBRANE FUSION WITH HYDROGEN BOND SURROGATE-BASED ARTIFICIAL HELICES

CROSS-REFERENCE

[0001] This application claims the benefit of U.S. Provisional Application No. 61/018,118, filed December 31, 2007, which application is incorporated herein by reference.

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with government support under grant numbers GM073943 and AI42382, both awarded by the National Institutes of Health. The government has certain rights in this invention.

BACKGROUND OF THE INVENTION

[0003] Enveloped viruses depend upon fusion between the viral membrane and a host cell membrane (the plasma membrane or an intracellular membrane, depending upon the specific virus) for delivery of viral genetic material to the host cell, thereby initiating infection of the host cell (Kielian & Ray, "Virus Membrane-fusion Proteins: More Than One Way to Make a Hairpin," *Nat. Rev.* 4:67–76 (2006), which is hereby incorporated by reference in its entirety). This membrane-fusion reaction relies on virus membrane-fusion proteins (Kielian & Ray, "Virus Membrane-fusion Proteins: More Than One Way to Make a Hairpin," *Nat. Rev.* 4:67–76 (2006), which is hereby incorporated by reference in its entirety). At least two classes of membrane-fusion proteins have been identified (Kielian & Ray, "Virus Membrane-fusion Proteins: More Than One Way to Make a Hairpin," *Nat. Rev.* 4:67–76 (2006), which is hereby incorporated by reference in its entirety). Class I fusion proteins contain two heptad repeat regions, termed the N-terminal heptad region and the C-terminal heptad region, between a hydrophobic "fusion peptide" region and a transmembrane domain (Dutch et al., "Virus Membrane Fusion Proteins: Biological Machines That Undergo a Metamorphosis," *Biosci. Rep.* 20(6):597–612 (2000), which is hereby incorporated by reference in its entirety). During membrane fusion, these heptad repeat regions ultimately adopt a highly stable coiled-coil assembly in which the N-terminal heptad repeat region forms an internal, trimeric coiled-coil buttressed by helices from the C-terminal heptad repeat region (Dutch et al., "Virus Membrane Fusion Proteins: Biological Machines That Undergo a Metamorphosis," *Biosci. Rep.* 20(6):597–612 (2000), which is hereby incorporated by reference in its entirety). Agents that interfere with the formation of this coiled-coil assembly can prevent viral–host cell membrane fusion, thereby inhibiting infection of the new host cell.

[0004] Viruses that use class I coiled-coil assemblies for viral infectivity include: *Orthomyxoviridae*, *e.g.*, influenza virus (Dutch et al., "Virus Membrane Fusion Proteins: Biological Machines That Undergo a Metamorphosis," *Biosci. Rep.* 20(6):597–612 (2000); Kielian & Ray, "Virus Membrane-fusion Proteins: More Than One Way to Make a Hairpin," *Nat. Rev.* 4:67–76 (2006), which are hereby incorporated by reference in their entirety); *Paramyxoviridae*, *e.g.*, Simian virus

5 (Dutch et al., "Virus Membrane Fusion Proteins: Biological Machines That Undergo a Metamorphosis," *Biosci. Rep.* 20(6):597–612 (2000); Kielian & Ray, "Virus Membrane-fusion Proteins: More Than One Way to Make a Hairpin," *Nat. Rev.* 4:67–76 (2006), which are hereby incorporated by reference in their entirety) and respiratory syncitial virus (Dutch et al., "Virus Membrane Fusion Proteins: Biological Machines That Undergo a Metamorphosis," *Biosci. Rep.* 20(6):597–612 (2000); Shepherd et al., "Modular α-Helical Mimetics with Antiviral Activity Against Respiratory Syncitial Virus," *J. Am. Chem. Soc.* 128:13284–9 (2006), which are hereby incorporated by reference in their entirety); *Filoviridae*, *e.g.*, Ebola virus (Dutch et al., "Virus Membrane Fusion Proteins: Biological Machines That Undergo a Metamorphosis," *Biosci. Rep.* 20(6):597–612 (2000); Kielian & Ray, "Virus Membrane-fusion Proteins: More Than One Way to Make a Hairpin," *Nat. Rev.* 4:67–76 (2006), which are hereby incorporated by reference in their entirety); *Retroviridae*, *e.g.*, Moloney murine leukemia virus, simian immunodeficiency virus, Human immunodeficiency virus ("HIV-1"), and human T cell leukemia virus (Dutch et al., "Virus Membrane Fusion Proteins: Biological Machines That Undergo a Metamorphosis," *Biosci. Rep.* 20(6):597–612 (2000); Kielian & Ray, "Virus Membrane-fusion Proteins: More Than One Way to Make a Hairpin," *Nat. Rev.* 4:67–76 (2006), which are hereby incorporated by reference in their entirety); *Coronaviridae*, *e.g.*, Mouse hepatitis virus and SARS virus (Kielian & Ray, "Virus Membrane-fusion Proteins: More Than One Way to Make a Hairpin," *Nat. Rev.* 4:67–76 (2006), which is hereby incorporated by reference in its entirety); and *Herpesviridae*, *e.g.*, human cytomegalovirus (English et al., "Rational Development of β-Peptide Inhibitors of Human Cytomegalovirus Entry," *J. Biol. Chem.* 281:2661–7 (2006), which is hereby incorporated by reference in its entirety).

[0005] HIV-1 is illustrative of viruses that use class I fusion proteins. HIV has been identified as the etiological agent responsible for acquired immune deficiency syndrome ("AIDS"), a fatal disease characterized by destruction of the immune system and the inability to fight off life threatening opportunistic infections. Recent statistics indicate that as many as 33 million people worldwide are infected with the virus (AIDS EPIDEMIC UPDATE at 1, United Nations Programme on HIV/AIDS (Dec. 2007)). In addition to the large number of individuals already infected, the virus continues to spread. Estimates from 2007 point to close to 2.5 million new infections in that year alone (AIDS EPIDEMIC UPDATE at 1, United Nations Programme on HIV/AIDS (Dec. 2007)). In the same year there were approximately 2.1 million deaths associated with HIV and AIDS (AIDS EPIDEMIC UPDATE at 1, United Nations Programme on HIV/AIDS (Dec. 2007)).

[0006] Entry of HIV-1 into its target cells to establish an infection is mediated by viral envelope glycoprotein ("Env") and cell surface receptors (CD4 and a coreceptor, such as CXCR4 or CCR5) (Eckert & Kim, "Mechanisms of Viral Membrane Fusion and Its Inhibition," *Annu. Rev. Biochem.* 70:777–810 (2001), which is hereby incorporated by reference in its entirety). The mature Env complex is a trimer, with three gp120 glycoproteins associated non-covalently with three viral membrane-anchored gp41 subunits. Binding of gp120/gp41 to cellular receptors

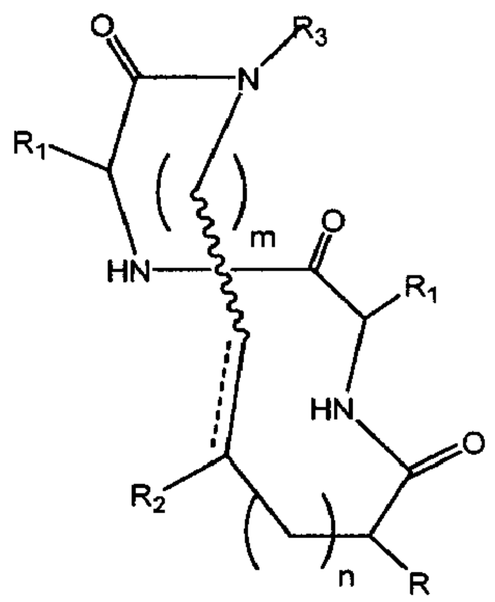
triggers a series of conformational changes in gp41 that ultimately leads to formation of a postfusion trimer-of-hairpins structure and membrane fusion (Chan et al., "Core Structure of gp41 from the HIV Envelope Glycoprotein," *Cell* 89:263–73 (1997); Weissenhorn et al., "Atomic Structure of the Ectodomain from HIV-1 gp41," *Nature* 387:426–30 (1997); Tan et al., "Atomic Structure of a Thermostable Subdomain of HIV-1 gp41," *Proc. Nat'l Acad. Sci. U.S.A.* 94:12303–8 (1997), which are hereby incorporated by reference in their entirety). As shown in Figures 1A and 1C, the core of the postfusion trimer-of-hairpins structure is a bundle of six α-helices: three N-peptide helices form an interior, parallel coiled-coil trimer, while three C-peptide helices pack in an antiparallel manner into hydrophobic grooves on the coiled-coil surface (Chan et al., "Core Structure of gp41 from the HIV Envelope Glycoprotein," *Cell* 89:263–73 (1997); Weissenhorn et al., "Atomic Structure of the Ectodomain from HIV-1 gp41," *Nature* 387:426–30 (1997); Tan et al., "Atomic Structure of a Thermostable Subdomain of HIV-1 gp41," *Proc. Nat'l Acad. Sci. U.S.A.* 94:12303–8 (1997), which are hereby incorporated by reference in their entirety). The N-peptide region features a hydrophobic pocket targeted by C-peptide residues W628, W631, and I635, as shown in Figure 1B (Chan et al., "Evidence That a Prominent Cavity in the Coiled Coil of HIV Type 1 gp41 Is an Attractive Drug Target," *Proc. Nat'l Acad. Sci. U.S.A.* 95:15613–7 (1998), which is hereby incorporated by reference in its entirety). Agents that interfere with the formation of the gp41 coiled-coil hexamer are primary targets for vaccine and drug development (Deng et al., "Protein Design of a Bacterially Expressed HIV-1 gp41 Fusion Inhibitor," *Biochem.* 46:4360–9 (2007), which is hereby incorporated by reference in its entirety). Peptides and synthetic molecules that bind to the N-terminal hydrophobic pocket and inhibit the formation of the six-helix bundle have been shown to effectively inhibit gp41-mediated HIV fusion (Wild et al., "Peptides Corresponding to a Predictive α-Helical Domain of Human Immunodeficiency Virus Type 1 gp41 Are Potent Inhibitors of Virus Infection," *Proc. Nat'l Acad. Sci. U.S.A.* 91:9770–4 (1994); Ferrer et al., "Selection of gp41-mediated HIV-1 Cell Entry Inhibitors from Biased Combinatorial Libraries of Non-natural Binding Elements," *Nat. Struct. Biol.* 6:953–60 (1999); Sia et al., "Short Constrained Peptides That Inhibit HIV-1 Entry," *Proc. Nat'l Acad. Sci. U.S.A.* 99:14664–9 (2002); Ernst et al., "Design of a Protein Surface Antagonist Based on α-Helix Mimicry: Inhibition of gp41 Assembly and Viral Fusion," *Angew. Chem. Int'l Ed. Engl.* 41:278–81 (2002), *originally published at Angew. Chem.* 114:282–91 (2002); Frey et al., "Small Molecules That Bind the Inner Core of gp41 and Inhibit HIV Envelope-mediated Fusion," *Proc. Nat'l Acad. Sci. U.S.A.* 103:13938–43 (2006); Stephens et al., "Inhibiting HIV Fusion with a β-Peptide Foldamer," *J. Am. Chem. Soc.* 127:13126–7 (2005); Deng et al., "Protein Design of a Bacterially Expressed HIV-1 gp41 Fusion Inhibitor," *Biochem.* 46:4360–9 (2007), which are hereby incorporated by reference in their entirety).

[0007] However, not all patients are responsive to existing therapies, and the virus develops resistance to most, if not all, known agents. Thus, there is a need for new antiviral agents against HIV and other viruses that use class I fusion proteins.

SUMMARY OF THE INVENTION

[0008] A first aspect of the present invention relates to a peptide having one or more stable, internally-constrained hydrogen bond surrogate ("HBS") α-helices, where the peptide mimics at least a portion of a C-peptide helix or at least a portion of an N-peptide helix of a viral coiled-coil assembly. In one embodiment, the invention provides a peptide having a stable, internally-constrained alpha-helix, wherein said alpha helix is constrained by a crosslink formed by a carbon-carbon bond-forming reaction, and further wherein the peptide mimics at least a portion of a class I C-peptide helix or at least a portion of a class I N-peptide helix, and wherein the peptide is an inhibitor of viral infectivity. The carbon-carbon bond-forming reaction may be, for example, metathesis. In other embodiments, the class I C-peptide helix or the class I N-peptide helix is derived from a virus selected from the group of *Orthomyxoviridae*, *Paramyxoviridae*, *Filoviridae*, *Retroviridae*, *Coronaviridae*, *Herpesviridae*, influenza virus, Simian virus 5, respiratory syncitial virus, Ebola virus, Moloney murine leukemia virus, simian immunodeficiency virus, human immunodeficiency virus, human T cell leukemia virus, Mouse hepatitis virus, SARS virus, and human cytomegalovirus. For example, the virus is human immunodeficiency virus. Peptides of the invention may mimic at least a portion of a gp41 C-peptide helix or at least a portion of a gp41 N-peptide helix. In one embodiment, a peptide mimics at least a portion of a gp41 C-peptide helix, such as the WWI region of a gp41 C-peptide helix.

[0009] In another embodiment, a peptide of the invention or a peptide for use in a method of the invention comprises the formula:



wherein

====== is a single or double carbon-carbon bond;

~~~~~ is a single bond and is *cis* or *trans* when ====== is a double bond;

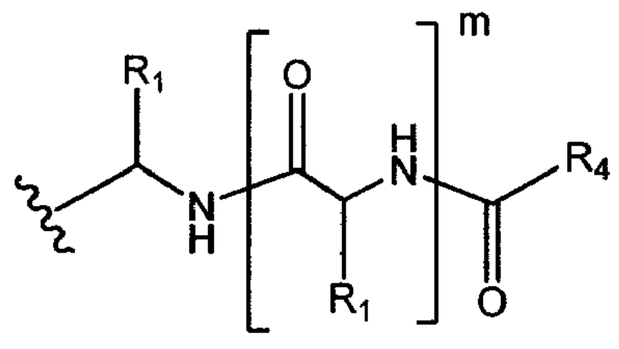
n is 1 or 2;

m is zero or any positive integer;

R is hydrogen, an amino acid side chain, an alkyl group, or an aryl group;

R_1 is an amino acid side chain, an alkyl group, or an aryl group;

R_2 is an amino acid, second peptide, –OR, $–CH_2NH_2$, an alkyl group, an aryl group, hydrogen, or a group having a formula
~~~~~



where R_4 is an amino acid, third peptide, –OR, $–NH_2$, an alkyl group, or an aryl group; and R_3 is a fourth peptide. R_3 may comprise, for example, the formula **–WXXWXXXIXXYXXXI**–R_4, where X is any amino acid.

[0010] A peptide may comprise the amino acid sequence of SEQ ID NO: 9, and may have an internally-constrained alpha-helix spanning residues 1 through 4 of SEQ ID NO: 9. In other embodiments, the gp41 C-peptide helix has an amino acid sequence of SEQ ID NO: 11. Alternatively, a peptide mimics at least a portion of a gp41 N-peptide helix, such as the hydrophobic pocket of a gp41 N-peptide helix. The gp41 N-peptide helix may have, for example, an amino acid sequence of SEQ ID NO: 12.

[0011] The invention also provides pharmaceutical compositions comprising a peptide of the invention and a pharmaceutically acceptable vehicle.

[0012] In another aspect, the invention provides a method of inhibiting infectivity of a virus in a subject, the method comprising administering to the subject an effective amount of a composition comprising a peptide having a stable, internally-constrained alpha-helix, wherein said alpha helix is constrained by a crosslink formed by a carbon-carbon bond-forming reaction, and further wherein the peptide mimics at least a portion of a class I C-peptide helix or at least a portion of a class I N-peptide helix. The carbon-carbon bond-forming reaction may be, for example, metathesis. In some embodiments, the virus is selected from the group of *Orthomyxoviridae*, *Paramyxoviridae*, *Filoviridae*, *Retroviridae*, *Coronaviridae*, *Herpesviridae*, influenza virus, Simian virus 5, respiratory syncitial virus, Ebola virus, Moloney murine leukemia virus, simian immunodeficiency virus, human immunodeficiency virus, human T cell leukemia virus, Mouse hepatitis virus, SARS virus, and human cytomegalovirus.

[0013] In yet another aspect, the invention provides a method of synthesizing a peptide which is an inhibitor of viral infectivity comprising: selecting a precursor peptide comprising at least a portion of a class I C-peptide helix or at least a portion of a class I N-peptide helix; and promoting formation of a carbon-carbon bond, wherein said bond formation results in a stable, internally-constrained alpha-helix. Such bond formation may, for example, introduce a non-native carbon-carbon bond. In one embodiment, the bond formation is effected by metathesis. In some embodiments, the virus is human immunodeficiency virus and the peptide mimics at least a portion of a gp41 C-peptide helix or at least a portion of a gp41 N-peptide helix. For example, the peptide may comprise the formula **WXXWXXXIXXYXXXI**–R_4, where X is any amino acid and R_4 is an amino acid, third peptide, –OR, $–NH_2$, an alkyl group, or an aryl group. For example, the peptide may comprise the amino acid sequence of SEQ ID NO: 9, and has an internally-constrained alpha-helix spanning residues 1 through 4 of SEQ ID NO: 9. In another embodiment,

the peptide comprises a gp41 C-peptide helix which has an amino acid sequence of SEQ ID NO: 11.

INCORPORATION BY REFERENCE

[0014] 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

[0015] 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:

[0016] Figures 1A–C are schematic diagrams. Figure 1A illustrates the HIV-1 gp41 core six-helix bundle. "N" denotes the N-peptide region; "C" denotes the C-peptide region. Figure 1B shows the interaction of C-peptide residues W628, W631, and I635 with the N-peptide (PDB code: 1AIK) (Chan et al., "Core Structure of gp41 from the HIV Envelope Glycoprotein," *Cell* 89:263–73 (1997), which is hereby incorporated by reference in its entirety). Figure 1C is a helical wheel diagram showing interactions between C- and N-peptide coiled-coil domains. The sequence spanning residues 624–642 (SEQ ID NO: 1) of the C-peptide region is also shown.

[0017] Figure 2 is a schematic diagram illustrating the HBS approach. HBS α-helices feature a carbon-carbon bond in place of an *i* and *i*+4 hydrogen bond. R = amino acid side chain.

[0018] Figure 3 is a schematic diagram of HBS α-helix **2**, which has an amino acid sequence of XMTWMEWDREINNYT (SEQ ID NO: 2, where X is a pentenoic acid residue).

[0019] Figure 4 is a schematic diagram of HBS α-helix **3**, which has an amino acid sequence of XWAAWDKKI (SEQ ID NO: 3, where X is a pentenoic acid residue).

[0020] Figure 5 is a schematic diagram of HBS α-helix **4**, which has an amino acid sequence of XAAAWEEWDKKI (SEQ ID NO: 4, where X is a pentenoic acid residue).

[0021] Figure 6 is a schematic diagram of HBS α-helix **5**, which has an amino acid sequence of XWAAWDREINNYT (SEQ ID NO: 5, where X is a pentenoic acid residue).

[0022] Figure 7 is a schematic diagram of HBS α-helix **6**, which has an amino acid sequence of XMTWEEWDKKIEEYT (SEQ ID NO: 6, where X is a pentenoic acid residue).

[0023] Figure 8 is a schematic diagram of HBS α-helix **7**, which has an amino acid sequence of XEMAWEEWDKKIEEYT (SEQ ID NO: 7, where X is a pentenoic acid residue).

[0024] Figure 9 is a schematic diagram of HBS α-helix **8**, which has an amino acid sequence of XNEMTWEEWDKKIEEYT (SEQ ID NO: 8, where X is a pentenoic acid residue).

[0025] Figure 10 is a schematic diagram of HBS α-helix **9**, which has an amino acid sequence of XMTWEEWDKKIEEYTKKI (SEQ ID NO: 9, where X is a pentenoic acid residue).

[0026] Figures 11A–L are analytical HPLC plots for peptides **1–12**. HPLC conditions: C_{18} reversed-phase column; 5% B to 15% B in 3 minutes, 15% B to 35% B in 20 minutes, 35% B to 100% B in 7 minutes; A: 0.1% aqueous TFA, B: acetonitrile; flow rate: 1.0 mL/min; monitored at 275 nm.

[0027] Figures 12A–J are CD spectra of peptides **1–10** in 10% TFE/PBS buffer.

[0028] Figure 13 is a schematic diagram of peptide **12** (*i.e.*, Suc-MTWMEWDERINNYTC^{Flu}-NH_2 (SEQ ID NO: 10)).

[0029] Figure 14 is a graph showing the saturation binding curve of fluorescein-labeled peptide **12** with **IZN17** in PBS buffer at 25 °C.

[0030] Figure 15 is a graph of the fraction of bound probe versus ligand concentration as a function of ligand binding affinity (see Example 3, *infra*).

[0031] Figures 16A–C are the circular dichroism spectra of **1**, **2**, **5**, and **9** in 10% TFE in PBS buffer (Figure 16A), a graph showing the determination of peptide binding to IZN17 by a fluorescence polarization assay (Figure 16B), and a graph of the inhibition of gp41-mediated cell-cell fusion by HBS α-helix **9** and peptide **10** (Figure 16C).

DETAILED DESCRIPTION OF THE INVENTION

[0032] The present invention relates to hydrogen bond surrogate ("HBS")-derived α-helices that inhibit the formation of viral class I coiled-coil assemblies. These HBS helices can potentially function as *in vivo* inhibitors of class I fusion protein–protein interactions involved in viral infectivity.

[0033] A first aspect of the present invention relates to a peptide having one or more stable, internally-constrained HBS α-helices, where the peptide mimics at least a portion of a class I C-peptide helix or at least a portion of a class I N-peptide helix of a viral coiled-coil assembly.

[0034] A peptide of this aspect of the present invention may mimic at least a portion of a class I C-peptide helix or a class I N-peptide helix of a virus selected from the group of *Orthomyxoviridae*, *Paramyxoviridae*, *Filoviridae*, *Retroviridae*, *Coronaviridae*, *Herpesviridae*, influenza virus, Simian virus 5, respiratory syncitial virus, Ebola virus, Moloney murine leukemia virus, simian immunodeficiency virus, HIV-1, human T cell leukemia virus, Mouse hepatitis virus, SARS virus, and human cytomegalovirus.

[0035] In a preferred embodiment, the peptide according to this aspect of the present invention mimics at least a portion of an HIV-1 gp41 C-peptide helix or at least a portion of an HIV-1 gp41 N-peptide helix. These peptides are expected to interfere with the formation of the gp41 coiled-coil assembly, which is known to mediate HIV-1 entry into host cells.

[0036] Suitable peptides that mimic a portion of a gp41 C-peptide helix include peptides that mimic the WWI region of a gp41 C-peptide helix. This region interacts with a hydrophobic pocket on a corresponding N-peptide helix during formation of the coiled-coil hexamer. Thus, artificial α-helical mimics of this region are expected to competitively interfere with the native gp41 C-peptide helices for binding with this hydrophobic region. Similarly, suitable peptides that mimic a portion of a gp41 N-peptide helix include those that mimic a hydrophobic pocket of a gp41 N-

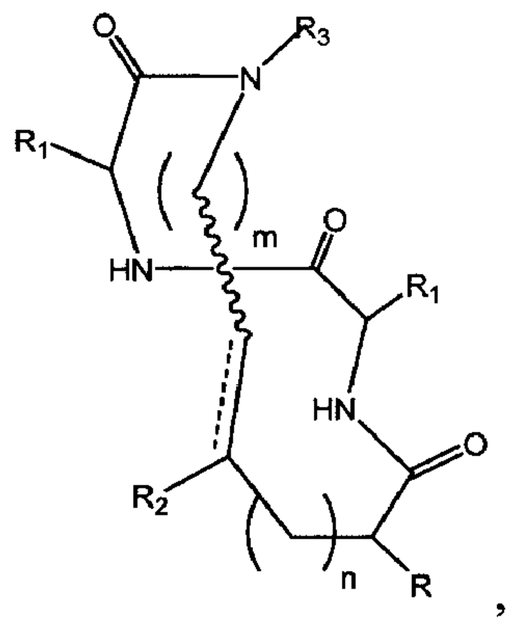
peptide helix. These artificial α-helices are expected to competitively interfere with gp41 C-peptide helices for binding with the native N-peptide helices.

[0037] By way of example, the artificial α-helices of the present invention can mimic at least a portion of the gp41 N-peptide and C-peptide helices shown in Table 1.

Table 1. Exemplary gp41 Helices.

Domain Name	Sequence
gp41 C-peptide	HTTWMEWDREINNYTSLIHSLIEESQNQQEKNEQELLE (SEQ ID NO: 11)
gp41 N-peptide	QLLSGIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQARILA (SEQ ID NO: 12)

[0001] Generally, suitable peptides of the present invention include those that include the formula:



where

====== is a single or double carbon-carbon bond;

~~~~~ is a single bond and is *cis* or *trans* when ====== is a double bond;

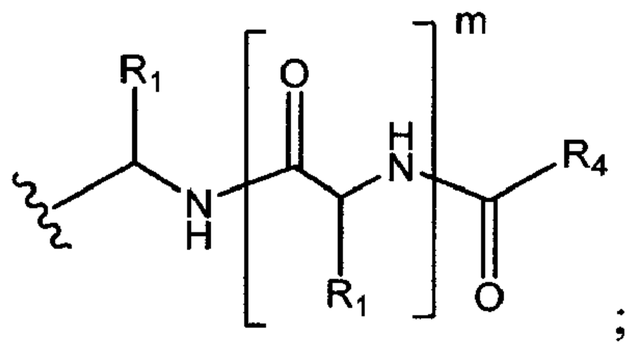
n is 1 or 2;

m is zero or any positive integer;

R is hydrogen, an amino acid side chain, an alkyl group, or an aryl group;

R_1 is an amino acid side chain, an alkyl group, or an aryl group;

R_2 is an amino acid, peptide, –OR, $–CH_2NH_2$, an alkyl group, an aryl group, hydrogen, or a group having a formula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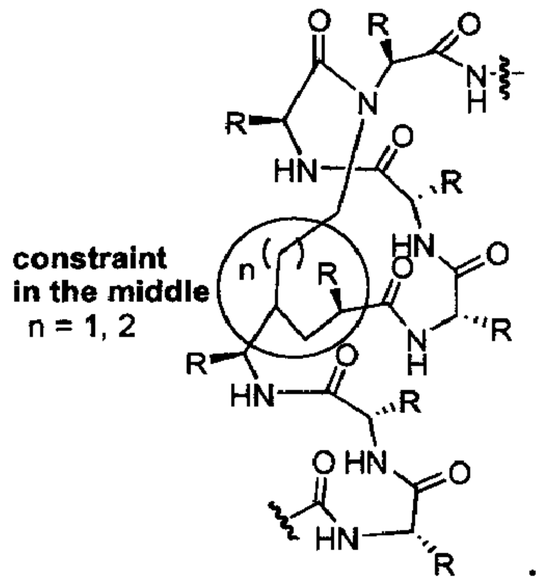
where R_4 is an amino acid, peptide, –OR, –NH_2, an alkyl group, or an aryl group and R3 is a fourth peptide.

[0038] In some embodiments, R_3 is a peptide comprising the formula –**WXXWXXXIXXYXXXI** (SEQ ID NO: 13)–R_4, where X is any amino acid. In another embodiment, a peptide of the present invention includes the amino acid sequence of SEQ ID NO: 9, and has an internally-constrained α-helical region spanning residues 1 through 4 of SEQ ID NO: 9.

[0039] As will be apparent to one of ordinary skill in the art, the methods of the present invention may be used to prepare peptides having highly stabilized, internally-constrained α-helices. The constraint may be placed anywhere within the peptide, not just at the N-terminus. For example, a compound prepared according to the methods of the present invention may have the formula



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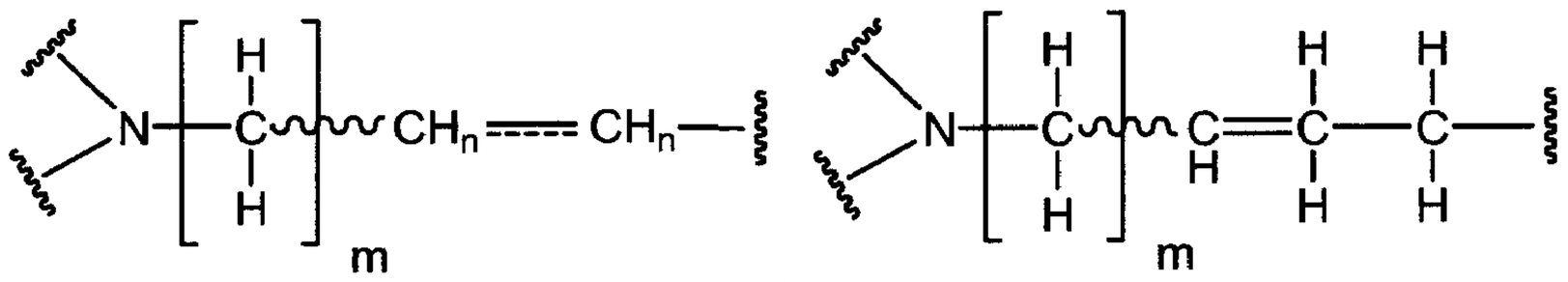
[0040] The peptides produced according to the methods of the present invention may, for example, be less than 15 amino acids, including, for example, less than 10 amino acid residues.

[0041] The present invention also relates to peptides having one or more stable, internally-constrained α-helices. The one or more stable, internally-constrained secondary structures includes the following motifs:



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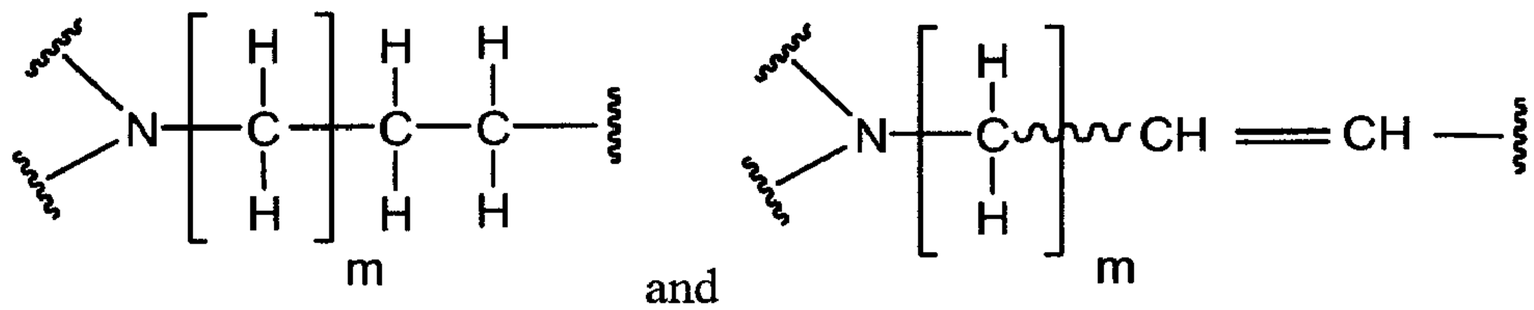
where ═══ is a single or double bond, ∿∿∿ is a single bond and is cis or trans when ═══ is a double bond; n is 1 or 2; and m is any number. Examples of such motifs include:

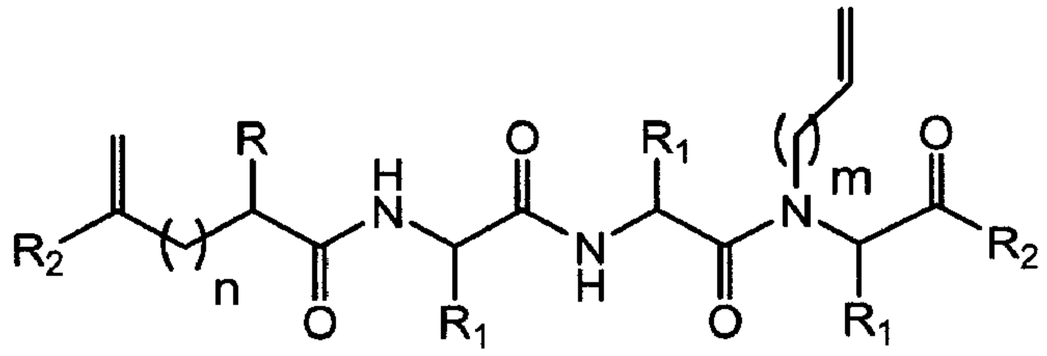


[0042] HBS α-helices of the present invention are obtained by replacing an *N*-terminal main-chain *i* and *i*+4 hydrogen bond with a carbon-carbon bond through a ring-closing metathesis reaction, as shown in Figure 2 (U.S. Patent No. 7,202,332 to Arora et al.; Chapman & Arora, "Optimized

Synthesis of Hydrogen-bond Surrogate Helices: Surprising Effects of Microwave Heating on the Activity of Grubbs Catalysts," *Org. Lett.* 8:5825–8 (2006); Chapman et al., "A Highly Stable Short α-Helix Constrained by a Main-chain Hydrogen-bond Surrogate," *J. Am. Chem. Soc.* 126:12252–3 (2004); Dimartino et al., "Solid-phase Synthesis of Hydrogen-bond Surrogate-derived α-Helices," *Org. Lett.* 7:2389–92 (2005), which are hereby incorporated by reference in their entirety). The hydrogen bond surrogate pre-organizes an α-turn and stabilizes the peptide sequence in an α-helical conformation. HBS α-helices have been shown to adopt stable α-helical conformations from a variety of short peptide sequences (Wang et al., "Evaluation of Biologically Relevant Short α-Helices Stabilized by a Main-chain Hydrogen-bond Surrogate," *J. Am. Chem. Soc.* 128:9248–56 (2006), which is hereby incorporated by reference in its entirety). It has also been shown that these artificial α-helices can target their expected protein receptor with high affinity (Wang et al., "Enhanced Metabolic Stability and Protein-binding Properties of Artificial α Helices Derived from a Hydrogen-bond Surrogate: Application to Bcl-xL," *Angew. Chem. Int'l Ed. Engl.* 44:6525–9 (2005), *originally published at Angew. Chem.* 117:6683–7 (2005), which is hereby incorporated by reference in its entirety).

[0043] In another aspect, preparing a compound of the invention involves providing a peptide precursor compound and promoting carbon-carbon bond formation to result in a stable, internally-constrained alpha-helix.

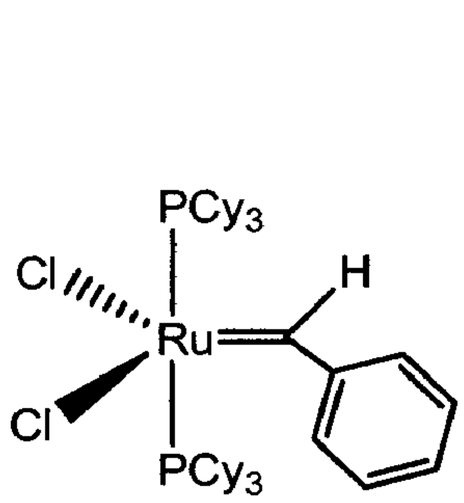
[0044] In one embodiment, the precursor has the formula:



[0045] The compound of the formula above may be reacted under conditions effective to promote formation of a carbon-carbon bond. Such a reaction may be, for example, metathesis. The exceptional functional group tolerance displayed by the olefin metathesis catalysts for the facile introduction of non-native carbon-carbon constraints in the preparation of peptidomimetics suggests that X and Y could be two carbon atoms connected through an olefin metathesis reaction, as shown in Scheme 2 (Hoveyda et al., "Ru Complexes Bearing Bidentate Carbenes: From Innocent Curiosity to Uniquely Effective Catalysts for Olefin Metathesis," *Org. Biomolec. Chem.* 2:8-23 (2004); Trnka et al., "The Development of L2X2Tu = CHR Olefin Metathesis Catalysts: An Organometallic Success Story," *Accounts Chem. Res.* 34:18-29 (2001), which are hereby incorporated by reference in their entirety).

[0046] This aspect of the present invention may, for example, involve a ring-closing olefin metathesis reaction. An olefin metathesis reaction couples two double bonds (olefins) to afford two new double bonds (one of which is typically ethylene gas). A ring-closing olefin metathesis utilizes an olefin metathesis reaction to form a macrocycle. In this reaction, two double bonds within a

chain are connected. The reaction may be performed with a metathesis catalyst, for example of the formula



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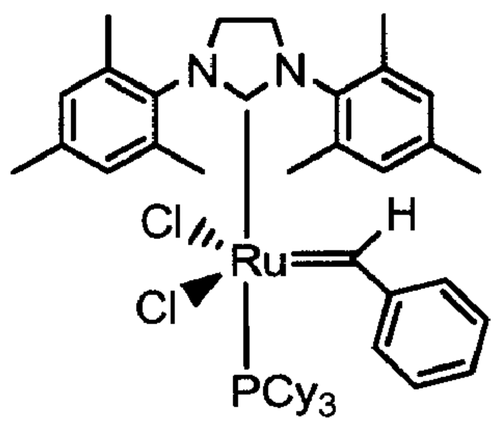
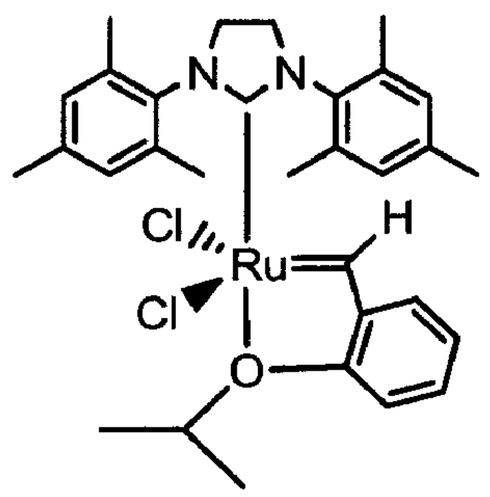


, or



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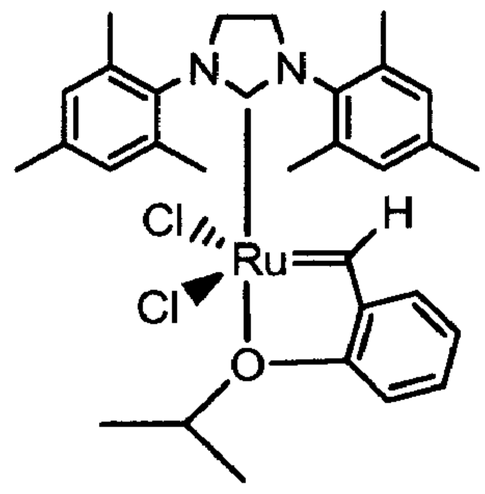
[0047] In other embodiments, the metathesis catalyst is of the formula



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[0048] The metathesis reaction may be performed, for example, at a temperature between about 25°C and 110°C, and more preferably, at a temperature of about 50°C.

[0049] The metathesis reaction may be performed with an organic solvent, such as dichloromethane, dichloroethane, trichloroethane, or toluene.

[0050] The reactions disclosed herein may, for example, be carried out on a solid support. Suitable solid supports include particles, strands, precipitates, gels, sheets, tubing, spheres, containers, capillaries, pads, slices, films, plates, slides, discs, membranes, etc. These solid supports can be made from a wide variety of materials, including polymers, plastics, ceramics, polysaccharides, silica or silica-based materials, carbon, metals, inorganic glasses, membranes, or composites thereof. The substrate is preferably flat but may take on a variety of alternative surface configurations. For example, the substrate may contain raised or depressed regions on which the synthesis takes place. The substrate and its surface preferably form a rigid support on which to carry out the reactions described herein. Other substrate materials will be readily apparent to those of ordinary skill in the art upon review of this disclosure.

[0051] The metathesis reaction performed may initially yield a compound in which the newly formed carbon-carbon bond is a double bond. This double bond can be subsequently converted to a single bond by hydrogenation methods known in the art.

[0052] A second aspect of the present invention relates to a method of inhibiting viral infectivity of a subject. This method involves administering to the subject an effective amount of a composition that includes a peptide of the present invention that mimics at least a portion of a class I N- or C-peptide helix. In a preferred embodiment, the method of this aspect of the present invention

inhibits HIV infectivity of a subject. In this embodiment, the composition includes a peptide of the present invention that mimics a gp41 N-peptide helix or gp41 C-peptide helix.

[0053] Inhibiting infectivity according to this aspect of the present invention refers to any decrease in the rate and/or degree of transmission of the virus. This includes, without limitation, inhibiting transmission of the virus from one individual to another, as well as inhibiting further transmission of the virus within an infected individual.

[0054] The composition may be administered to individuals already infected with the virus (to inhibit transmission of the virus to others, and/or to inhibit further transmission of the virus within the infected individual), as well as to individuals not already infected with the virus (to inhibit transmission of the virus to the individual).

[0055] As will be apparent to one of ordinary skill in the art, administering may be carried out using generally known methods.

[0056] Administration can be accomplished either via systemic administration to the subject or via targeted administration to affected cells. Exemplary routes of administration include, without limitation, by intratracheal inoculation, aspiration, airway instillation, aerosolization, nebulization, intranasal instillation, oral or nasogastric instillation, intraperitoneal injection, intravascular injection, topically, transdermally, parenterally, subcutaneously, intravenous injection, intra-arterial injection (such as via the pulmonary artery), intramuscular injection, intrapleural instillation, intraventricularly, intralesionally, by application to mucous membranes (such as that of the nose, throat, bronchial tubes, genitals, and/or anus), or implantation of a sustained release vehicle.

[0057] Typically, the peptide of the present invention will be administered to a mammal as a pharmaceutical formulation that includes the therapeutic agent and any pharmaceutically acceptable adjuvants, carriers, excipients, and/or stabilizers, and can be in solid or liquid form, such as tablets, capsules, powders, solutions, suspensions, or emulsions. The compositions preferably contain from about 0.01 to about 99 weight percent, more preferably from about 2 to about 60 weight percent, of therapeutic agent together with the adjuvants, carriers and/or excipients. The amount of active compound in such therapeutically useful compositions is such that a suitable dosage unit will be obtained.

[0058] The agents may be orally administered, for example, with an inert diluent, or with an assimilable edible carrier, or they may be enclosed in hard or soft shell capsules, or they may be compressed into tablets, or they may be incorporated directly with the food of the diet. For oral therapeutic administration, these active compounds may be incorporated with excipients and used in the form of tablets, capsules, elixirs, suspensions, syrups, and the like. Such compositions and preparations should contain at least 0.1% of the agent. The percentage of the agent in these compositions may, of course, be varied and may conveniently be between about 2% to about 60% of the weight of the unit. The amount of the agent in such therapeutically useful compositions is such that a suitable dosage will be obtained.

[0059] The tablets, capsules, and the like may also contain a binder such as gum tragacanth, acacia, corn starch, or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, or alginic acid; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose, or saccharin. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier, such as a fatty oil.

[0060] Various other materials may be present as coatings or to modify the physical form of the dosage unit. For instance, tablets may be coated with shellac, sugar, or both. A syrup may contain, in addition to active ingredient(s), sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye, and flavoring such as cherry or orange flavor.

[0061] The agents may also be administered parenterally. Solutions or suspensions of the agent can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof in oils. Illustrative oils are those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, or mineral oil. In general, water, saline, aqueous dextrose and related sugar solutions, and glycols such as propylene glycol or polyethylene glycol, are preferred liquid carriers, particularly for injectable solutions. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

[0062] The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol), suitable mixtures thereof, and vegetable oils.

[0063] The agents according to this aspect of the present invention may also be administered directly to the airways in the form of an aerosol. For use as aerosols, the compounds of the present invention in solution or suspension may be packaged in a pressurized aerosol container together with suitable propellants, for example, hydrocarbon propellants like propane, butane, or isobutane with conventional adjuvants. The materials of the present invention also may be administered in a non-pressurized form such as in a nebulizer or atomizer.

[0064] The agents of the present invention may be administered directly to a targeted tissue, *e.g.*, tissue that is susceptible to infection by the virus. Additionally and/or alternatively, the agent may be administered to a non-targeted area along with one or more agents that facilitate migration of the agent to (and/or uptake by) a targeted tissue, organ, or cell. While the targeted tissue can be any tissue subject to infection by the virus, preferred target tissues in the case of inhibiting HIV-1 infection include mucous membranes of the mouth, genitals, and rectum. As will be apparent to

one of ordinary skill in the art, the therapeutic agent itself be modified to facilitate its transport to (and uptake by) the desired tissue, organ, or cell.

[0065] Exemplary delivery devices include, without limitation, nebulizers, atomizers, liposomes, transdermal patches, implants, implantable or injectable protein depot compositions, and syringes. Other delivery systems which are known to those of skill in the art can also be employed to achieve the desired delivery of the therapeutic agent to the desired organ, tissue, or cells *in vivo* to effect this aspect of the present invention.

[0066] Any suitable approach for delivery of the agents can be utilized to practice this aspect of the present invention. Typically, the agent will be administered to a patient in a vehicle that delivers the agent(s) to the target cell, tissue, or organ.

[0067] One approach for delivering agents into cells involves the use of liposomes. Basically, this involves providing a liposome which includes agent(s) to be delivered, and then contacting the target cell, tissue, or organ with the liposomes under conditions effective for delivery of the agent into the cell, tissue, or organ.

[0068] Liposomes are vesicles comprised of one or more concentrically ordered lipid bilayers which encapsulate an aqueous phase. They are normally not leaky, but can become leaky if a hole or pore occurs in the membrane, if the membrane is dissolved or degrades, or if the membrane temperature is increased to the phase transition temperature. Current methods of drug delivery via liposomes require that the liposome carrier ultimately become permeable and release the encapsulated drug at the target site. This can be accomplished, for example, in a passive manner where the liposome bilayer degrades over time through the action of various agents in the body. Every liposome composition will have a characteristic half-life in the circulation or at other sites in the body and, thus, by controlling the half-life of the liposome composition, the rate at which the bilayer degrades can be somewhat regulated.

[0069] In contrast to passive drug release, active drug release involves using an agent to induce a permeability change in the liposome vesicle. Liposome membranes can be constructed so that they become destabilized when the environment becomes acidic near the liposome membrane (*see, e.g.*, Wang & Huang, "pH-Sensitive Immunoliposomes Mediate Target-cell-specific Delivery and Controlled Expression of a Foreign Gene in Mouse," *Proc. Nat'l Acad. Sci. USA* 84:7851–5 (1987), which is hereby incorporated by reference in its entirety). When liposomes are endocytosed by a target cell, for example, they can be routed to acidic endosomes which will destabilize the liposome and result in drug release.

[0070] Alternatively, the liposome membrane can be chemically modified such that an enzyme is placed as a coating on the membrane, which enzyme slowly destabilizes the liposome. Since control of drug release depends on the concentration of enzyme initially placed in the membrane, there is no real effective way to modulate or alter drug release to achieve "on demand" drug delivery. The same problem exists for pH-sensitive liposomes in that as soon as the liposome vesicle comes into contact with a target cell, it will be engulfed and a drop in pH will lead to drug release.

[0071] This liposome delivery system can also be made to accumulate at a target organ, tissue, or cell via active targeting (*e.g.*, by incorporating an antibody or hormone on the surface of the liposomal vehicle). This can be achieved according to known methods.

[0072] Different types of liposomes can be prepared according to Bangham et al., "Diffusion of Univalent Ions Across the Lamellae of Swollen Phospholipids," *J. Mol. Biol.* 13:238–52 (1965); U.S. Patent No. 5,653,996 to Hsu; U.S. Patent No. 5,643,599 to Lee et al.; U.S. Patent No. 5,885,613 to Holland et al.; U.S. Patent No. 5,631,237 to Dzau & Kaneda; and U.S. Patent No. 5,059,421 to Loughrey et al., each of which is hereby incorporated by reference in its entirety.

[0073] These liposomes can be produced such that they contain, in addition to the therapeutic agents of the present invention, other therapeutic agents, such as anti-inflammatory agents, which would then be released at the target site (*e.g.*, Wolff et al., "The Use of Monoclonal Anti-Thy1 IgG1 for the Targeting of Liposomes to AKR-A Cells *in Vitro* and *in Vivo*," *Biochim. Biophys. Acta* 802:259–73 (1984), which is hereby incorporated by reference in its entirety).

[0074] An alternative approach for delivery of proteins or polypeptide agents (*e.g.*, peptides of the present invention) involves the conjugation of the desired protein or polypeptide to a polymer that is stabilized to avoid enzymatic degradation of the conjugated protein or polypeptide. Conjugated proteins or polypeptides of this type are described in U.S. Patent No. 5,681,811 to Ekwuribe, which is hereby incorporated by reference in its entirety.

[0075] Yet another approach for delivery of proteins or polypeptide agents involves preparation of chimeric proteins according to U.S. Patent No. 5,817,789 to Heartlein et al., which is hereby incorporated by reference in its entirety. The chimeric protein can include a ligand domain and the polypeptide agent (*e.g.*, the artificial α-helix of the present invention). The ligand domain is specific for receptors located on a target cell. Thus, when the chimeric protein is delivered intravenously or otherwise introduced into blood or lymph, the chimeric protein will adsorb to the targeted cell, and the targeted cell will internalize the chimeric protein.

[0076] Administration can be carried out as frequently as required and for a duration that is suitable to provide effective treatment against viral infection. For example, administration can be carried out with a single sustained-release dosage formulation or with multiple daily doses. Administration can be carried out before, concurrently with, and/or after exposure of the subject to the virus.

[0077] The amount to be administered will, of course, vary depending upon the treatment regimen. Generally, an agent is administered to achieve an amount effective for a reduction in infectivity of the virus (*i.e.*, a therapeutically effective amount). Thus, a therapeutically effective amount can be an amount which is capable of at least partially preventing transmission of the virus to the subject, or spread of the virus within the subject. The dose required to obtain an effective amount may vary depending on the agent, formulation, virus, and individual to whom the agent is administered.

[0078] Determination of effective amounts may also involve *in vitro* assays in which varying doses of agent are administered to cells in culture and the concentration of agent effective for inhibiting

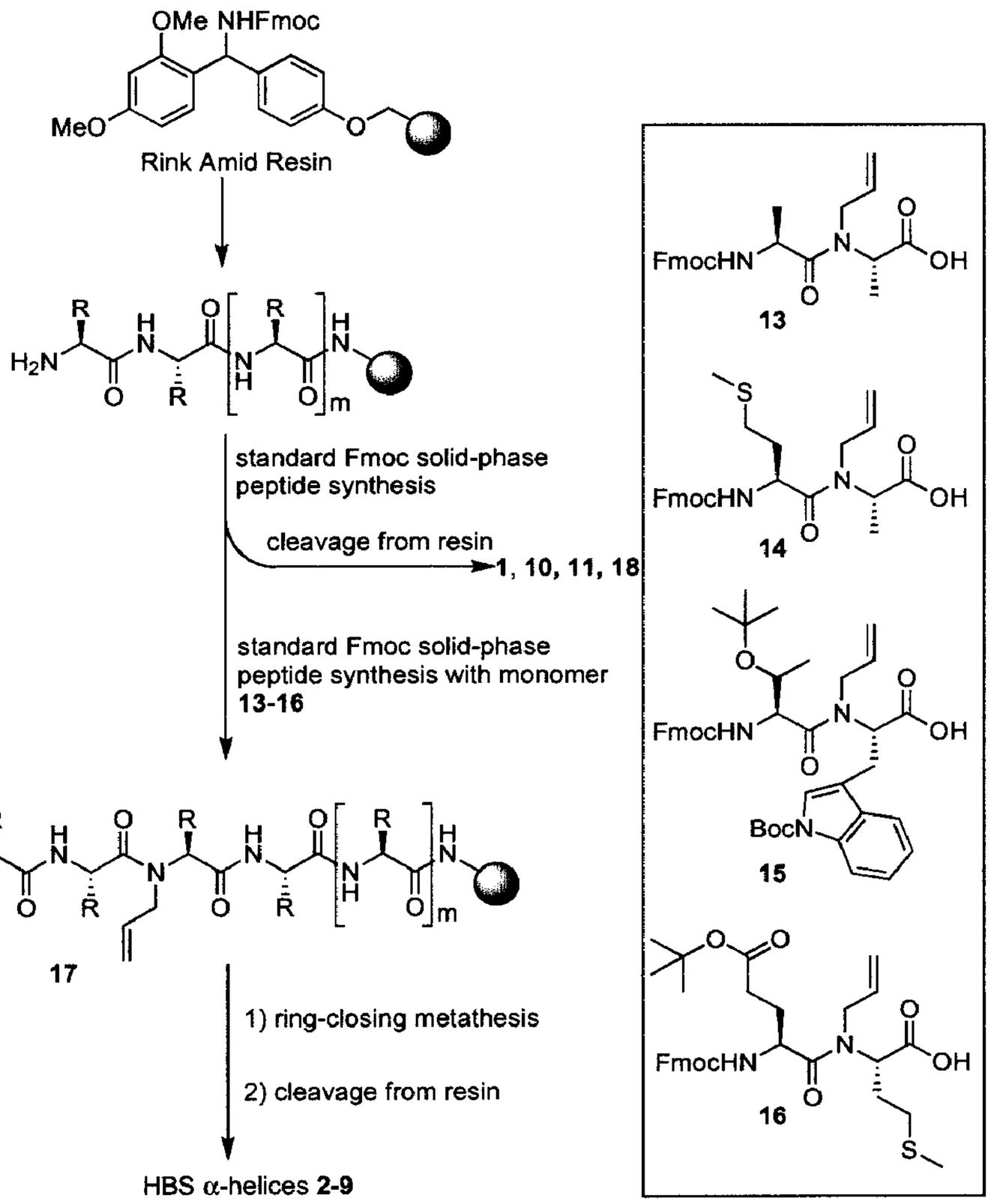
infectivity is determined in order to calculate the concentration required *in vivo*. Effective amounts may also be based on *in vivo* animal studies. A therapeutically effective amount can be determined empirically by those of skill in the art.

EXAMPLES

Example 1 — Synthesis of Peptides 1–11 and Characterization of Peptides 1–12.

[0079] Peptides **1–11** were synthesized as shown in Scheme 1. Peptides **1** (*i.e.*, AcMTWMEWDREINNYT-NH_2 (SEQ ID NO: 14)), **10** (*i.e.*, AcMTWEEWDKKIEEYTKKI-NH_2 (SEQ ID NO: 15)), and **11** (*i.e.*, peptide IZN17, AcIKKEIEAIKKEQEAIKKKIEAIEKLLQLTVWGIKQLQARIL-NH_2 (SEQ ID NO: 16)), and resin-bound bis-olefins **(17)** were synthesized by conventional Fmoc solid phase chemistry on Rink amide HMBA resin (NovaBiochem), 0.05–0.15 mmol scale, with appropriate substitutions of *N*(allyl)-dipeptides **13–16** and 4-pentenoic acid. In each coupling step, the Fmoc group was removed by treatment with 20% piperidine in NMP (2×20 min). The next Fmoc amino acid (4 equiv.) in the sequence was activated with HBTU (3.6 equiv.) in a 5% DIPEA/NMP solution for 15 minutes, added to the resin bearing the free amine. The resulting mixture was shaken for 60 minutes. The coupling efficiency for each step was monitored by ninhydrin test. After the peptide was assembled on the resin, the resin was thoroughly washed with DMF, methanol, and dichloromethane, respectively, and dried under vacuum overnight.

Scheme 1. Synthesis of Peptides **1–10**



[0080] Microwave-assisted ring-closing metathesis reactions on resin-bound bis-olefins (**17**) were performed with the Hoveyda-Grubbs catalyst (0.15 equiv.) in dichloroethane as described in Chapman & Arora, "Optimized Synthesis of Hydrogen-bond Surrogate Helices: Surprising Effects of Microwave Heating on the Activity of Grubbs Catalysts," *Org. Lett.* 8:5825–8 (2006), which is hereby incorporated by reference in its entirety. The reaction mixture was irradiated with these settings: 250 W maximum power, 120 °C, 5 minute ramp time, and 10 minute hold time. Resin bound peptides were cleaved from the resin by treatment with a cleavage cocktail (CF_3CO_2H:H_2O:triisopropylsilane, 95:2.5:2.5) for 1.5 hours, and purified by reversed-phase HPLC to afford HBS α-helices **2–9**, which are shown in Figures 3–10. HPLC plots for peptides **1–12** are shown in Figures 11A–L.

[0081] Peptides **1–12** were examined using liquid chromatography-mass spectrometry ("LCMS"). LCMS data were obtained on an Agilent 1100 series. The LCMS results are shown in Table 2.

Table 2. Mass spectrometry results for Peptides 1–12 (LC/MSD (XCT) electrospray trap).

peptide	Expected $[M+H]^+$	Found $[M+H]^+$
1	1929.8	1929.6
2	2068.0	2068.6
3	1110.6	1111.1
4	1439.7	1440.5

5	1631.8	1632.2
6	1981.0	1981.8
7	2080.0	2080.0
8	2224.0	2225.0
9	2350.2	2351.2
10	2298.2	2299.0
11	2478.0	2478.6
12	4855.9	4854.7

Example 2 - Circular Dichroism Spectroscopy of Peptides 1–10.

[0082] CD spectra were recorded on an AVIV 202SF CD spectrometer equipped with a temperature controller using 1 mm length cells and a scan speed of 5 nm/min. The spectra were averaged over 10 scans with the baseline subtracted from analogous conditions as that for the samples. The samples were prepared in 0.1x phosphate buffered saline (13.7 mM NaCl, 1 mM phosphate, 0.27 mM KCl, pH 7.4), containing 10% trifluoroethanol, with the final peptide concentration of 50–100 μM. The concentrations of unfolded peptides were determined by the UV absorption of the tyrosine residue at 276 nm in 6.0 M guanidinium hydrochloride aqueous solution. The helix content of each peptide was determined from the mean residue CD at 222 nm, $[\theta]_{222}$ (deg cm^2 $dmol^{-1}$) corrected for the number of amino acids. Percent helicity was calculated from the ratio $[\theta]_{222}/[\theta]_{max}$, where $[\theta]_{max} = (-44000 + 250T)(1 - k/n)$, with $k = 4.0$ and n = number of residues. For details on θ_{max} calculations for HBS helices, see Wang et al., "Evaluation of Biologically Relevant Short α-Helices Stabilized by a Main-chain Hydrogen-bond Surrogate," *J. Am. Chem. Soc.* 128:9248–56 (2006), which is hereby incorporated by reference in its entirety. The CD spectra for peptides **1–10** are shown in Figures 12A–J.

Example 3 — Affinity of Peptide 12 for IZN17.

[0083] The relative affinity of peptides **1–10** for **IZN17** (*i.e.*, peptide **11**) was determined using a fluorescence polarization-based competitive binding assay (Eckert & Kim, "Design of Potent Inhibitors of HIV-1 Entry from the gp41 N-Peptide Region," *Proc. Nat'l Acad. Sci. U.S.A.* 98:11187–92 (2001); Stephens et al., "Inhibiting HIV Fusion with a β-Peptide Foldamer," *J. Am. Chem. Soc.* 127:13126–7 (2005), which are hereby incorporated by reference in their entirety) with fluorescein-labeled peptide **12**, shown in Figure 13. Figure 14 is a graph showing the saturation binding curve of fluorescein-labeled peptide **12** with **IZN17** in PBS buffer at 25 °C.

[0084] All samples were prepared in 96-well plates in 1x phosphate buffered saline (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4) with 0.1% pluronic F-68 (Sigma). A solution of 25 μM **IZN17** and 15 nM fluorescein-labeled peptide **12** was incubated at 25 °C. After 1 hour, appropriate concentrations (10 nM to 500 μM) of the antagonists (peptides **1–10**) were added. The total volume of the incubation solution was 60 μL. After 1 hour, the amount of dissociated fluorescent probe **12** was determined using a DTX 880 Multimode Detector (Beckman) at 25 °C, with excitation and emission wavelengths of 485 and 525 nm, respectively.

[0085] The binding affinity (K_D) values reported in Table 3 (see Example 6) for each peptide are the averages of 3~5 individual measurements, and were determined by fitting the experimental data to a sigmoidal dose-response nonlinear regression model on GraphPad Prism 4.0.

[0086] It is noted that this competitive binding assay does not allow accurate estimates of K_d values much lower than the binding values of the fluorescent probe. Figure 15 shows a graph of the fraction of bound probe versus ligand concentration as a function of ligand binding affinity. The narrowing of the space between curves below K_d of 5 μM illustrates the limits of this assay.

Example 4 — Cell–Cell Fusion Inhibition Assay.

[0087] Cell–cell fusion (i.e., syncytium formation) was assayed by coculturing CHO[HIVe] (clone 7d2) cells expressing HXB2 envelope and tat with U373-MAGI cells (M. Emerman and A. Geballe, National Institutes of Health AIDS Research and Reference Reagent Program) in the presence of different concentrations of peptides **1–10**. Cell fusion allows the expression of nuclear β-galactosidase from the U373-MAGI indicator cell line and can be quantitated by monitoring β-galactosidase activity. After an overnight incubation at 37°C after coculture, β-galactosidase enzymatic activity was measured with the Mammalian β-galactosidase Chemiluminescent Assay Kit (Gal-Screen from Applied Biosystems). The peptide inhibitor concentrations at which activities were reduced by 50% (IC_{50}) relative to control samples lacking peptide inhibitor were calculated by fitting data to the variable-slope-sigmoid equation using the Prism program.

Example 5 — Cytotoxity of Peptides on U373-MAGI.

[0088] The cytotoxic effect of peptides **1–10** on U373-MAGI cells was measured in the presence of a series of diluted inhibitors for 6 days, and cell viability was quantitated with an MTT assay (Mosmann, "Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays," *J. Immunol. Meth.* 65:55–63 (1983), which is hereby incorporated by reference in its entirety). No cytotoxicity was observed for peptides up to 200 μM concentrations.

Example 6 — Peptide Design.

[0089] The present gp41-targeting studies were begun by mimicking the 14-residue C-peptide (**1**) derived from gp41 that contains residues W628, W631, and I635, as shown in Table 3. This sequence would be expected to bind the gp41 hydrophobic pocket based on its crystal structure (shown in Figure 1B) (Chan et al., "Core Structure of gp41 from the HIV Envelope Glycoprotein," *Cell* 89:263–73 (1997), which is hereby incorporated by reference in its entirety), but has been previously shown to be ineffective in cell-cell fusion assays (Sia et al., "Short Constrained Peptides That Inhibit HIV-1 Entry," *Proc. Nat'l Acad. Sci. U.S.A.* 99:14664–9 (2002), which is hereby incorporated by reference in its entirety).

Table 3. Binding affinities and cell fusion inhibition properties of peptides and HBS α-helices.

Compound	Sequence[a]		K_d (μM)[b]	EC_{50} (μM)[c]
C-peptide	NNMT**W**ME**W**DREINNYTSLI	(SEQ ID NO: 1)	----	----
1	AcMT**W**ME**W**DREINNYT	(SEQ ID NO: 14)	37.4±14.8[d]	----
2	XMT**W**ME**W**DREINNYT	(SEQ ID NO: 2)	46.6±14.6[d]	>>200
3	X**W**AA**W**DKKI	(SEQ ID NO: 3)	>500	>>200
4	XAAA**W**EE**W**DKKI	(SEQ ID NO: 4)	>500	>>200
5	X**W**AA**W**DREINNYT	(SEQ ID NO: 5)	>500	>>200
6	XMT**W**EE**W**DKKIEEYT	(SEQ ID NO: 6)	7.50±1.70	>>200
7	XEMA**W**EE**W**DKKIEEYT	(SEQ ID NO: 7)	146±47.3	>>200
8	XNEMT**W**EE**W**DKKIEEYT	(SEQ ID NO: 8)	<5.00[e]	>>200
9	XMT**W**EE**W**DKKIEEYTKKI	(SEQ ID NO: 9)	<5.00[e]	42.7±7.50
10	AcMT**W**EE**W**DKKIEEYTKKI	(SEQ ID NO: 15)	<5.00[e]	>>200

[a] X denotes pentenoic acid residue. Residues that occupy *a* or *d* positions in the heptad are shown in bold.
[b] Binding affinity for IZN17 as calculated from a fluorescence polarization assay (Example 3) (Eckert & Kim, "Design of Potent Inhibitors of HIV-1 Entry from the gp41 N-Peptide Region," *Proc. Nat'l Acad. Sci. U.S.A.* 98:11187–92 (2001), which is hereby incorporated by reference in its entirety).
[c] Inhibitory activity of peptides in a gp41-mediated cell-cell fusion assay monitoring syncytia formation (Eckert et al., "Inhibiting HIV-1 Entry: Discovery of D-Peptide Inhibitors That Target the gp41 Coiled-coil Pocket," *Cell* 99:103–15 (1999), which is hereby incorporated by reference in its entirety).
[d] Precise measurements of the K_d value not possible because the peptide aggregates at high concentrations.
[e] The competitive binding assay (Example 3) does not allow accurate estimates of K_d values much lower than the binding values of the fluorescent probe.

[0090] A previously described fluorescence polarization assay was utilized to determine the *in vitro* binding affinity of unconstrained peptides and HBS helices for a stable model of the gp41 N-terminal three strand coiled-coil, IZN17, which contains the binding site for residues W628, W631, and I635 (see Example 3, *supra*) (Eckert & Kim, "Design of Potent Inhibitors of HIV-1 Entry from the gp41 N-Peptide Region," *Proc. Nat'l Acad. Sci. U.S.A.* 98:11187–92 (2001), which is hereby incorporated by reference in its entirety). A fluorescein-labeled C-peptide derivative (*i.e.*, suc-MTWMEWDREINNYTC^{Flu} (SEQ ID NO: 10); peptide **12**) containing residues 628–641 of gp41 was used as a probe. The competitive displacement of this probe by HBS helices afforded the K_d value for each peptide shown in Table 3 and Figure 16B (Stephens et al., "Inhibiting HIV Fusion with a β-Peptide Foldamer," *J. Am. Chem. Soc.* 127:13126–7 (2005), which is hereby incorporated by reference in its entirety). Peptide **12** bound IZN17 with a K_d value of 24 μM, within range of the previously reported values (Sia et al., "Short Constrained Peptides That Inhibit HIV-1 Entry," *Proc. Nat'l Acad. Sci. U.S.A.* 99:14664–9 (2002); Stephens et al., "Inhibiting HIV Fusion with a β-Peptide Foldamer," *J. Am. Chem. Soc.* 127:13126–7 (2005), which are hereby incorporated by reference in their entirety). The competition assay provided a binding affinity of 37 μM for peptide **1**, which is also in range of reported values, although aggregation of the peptide made it difficult to obtain accurate K_d values.

[0091] Circular dichroism spectroscopy (see Example 2, *supra*) suggested that **1** is unstructured or very weakly helical in 10% trifluoroethanol ("TFE") in PBS buffer (Sia et al., "Short Constrained Peptides That Inhibit HIV-1 Entry," *Proc. Nat'l Acad. Sci. U.S.A.* 99:14664–9 (2002), which is

hereby incorporated by reference in its entirety). It was conjectured that stabilization of this peptide in helical conformation with the HBS approach may increase its helicity and affinity for IZN17. HBS α-helix **2** is roughly four times more helical than **1**, as shown in Figure 16A, but did not bind the target protein with higher affinity, as shown in Table 3. This suggests that there may be a complex interplay between helicity of the peptide and its binding affinity for the target (Martin, "Preorganization in Biological Systems: Are Conformational Constraints Worth the Energy?," *Pure Appl. Chem.* 79:193–200 (2007); Benfield et al., "Ligand Preorganization May Be Accompanied by Entropic Penalties in Protein-ligand Interactions," *Angew. Chem. Int'l Ed. Engl.* 45:6830–5 (2006), *originally published at Angew. Chem.* 118:6984–9 (2006), which are hereby incorporated by reference in their entirety). Results with the short peptide and constrained helix mirror those observed by Kim and coworkers (Sia et al., "Short Constrained Peptides That Inhibit HIV-1 Entry," *Proc. Nat'l Acad. Sci. U.S.A.* 99:14664–9 (2002), which is hereby incorporated by reference in its entirety), and prompted the design of a small library of HBS α-helices to identify sequences that bind IZN17 with higher affinity. A representative selection (*i.e.*, peptides **2–9**) of these HBS helices is shown in Table 3.

[0092] The limited solubility of short C-peptides in aqueous solutions has been proposed as a key reason for their inactivity (Otaka et al., "Remodeling of gp41-C34 Peptide Leads to Highly Effective Inhibitors of the Fusion of HIV-1 with Target Cells," *Angew. Chem. Int'l Ed. Engl.* 41:2937–40 (2002), *originally published at Angew. Chem.* 114:3061–4 (2002), which is hereby incorporated by reference in its entirety). To improve the solubility and helicity of constrained C-peptides, charged residues were incorporated at positions not expected to be involved in binding to IZN17 (Otaka et al., "Remodeling of gp41-C34 Peptide Leads to Highly Effective Inhibitors of the Fusion of HIV-1 with Target Cells," *Angew. Chem. Int'l Ed. Engl.* 41:2937–40 (2002), *originally published at Angew. Chem.* 114:3061–4 (2002), which is hereby incorporated by reference in its entirety). HBS α-helix **3** is the shortest peptide designed to contain the Trp/Trp/Ile motif necessary for binding. Two lysine residues were added within this sequence to improve solubility of the peptide. HBS α-helix **4** was designed such that the Trp/Trp/Ile motif would lie outside of the constraint in order to probe potential steric effects of the HBS constraints. A pair of glutamic acid and lysine residues were placed at *i* and *i*+4 positions to further stabilize the α-helical conformation through potential salt-bridging interactions (Marqusee & Baldwin, "Helix Stabilization by $Glu^{-}\cdots Lys^{+}$ Salt Bridges in Short Peptides of *de Novo* Design," *Proc. Nat'l Acad. Sci. U.S.A.* 84:8898–902 (1987); Shi et al., "Stabilization of α-Helix Structure by Polar Side-chain Interactions: Complex Salt Bridges, Cation-pi Interactions, and C-H Em Leader O H-bonds," *Biopolymers* 60:366–80 (2001), which are hereby incorporated by reference in their entirety). Neither of these two HBS helices bound IZN17, suggesting that more contact points are needed to target the coiled-coil complex. HBS α-helix **5** contains tyrosine-638 in addition to the Trp/Trp/Ile residues. Tyr-638 occupies the *d* position in the heptad and is expected to directly contact the N-terminal three strand coiled-coil, as shown in Figure 1C. The sequence of HBS α-

helix 5 is similar to that of the parent compounds **1** and **2** but is missing N-terminal methionine-626 and threonine-627 residues. This compound also showed negligible affinity for IZN17, which was surprising because it was expected that **5** would bind IZN17 with similar affinity to that observed for **2**. CD spectroscopy suggests that **2** and **5** are equally helical, as shown in Figure 16A. The low binding affinity of **5** relative to **2** highlights the potential role of threonine-627, which occupies the *g* position in the heptad, as shown in Figure 1C, and was not projected to contribute significantly to binding. Reintroduction of the Met-626 and Thr-627 residues and incorporation of charged residues at positions not expected to be involved in binding provided HBS α-helix **6**, which bound IZN17 with a higher affinity than the parent HBS α-helix **2**. The role of Thr-627 in the binding of HBS helices is also highlighted by HBS α-helix **7**, in which this residue is replaced with an alanine residue. HBS α-helix **7** bound IZN17 by twenty-fold lower affinity than did **6**.

[0093] Glutamic acid residues were placed at the N-terminus of HBS α-helices **7** and **8** to potentially stabilize the helix macrodipole. However, incorporation of this residue did not affect the binding affinity of these peptides.

[0094] HBS α-helices **8** and **9** were prepared to explore the effect of additional contact points (residues that occupy *a* or *d* positions in the heptad) on the amino and carboxy ends. Both HBS α-helices **8** and **9** consist of five residues that occupy *a* or *d* positions in the heptad and bind IZN17 with K_d values <5 μM. Values for **8** and **9** represent upper limits in the present 1ZN17 assay, because competitive binding analysis does not allow accurate estimates of K_d values much lower than the binding affinity of the fluorescent probe (see Example 3, *supra*) (Roehrl et al., "A General Framework for Development and Data Analysis of Competitive High-throughput Screens for Small-molecule Inhibitors of Protein-protein Interactions by Fluorescence Polarization," *Biochemistry* 43:16056–66 (2004), which is hereby incorporated by reference in its entirety). Although the 1ZN17 assay did not provide accurate values for the best HBS helices, it allowed for the qualitative evaluation of various constructs described herein. It was determined that a combination of the 1ZN17 binding assay and a gp41-mediated cell-cell fusion assay would provide a better gauge of the effectiveness of HBS helices as inhibitors of HIV fusion (Eckert et al., "Inhibiting HIV-1 Entry: Discovery of D-Peptide Inhibitors That Target the gp41 Coiled-coil Pocket," *Cell* 99:103–15 (1999), which is hereby incorporated by reference in its entirety).

[0095] Cell-cell fusion (*i.e.*, syncytium formation) was assayed by co-culturing CHO[HIVe] (clone 7d2) cells expressing HXB2 envelope and tat (Kozarsky et al., "Glycosylation and Processing of the Human Immunodeficiency Virus Type 1 Envelope Protein," *J. Acquir. Immune Defic. Syndr.* 2:163–9 (1989), which is hereby incorporated by reference in its entirety) with U373-MAGI cells in the presence of different concentrations of the peptide inhibitors. Cell fusion allows the expression of nuclear β-galactosidase from the U373-MAGI indicator cell line and can be quantitated by monitoring β-galactosidase activity.

[0096] Inhibition of gp41-mediated cell fusion by short peptides is a challenging feat and has only been accomplished with a handful of synthetic peptides (Sia et al., "Short Constrained Peptides That Inhibit HIV-1 Entry," *Proc. Nat'l Acad. Sci. U.S.A.* 99:14664–9 (2002); Stephens et al., "Inhibiting HIV Fusion with a β-Peptide Foldamer," *J. Am. Chem. Soc.* 127:13126–7 (2005); Eckert et al., "Inhibiting HIV-1 Entry: Discovery of D-Peptide Inhibitors That Target the gp41 Coiled-coil Pocket," *Cell* 99:103–15 (1999), which are hereby incorporated by reference in their entirety). It was found that only HBS α-helix **9** inhibited cell fusion with an EC_{50} value of 43 μM, as shown in Figure 16C. This value is comparable to those measured for side chain constrained α-helices (Sia et al., "Short Constrained Peptides That Inhibit HIV-1 Entry," *Proc. Nat'l Acad. Sci. U.S.A.* 99:14664–9 (2002), which is hereby incorporated by reference in its entirety), cyclic D-peptides (Eckert et al., "Inhibiting HIV-1 Entry: Discovery of D-Peptide Inhibitors That Target the gp41 Coiled-coil Pocket," *Cell* 99:103–15 (1999), which is hereby incorporated by reference in its entirety), aromatic foldamers (Ernst et al., "Design of a Protein Surface Antagonist Based on α-Helix Mimicry: Inhibition of gp41 Assembly and Viral Fusion," *Angew. Chem. Int'l Ed. Engl.* 41:278–81 (2002), *originally published at Angew. Chem.* 114:282–91 (2002), which is hereby incorporated by reference in its entirety), and β-peptide foldamers (Stephens et al., "Inhibiting HIV Fusion with a β-Peptide Foldamer," *J. Am. Chem. Soc.* 127:13126–7 (2005), which is hereby incorporated by reference in its entirety). Other HBS α-helices (**2–8**) did not provide any hints of cell fusion inhibition at concentrations up to 200 μM. Unconstrained peptide **10**, which bound IZN17 with a similar affinity as **9**, remained ineffective in the cell culture assay. This result potentially reflects the proteolytic instability of the unconstrained peptide, as stabilization of peptides in α-helical conformation is expected to enhance their resistance to proteases (Tyndall et al., "Proteases Universally Recognize β Strands in Their Active Sites," *Chem. Rev.* 105:973–99 (2005), which is hereby incorporated by reference in its entirety). Improvements in the proteolytic stability of HBS α-helices as compared to their unconstrained counterparts has previously been reported (Wang et al., "Enhanced Metabolic Stability and Protein-binding Properties of Artificial α Helices Derived from a Hydrogen-bond Surrogate: Application to Bcl-xL," *Angew. Chem. Int'l Ed. Engl.* 44:6525–9 (2005), *originally published at Angew. Chem.* 117:6683–7 (2005), which is hereby incorporated by reference in its entirety).

[0097] In summary, through rational design and synthesis, an artificial α-helix (**9**) that inhibits gp41-mediated cell fusion has been developed. As formation of coiled-coil assemblies is a prerequisite for the fusion of several classes of viruses to their host cells (Dimitrov, "Virus Entry: Molecular Mechanisms and Biomedical Applications," *Nat. Rev. Microbiol.* 2:109–22 (2004) which is hereby incorporated by reference in its entirety), this work suggests that HBS helices may be effective scaffolds for the generation of small molecule inhibitors or antigens against these viruses (English et al., "Rational Development of β-Peptide Inhibitors of Human Cytomegalovirus Entry," *J. Biol. Chem.* 281:2661–7 (2006); Shepherd et al., "Modular α-Helical

Mimetics with Antiviral Activity Against Respiratory Syncitial Virus," *J. Am. Chem. Soc.* 128:13284–9 (2006), which are hereby incorporated by reference in their entirety).

[0098] 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 peptide having a stable, internally-constrained alpha-helix, wherein said alpha helix is constrained by a crosslink formed by a carbon-carbon bond-forming reaction, and further wherein the peptide mimics at least a portion of a class I C-peptide helix or at least a portion of a class I N-peptide helix, and wherein the peptide is an inhibitor of viral infectivity.

2. The peptide of claim 1, wherein the carbon-carbon bond-forming reaction is metathesis.

3. The peptide according to claim 1, wherein the class I C-peptide helix or the class I N-peptide helix is derived from a virus selected from the group of *Orthomyxoviridae*, *Paramyxoviridae*, *Filoviridae*, *Retroviridae*, *Coronaviridae*, *Herpesviridae*, influenza virus, Simian virus 5, respiratory syncitial virus, Ebola virus, Moloney murine leukemia virus, simian immunodeficiency virus, human immunodeficiency virus, human T cell leukemia virus, Mouse hepatitis virus, SARS virus, and human cytomegalovirus.

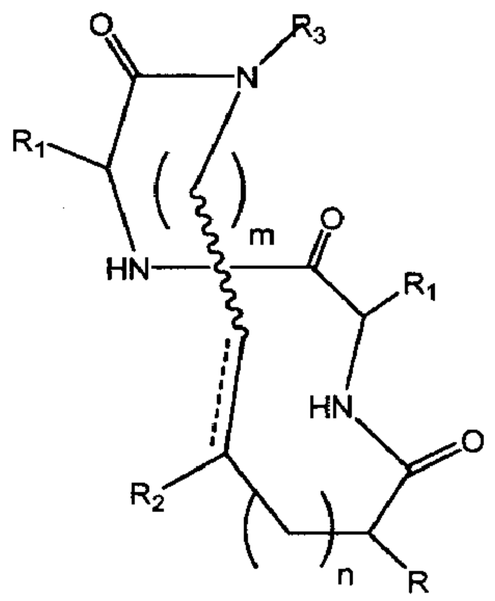
4. The peptide according to claim 3, wherein the virus is human immunodeficiency virus.

5. The peptide according to claim 4, wherein the peptide mimics at least a portion of a gp41 C-peptide helix or at least a portion of a gp41 N-peptide helix.

6. The peptide according to claim 5, wherein the peptide mimics at least a portion of a gp41 C-peptide helix.

7. The peptide according to claim 6, wherein the peptide mimics the WWI region of a gp41 C-peptide helix.

8. The peptide according to claim 1, wherein the peptide comprises the formula:



wherein

⚌ is a single or double carbon-carbon bond;

∿ is a single bond and is *cis* or *trans* when ⚌ is a double bond;

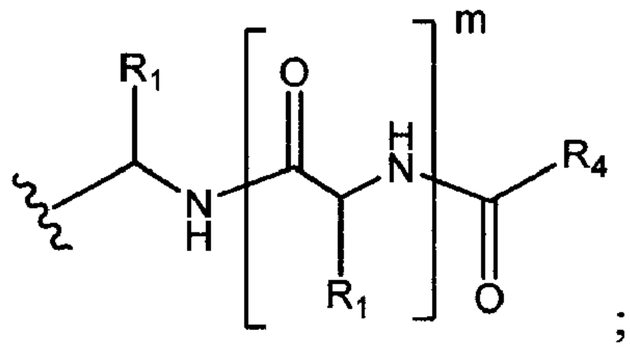
n is 1 or 2;

m is zero or any positive integer;

R is hydrogen, an amino acid side chain, an alkyl group, or an aryl group;

R_1 is an amino acid side chain, an alkyl group, or an aryl group;

R_2 is an amino acid, second peptide, –OR, $–CH_2NH_2$, an alkyl group, an aryl group, hydrogen, or a group having a formula



where R_4 is an amino acid, third peptide, –OR, $–NH_2$, an alkyl group, or an aryl group; and R_3 is a fourth peptide.

9. The peptide according to claim 8, wherein R_3 comprises the formula – **WXXWXXXIXXYXXXI**–R_4, where X is any amino acid.

10. The peptide according to claim 6, wherein the peptide comprises the amino acid sequence of SEQ ID NO: 9, and has an internally-constrained alpha-helix spanning residues 1 through 4 of SEQ ID NO: 9.

11. The peptide according to claim 6, wherein the gp41 C-peptide helix has an amino acid sequence of SEQ ID NO: 11.

12. The peptide according to claim 5, wherein the peptide mimics at least a portion of a gp41 N-peptide helix.

13. The peptide according to claim 12, wherein the peptide mimics a hydrophobic pocket of a gp41 N-peptide helix.

14. The peptide according to claim 12, wherein the gp41 N-peptide helix has an amino acid sequence of SEQ ID NO: 12.

15. A pharmaceutical composition comprising a peptide according to claim 1 and a pharmaceutically acceptable vehicle.

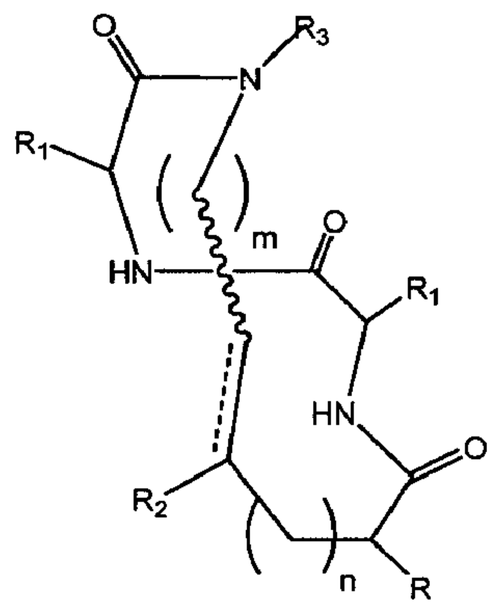
16. A method of inhibiting infectivity of a virus in a subject, said method comprising administering to the subject an effective amount of a composition comprising a peptide having a stable, internally-constrained alpha-helix, wherein said alpha helix is constrained by a crosslink formed by a carbon-carbon bond-forming reaction, and further wherein the peptide mimics at least a portion of a class I C-peptide helix or at least a portion of a class I N-peptide helix.

17. The method according to claim 16, wherein the carbon-carbon bond-forming reaction is metathesis.

18. The method according to claim 16, wherein the virus is selected from the group of *Orthomyxoviridae*, *Paramyxoviridae*, *Filoviridae*, *Retroviridae*, *Coronaviridae*, *Herpesviridae*, influenza virus, Simian virus 5, respiratory syncitial virus, Ebola virus, Moloney murine leukemia virus, simian immunodeficiency virus, human immunodeficiency virus, human T cell leukemia virus, Mouse hepatitis virus, SARS virus, and human cytomegalovirus.

19. The method according to claim 17, wherein the virus is human immunodeficiency virus and the peptide mimics at least a portion of a gp41 C-peptide helix or at least a portion of a gp41 N-peptide helix.

20. The method according to claim 16, wherein the peptide comprises the formula:



wherein

===== is a single or double carbon-carbon bond;

~~~~ is a single bond and is *cis* or *trans* when ===== is a double bond;

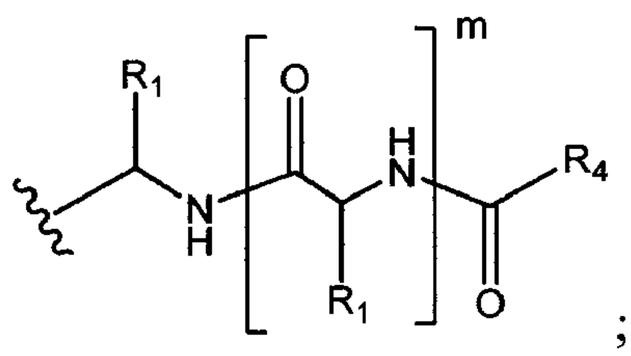
n is 1 or 2;

m is zero or any positive integer;

R is hydrogen, an amino acid side chain, an alkyl group, or an aryl group;

R_1 is an amino acid side chain, an alkyl group, or an aryl group;

R_2 is an amino acid, second peptide, –OR, $–CH_2NH_2$, an alkyl group, an aryl group, hydrogen, or a group having a formula



;

where R_4 is an amino acid, third peptide, –OR, $–NH_2$, an alkyl group, or an aryl group; and

R_3 is a fourth peptide.

21. The method according to claim 20, wherein R_3 comprises the formula –**WXXWXXXIXXYXXXI**–R_4, where X is any amino acid.

22. The method according to claim 16, wherein the peptide comprises the amino acid sequence of SEQ ID NO: 9, and has an internally-constrained alpha-helix spanning residues 1 through 4 of SEQ ID NO: 9.

23. The method according to claim 19, wherein the gp41 C-peptide helix has an amino acid sequence of SEQ ID NO: 11.

24. A method of synthesizing a peptide which is an inhibitor of viral infectivity comprising: selecting a precursor peptide comprising at least a portion of a class I C-peptide helix or at least a portion of a class I N-peptide helix; and promoting formation of a carbon-carbon bond, wherein said bond formation results in a stable, internally-constrained alpha-helix.

25. The method according to claim 24, wherein said bond formation introduces a non-native carbon-carbon bond.
~~~~

26. The method according to claim 24, wherein said carbon-carbon bond is formed by metathesis.

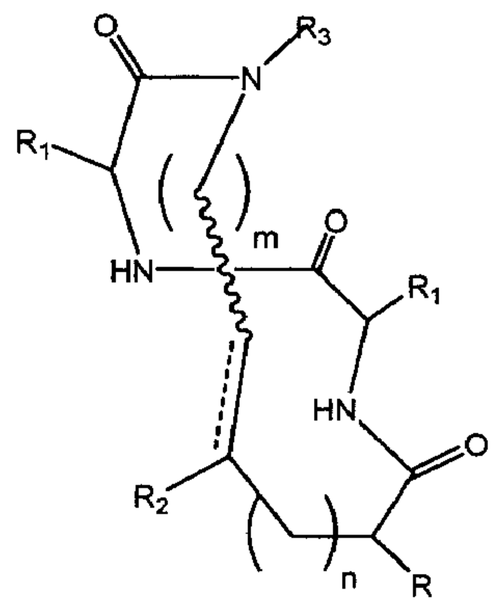
27. The method according to claim 24, wherein the virus is human immunodeficiency virus and the peptide mimics at least a portion of a gp41 C-peptide helix or at least a portion of a gp41 N-peptide helix.

28. The method according to claim 24, wherein the peptide comprises the formula **WXXWXXXIXXYXXXI**–R_4, where X is any amino acid and R_4 is an amino acid, third peptide, –OR, –NH_2, an alkyl group, or an aryl group.

29. The method according to claim 28, wherein the peptide comprises the amino acid sequence of SEQ ID NO: 9, and has an internally-constrained alpha-helix spanning residues 1 through 4 of SEQ ID NO: 9.

30. The method according to claim 27, wherein the gp41 C-peptide helix has an amino acid sequence of SEQ ID NO: 11.

31. The method according to claim 24, wherein the peptide comprises the formula:



wherein

⚌ is a single or double carbon-carbon bond;

∿ is a single bond and is *cis* or *trans* when ⚌ is a double bond;

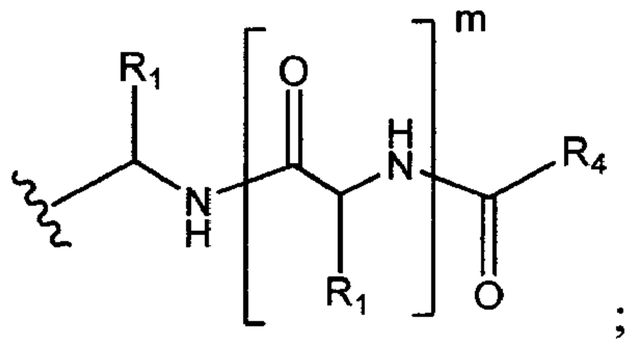
n is 1 or 2;

m is zero or any positive integer;

R is hydrogen, an amino acid side chain, an alkyl group, or an aryl group;

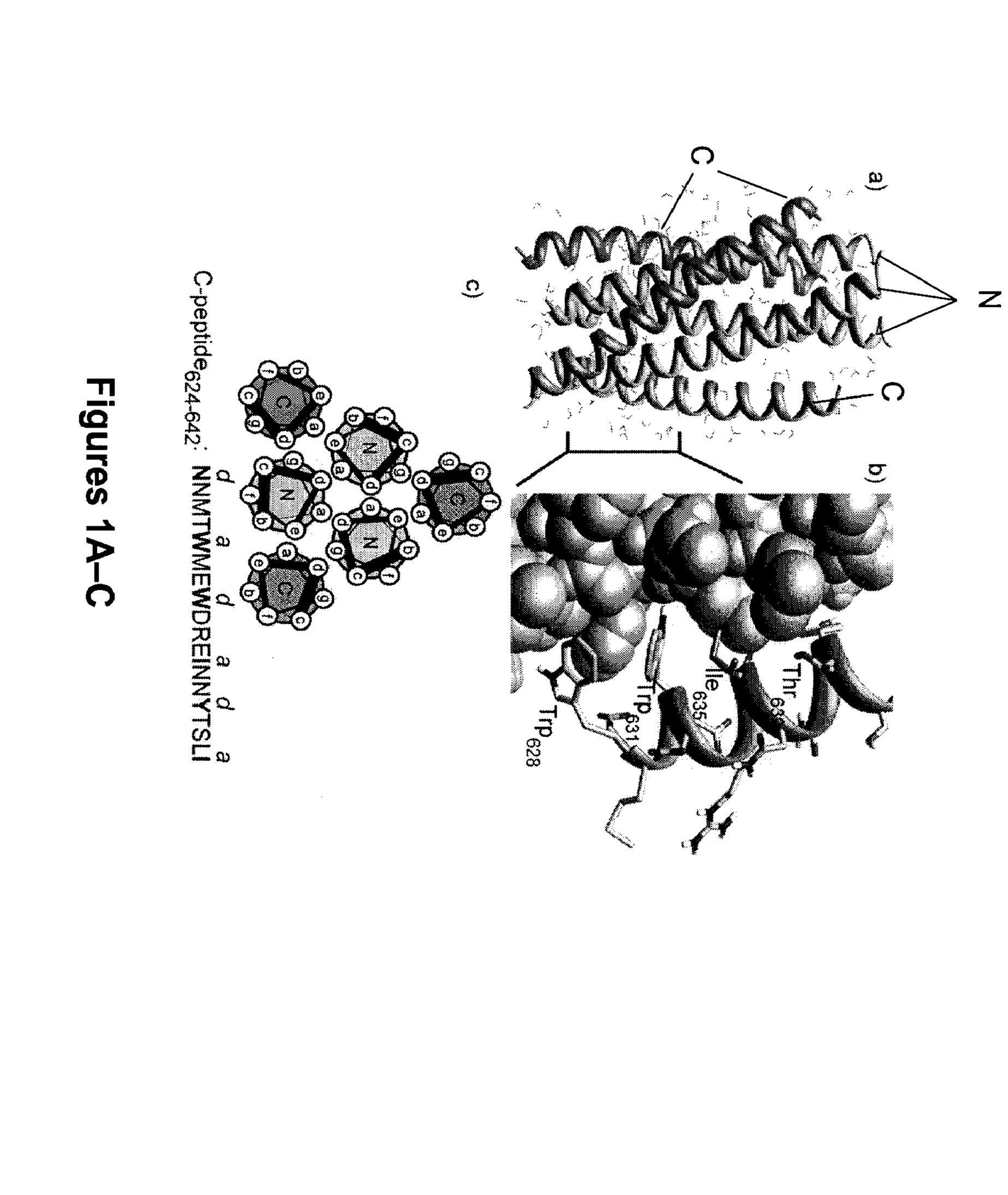
R_1 is an amino acid side chain, an alkyl group, or an aryl group;

R_2 is an amino acid, second peptide, –OR, –CH_2NH_2, an alkyl group, an aryl group, hydrogen, or a group having a formula



;

where R_4 is an amino acid, third peptide, –OR, –NH_2, an alkyl group, or an aryl group; and R_3 is a fourth peptide.


a)
C
N
C
b)
Trp628
Trp631
Ile635
Thr
c)
C-peptide624-642: NNMTWMEWDREINNYTSLI
d a d a d a d a

Figures 1A–C

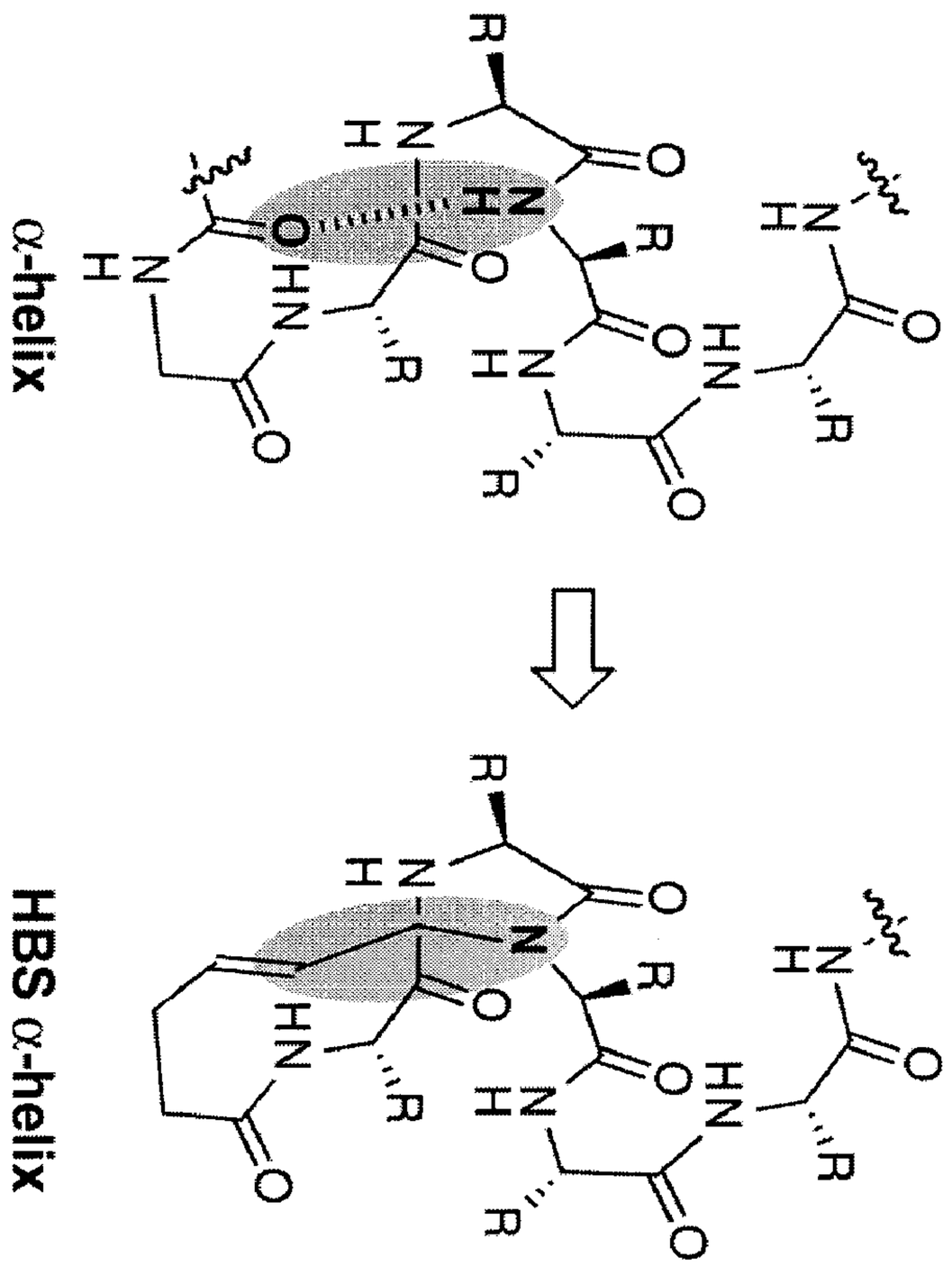


Figure 2

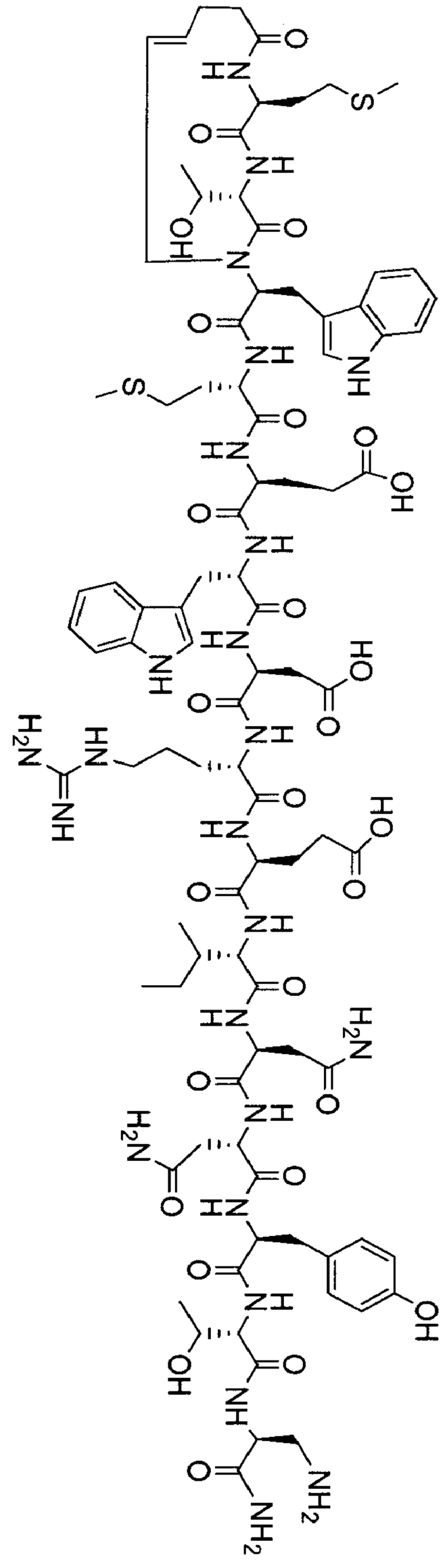


Figure 3

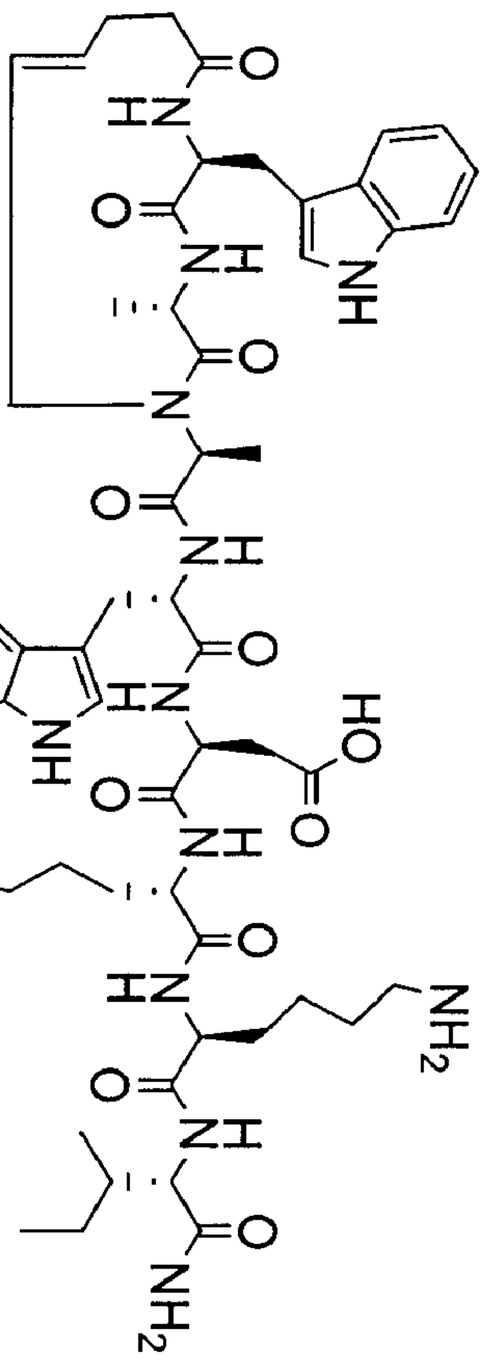


Figure 4

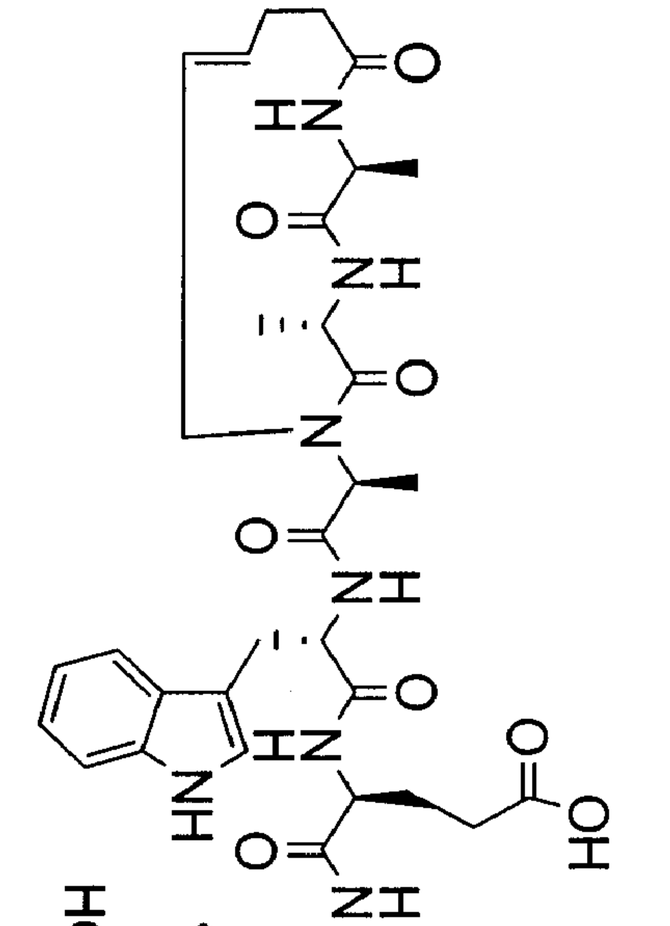


Figure 5

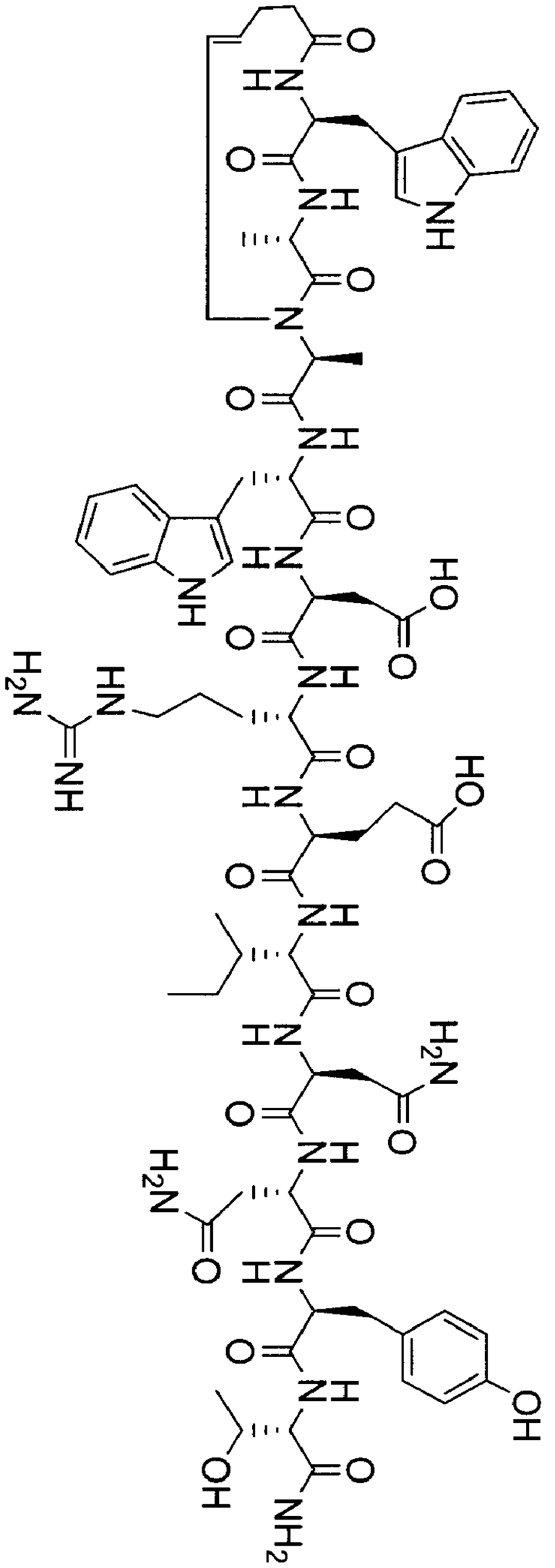


Figure 6

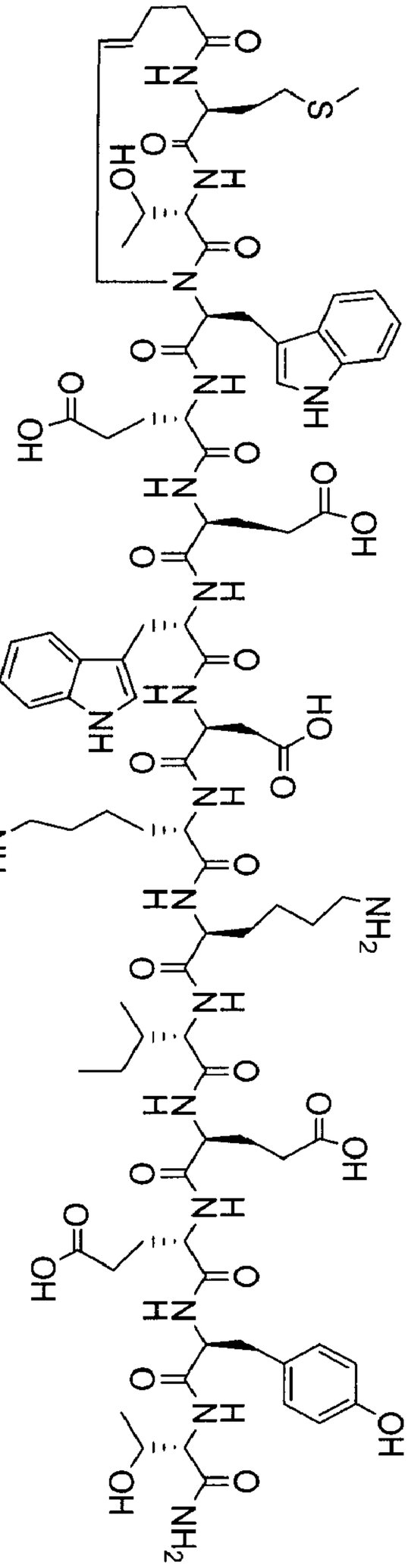


Figure 7

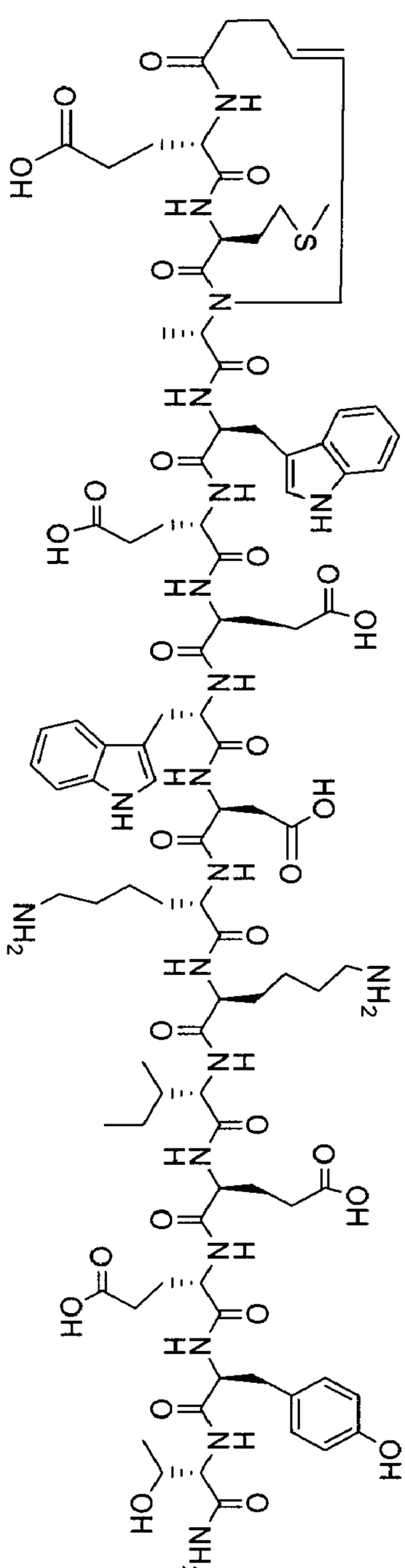


Figure 8

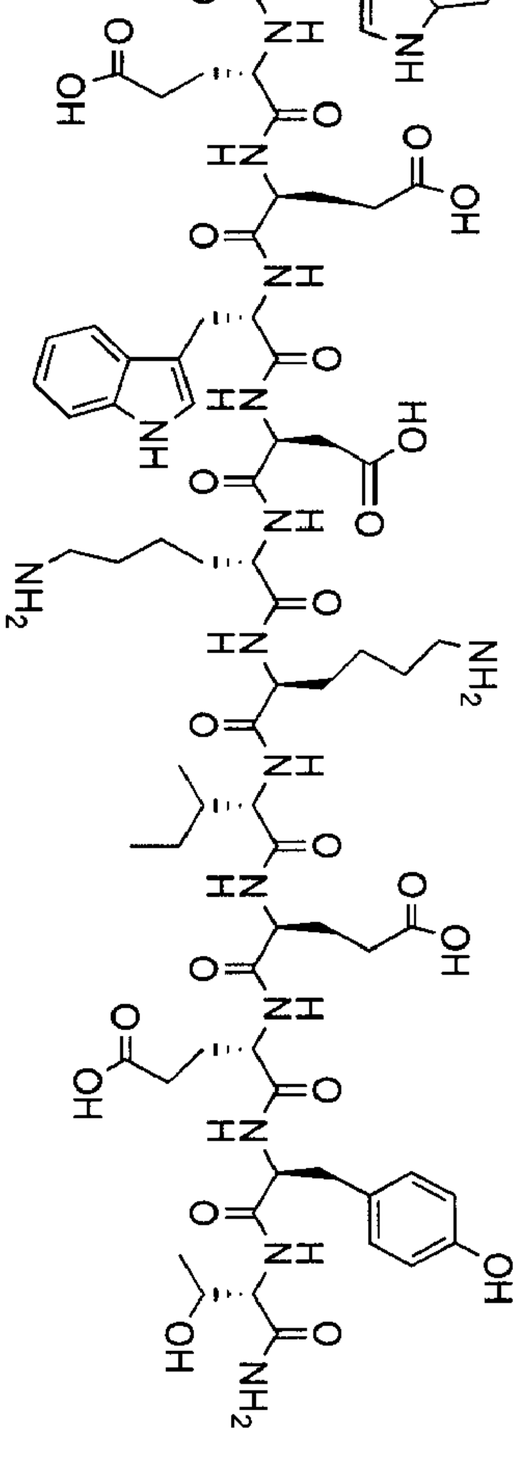


Figure 9

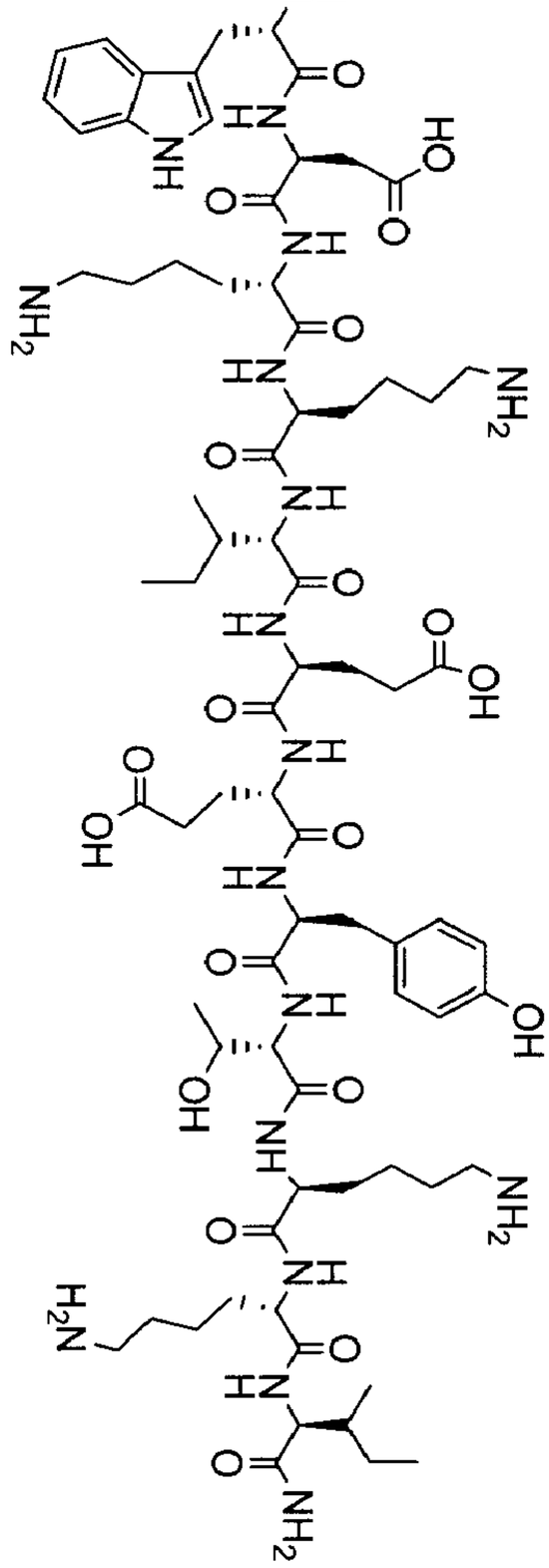
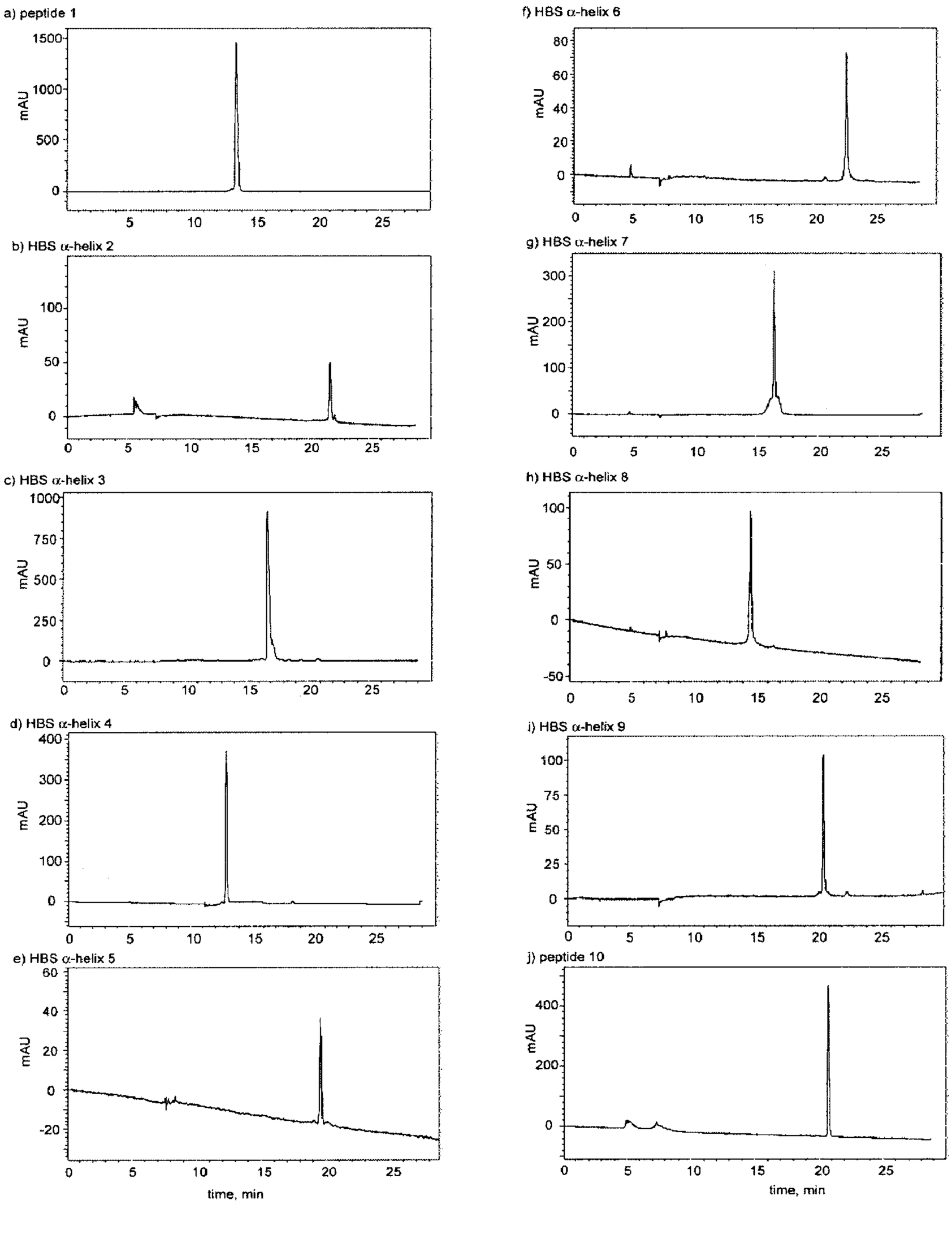
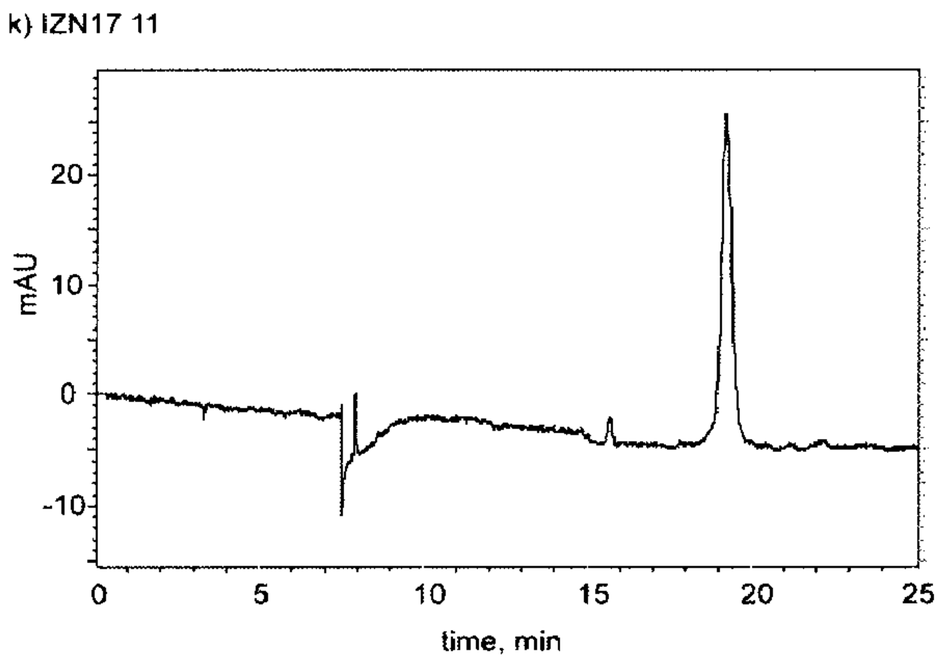
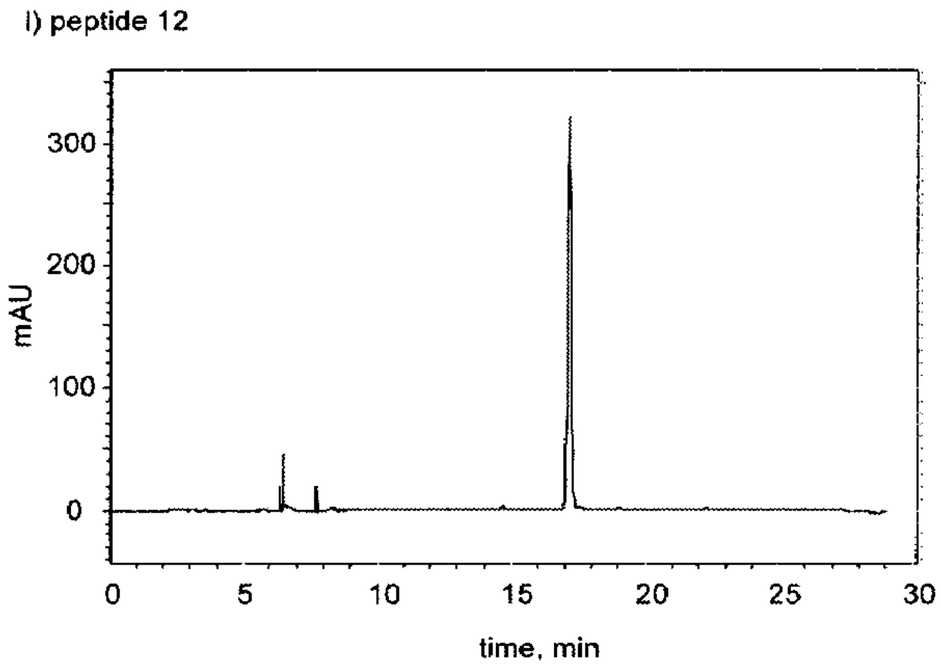


Figure 10



Figures 11A–J





Figures 11K–L

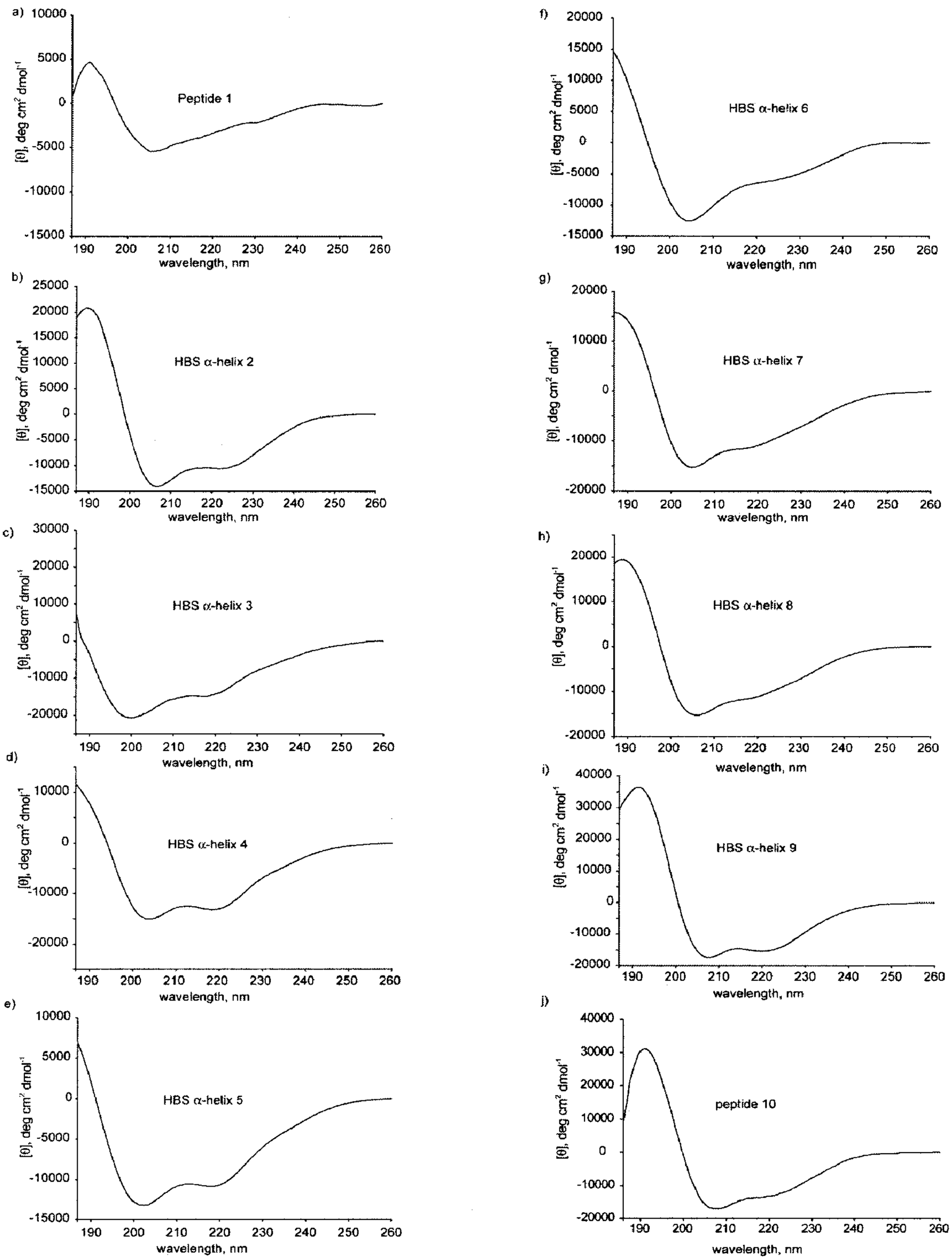


Figure 125A–J

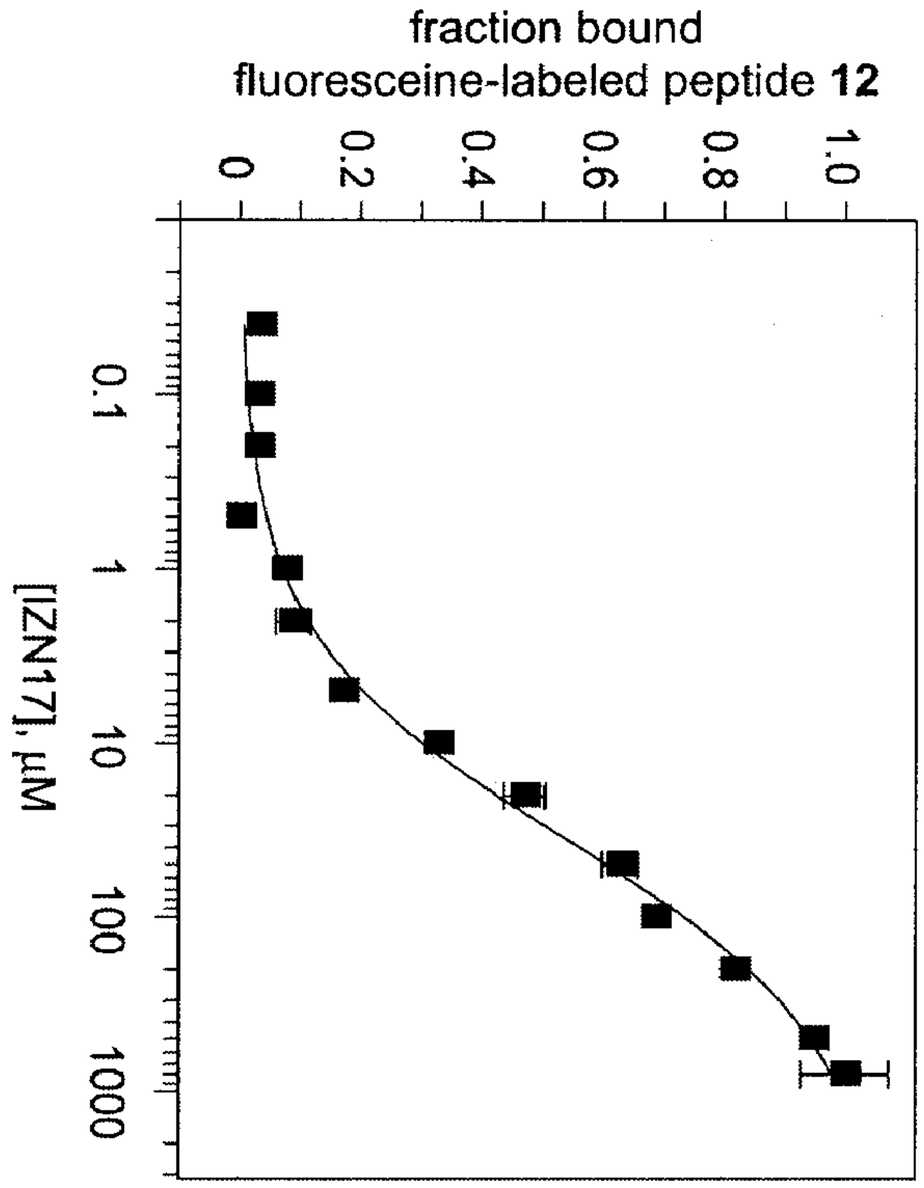


Figure 14

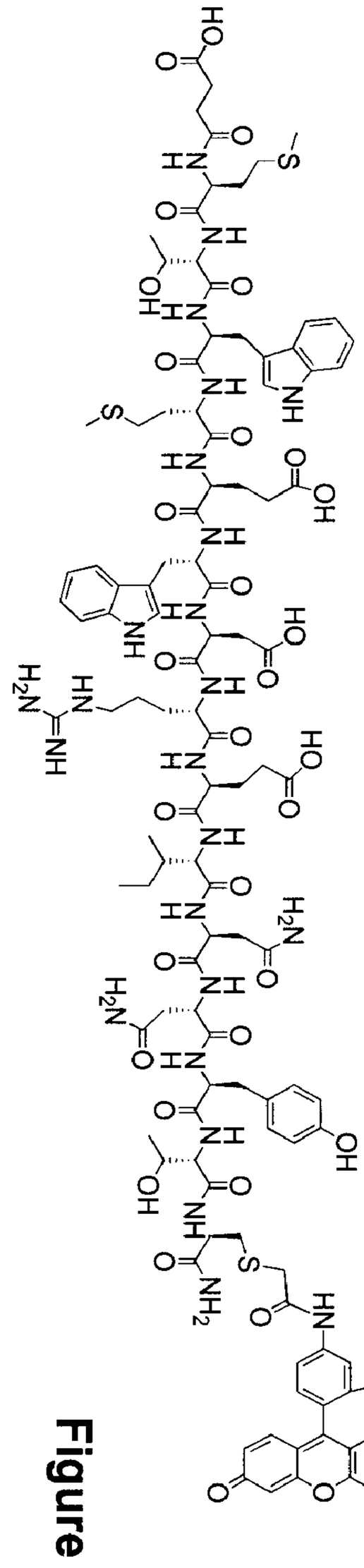


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Figure 13

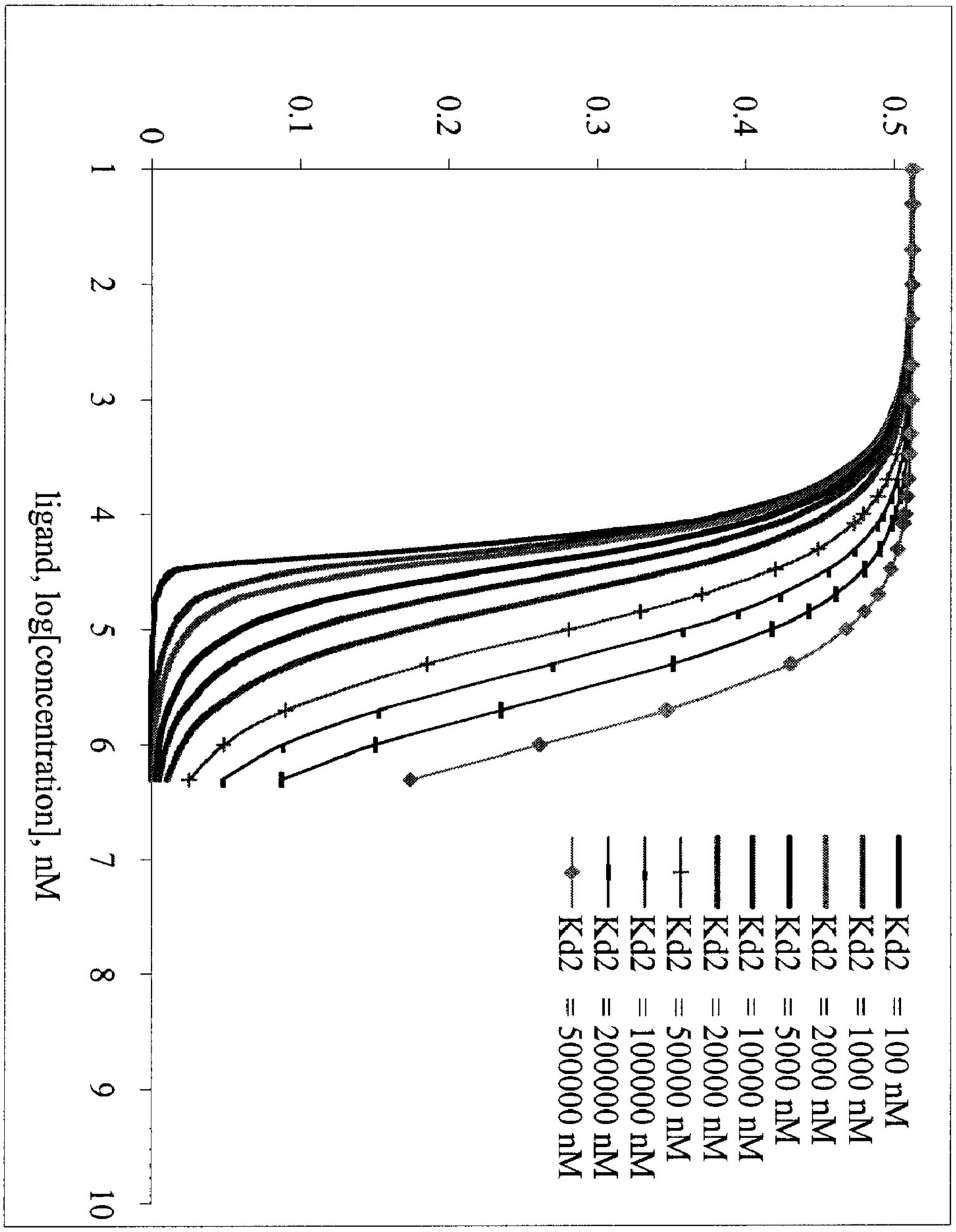
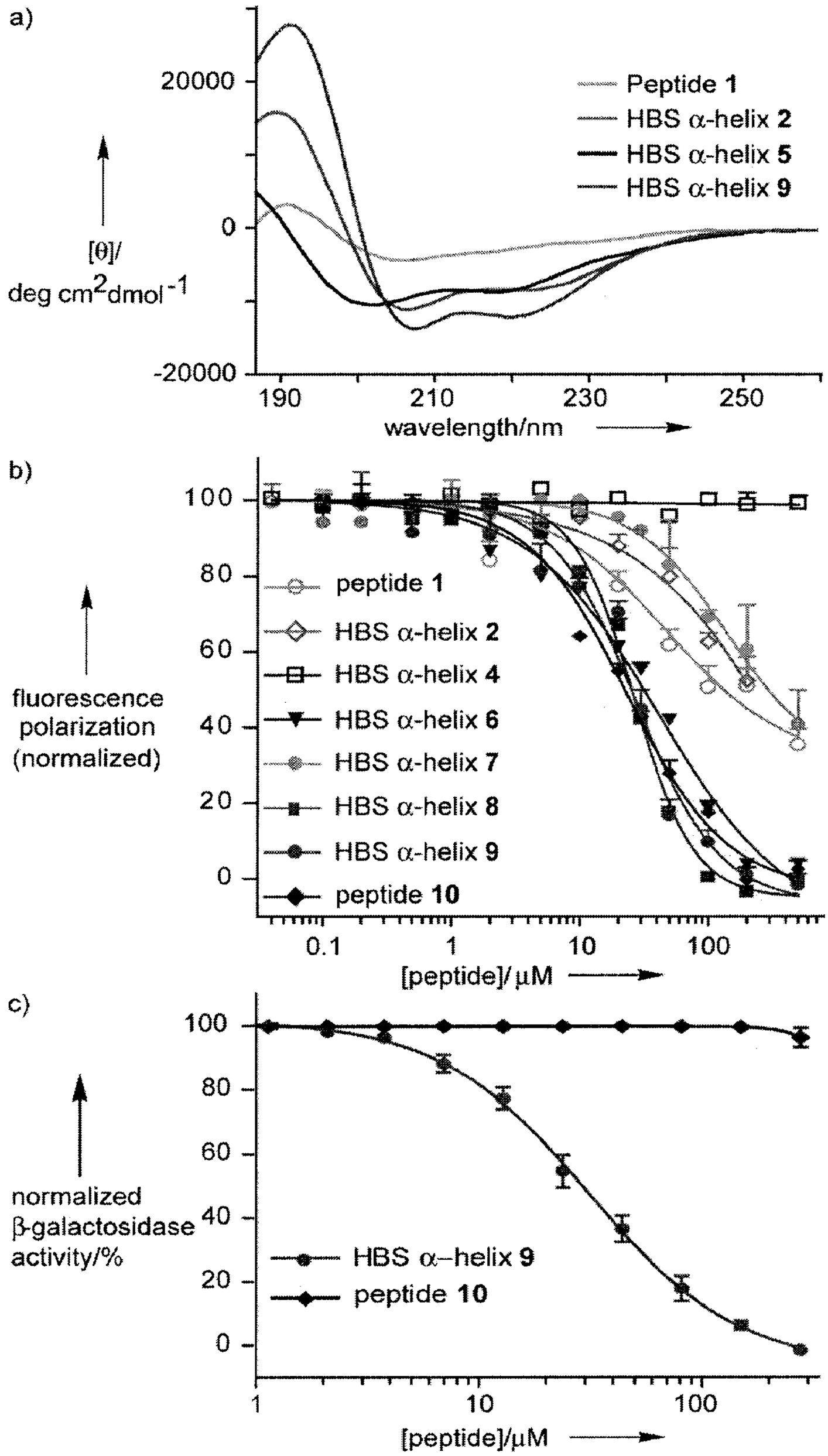

0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
7
8
9
10
ligand, log[concentration], nM
Kd2 = 100 nM
Kd2 = 1000 nM
Kd2 = 2000 nM
Kd2 = 5000 nM
Kd2 = 10000 nM
Kd2 = 20000 nM
Kd2 = 50000 nM
Kd2 = 100000 nM
Kd2 = 200000 nM
Kd2 = 500000 nM

Figure 15



Figures 16A–C