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(54) Title: METHODS FOR PREPARING INTERNALLY CONSTRAINED PEPTIDES AND PEPTIDOMIMETICS

(57) Abstract: The present invention relates to a method for preparing a peptide having a stable, internally constrained alpha-helical, beta-sheet/beta-turn, ₃₁₀-helical, or pi-helical region and a method of stabilizing an alpha-helical, beta-sheet/beta-turn, ₃₁₀-helical, or pi-helical region within a peptide structure. The resulting peptides and methods of using them are also disclosed.

METHODS FOR PREPARING INTERNALLY CONSTRAINED PEPTIDES AND PEPTIDOMIMETICS

[0001] This application claims the benefit of U.S. Provisional Patent Application Serial No. 60/574,964, filed May 27, 2004, which is hereby incorporated
5 by reference in its entirety.

[0002] The present invention was made, at least in part, with funding received from the National Institutes of Health, grant number GM073943, and the National Science Foundation, grant numbers MRI-0116222 and CHE-0234863. The U.S. government may have certain rights in this invention.

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FIELD OF THE INVENTION

[0003] This invention is directed generally to methods for preparing internally constrained peptides and peptidomimetics.

BACKGROUND OF THE INVENTION

[0004] Protein secondary structures include β -sheets/ β -turns, π -helices, 3_{10} -
15 helices, and α -helices.

[0005] The α -helix is the most common element of protein secondary structure and participates widely in fundamental biological processes, including highly specific protein-protein and protein-nucleic acids interactions. Molecules that can predictably and specifically disrupt these interactions would be invaluable as tools
20 in molecular biology, and, potentially, as leads in drug development (Kelso et al., *J. Am. Chem. Soc.* 126:4828-4842 (2004); Schafmeister et al., *J. Am. Chem. Soc.*, 122:5891-5892 (2000); Austin et al., *J. Am. Chem. Soc.* 119:6461-6472 (1997); Phelan et al., *J. Am. Chem. Soc.* 119:455-460 (1997); Osapay et al., *J. Am. Chem. Soc.* 114:6966-6973 (1992); Kemp et al., *J. Org. Chem.* 56:6672-6682(1991); Jackson et al., *J. Am. Chem. Soc.* 113:9391-9392 (1991); Ghadiri et al., *J. Am. Chem. Soc.* 112:1630-1632 (1990); Felix et al., *Int. J. Pept. Protein Res.* 32:441-454 (1988)).
25 Exposed α -helices on the surfaces of proteins are also often involved in recognition of other biomolecules. Peptides composed of less than fifteen residues corresponding to these α -helical regions typically do not remain helical once excised from the protein

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environment. Short peptides (<15 residues) that can adopt α -helical structure are expected to be useful models for the design of bioactive molecules and for studying aspects of protein folding.

[0006] Several strategies have been reported for the preparation of stabilized
5 α -helices (Andrews et al., "Forming Stable Helical Peptides Using Natural and Artificial Amino Acids," *Tetrahedron* 55:11711-11743 (1999)). These methods include incorporation of nonnatural amino acids (Lyu et al., "Alpha-helix Stabilization by Natural and Unnatural Amino Acids with Alkyl Side Chains," *Proc. Nat'l Acad. Sci.* 88:5317-5320 (1991); Kaul et al., "Stereochemical Control of Peptide
10 Folding," *Bioorg. Med. Chem.* 7:105-117 (1999)), capping motifs (Austin et al., "Template for Stabilization of a Peptide Alpha-helix: Synthesis and Evaluation of Conformational Effects by Circular Dichroism and NMR," *J. Am. Chem. Soc.* 119:6461-6472 (1997); Lyu et al., "Capping Interactions in Isolated Alpha Helices: Position-dependent Substitution Effects and Structure of a Serine-capped Peptide
15 Helix," *Biochemistry* 32:421-425 (1993); Chakrabartty et al., "Helix Capping Propensities in Peptides Parallel Those in Proteins," *Proc. Nat'l Acad. Sci. U.S.A.* 90:11332-11336 (1993); Kemp et al., "Studies of *N*-Terminal Templates for Alpha-helix Formation - Synthesis and Conformational-analysis of (2s,5s,8s,11s)-1-acetyl-1,4-diaza-3-keto-5-carboxy-10-thiatricyclo[2.8.1.0(4,8)]tridecane (Ac-Hel1-Oh)," *J.*
20 *Org. Chem.* 56:6683-6697 (1991)), salt-bridges (Bierzynski et al., "A Salt Bridge Stabilizes the Helix Formed by Isolated C-Peptide of RNase A," *Proc. Nat'l Acad. Sci. U.S.A.* 79:2470-2474 (1982)), metal ion chelation (Kelso et al., *J. Am. Chem. Soc.*, 126:4828-4842 (2004); Kelso et al., "A Cyclic Metallopeptide Induces Alpha Helicity in Short Peptide Fragments of Thermolysin," *Angew. Chem. Int. Ed. Engl.*
25 42:421-424 (2003); Ruan et al., "Metal-ion Enhanced Helicity in Synthetic Peptides Containing Unnatural, Metal-ligating Residues," *J. Am. Chem. Soc.* 112:9403-9404 (1990); Ghadiri, *J. Am. Chem. Soc.*, 112:1630-1632 (1990)), and covalent side chain linkers such as disulfide (Jackson et al., "A General Approach to the Synthesis of Short Alpha-helical Peptides," *J. Am. Chem. Soc.* 113:9391-9392 (1991)), lactam
30 (Phelan et al., "A General Method for Constraining Short Peptides to an Alpha-helical Conformation," *J. Am. Chem. Soc.* 119:455-460 (1997); Bracken et al., *J. Am. Chem. Soc.* 116:6431-6432 (1994); Osapay et al., *J. Am. Chem. Soc.*, 114:6966-6973 (1992);

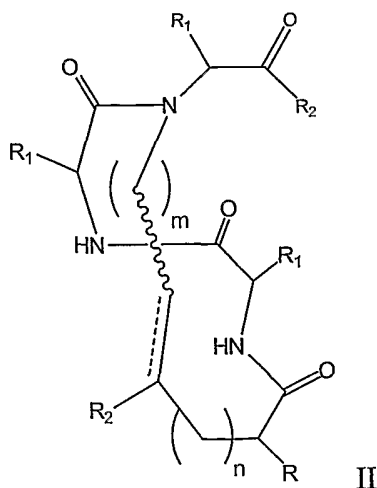
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Felix et al., *Int. J. Pept. Protein Res.* 32:441-454 (1988)), and hydrocarbon bridges (Schafmeister et al., "An All-hydrocarbon Cross-linking System for Enhancing the Helicity and Metabolic Stability of Peptides," *J. Am. Chem. Soc.* 122:5891-5892 (2000); Blackwell et al., "Highly Efficient Synthesis of Covalently Cross-linked Peptide Helices by Ring-closing Metathesis," *Angew. Chem. Int. Ed. Engl.* 37:3281-3284 (1998)). Stabilization of the α -helix structure with these strategies is typically context dependent (Geistlinger et al., "An Inhibitor of the Interaction of Thyroid Hormone Receptor Beta and Glucocorticoid Interacting Protein," *J. Am. Chem. Soc.* 123:1525-1526 (2001); McNamara et al., "Peptides Constrained by an Aliphatic Linkage between Two C(alpha) Sites: Design, Synthesis, and Unexpected Conformational Properties of an $i,(i+4)$ -Linked Peptide," *Org. Chem.* 66:4585-4594 (2001)). More importantly, however, these strategies typically block solvent-exposed surfaces of the target α -helices, or restrict or replace important side chain functionalities from the putative α -helices.

15 [0007] Thus, there remains a need for identifying a general method for the synthesis of highly stable internally-constrained peptide structures, such as short α -helical peptides, with strict preservation of the helix surfaces. The present invention is directed to overcoming these and other deficiencies in the art.

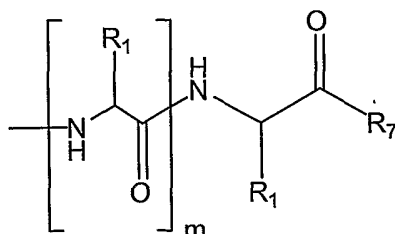
SUMMARY OF THE INVENTION

20 [0008] One aspect of the present invention relates to a method of preparing a compound of Formula II:

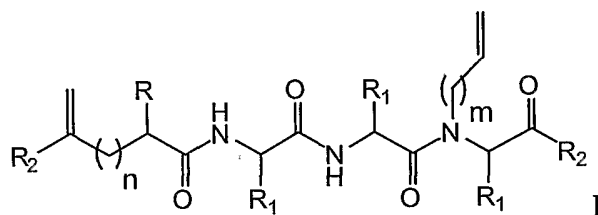


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where R is hydrogen, an amino acid side chain, an alkyl group, or an aryl group; R₁ is an amino acid side chain, an alkyl group, or an aryl group; R₂ is an amino acid, peptide, OR, CH₂NH₂, an alkyl group, an aryl group, or a group of formula

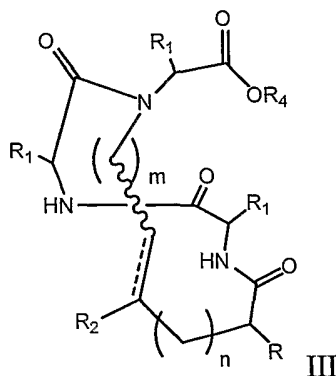


- 5 where R₇ is an amino acid, peptide, OR, CH₂NH₂, an alkyl group, or an aryl group; ----- is a single or double carbon-carbon bond; ~~~~~ is a single bond and is cis or trans when ----- is a double bond; n is 1 or 2; and m is any number. This method involves providing a compound of Formula I:



- 10 and reacting the compound of Formula I under conditions effective to produce a compound of Formula II.

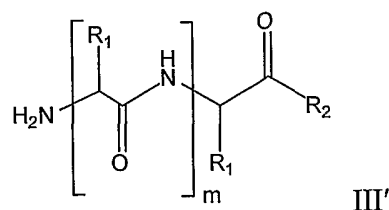
[0009] Another aspect of the present invention relates to a second method of preparing a compound of Formula II. This method involves providing a compound of Formula III or a salt thereof



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where R₄ is a carboxyl protecting group. The compound of Formula III or a salt thereof is reacted with a peptide coupling reagent of Formula III':

- 5 -



III'

under conditions effective to produce a compound of Formula II.

[0010] Another aspect of the present invention relates to peptides having one or more stable, internally-constrained alpha-helical, beta-sheet/beta-turn, 3₁₀-helical, or pi-helical regions.

[0011] Yet another aspect of the present invention relates to a method for promoting cell death. This method involves contacting a cell with one or more Bak BH3 peptides having a stable, internally-constrained alpha-helical region under conditions effective for the one or more Bak BH3 peptides to promote cell death. In this aspect of the present invention, one or more hydrogen bonds within the one or more Bak BH3 peptides are replaced with a carbon-carbon bond.

[0012] Yet another aspect of the present invention relates to a method for promoting cell death. This method involves contacting a cell with one or more peptides which inhibits p53/hDM2 interaction and has a stable, internally-constrained alpha-helical region, under conditions effective for the one or more peptides to promote cell death. In this aspect of the present invention, one or more hydrogen bonds within the one or more peptides are replaced with a carbon-carbon bond.

[0013] Yet another aspect of the present invention relates to a method for promoting cell death. This method involves contacting the cell with one or more peptides which inhibits Jun-Jun and/or Jun-Fos interactions and has a stable, internally-constrained alpha-helical region under conditions effective for the one or more peptides to promote cell death. In this aspect of the present invention, one or more hydrogen bonds within the one or more peptides are replaced with a carbon-carbon bond.

[0014] Yet another aspect of the present invention relates to a method for inhibiting cell proliferation. This method involves contacting the cell with one or more peptides which inhibits Jun-Jun and/or Jun-Fos interactions and has a stable,

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internally-constrained alpha-helical region under conditions effective for the one or more peptides to inhibit cell proliferation. In this aspect of the present invention, one or more hydrogen bonds within the one or more peptides are replaced with a carbon-carbon bond.

5 [0015] Yet another aspect of the present invention relates to a method for inhibiting cell transformation. This method involves contacting the cell with one or more peptides which inhibits Jun-Jun and/or Jun-Fos interactions and has a stable, internally-constrained alpha-helical region under conditions effective for the one or more peptides to inhibit cell transformation. In this aspect of the present invention,
10 one or more hydrogen bonds within the one or more peptides are replaced with a carbon-carbon bond.

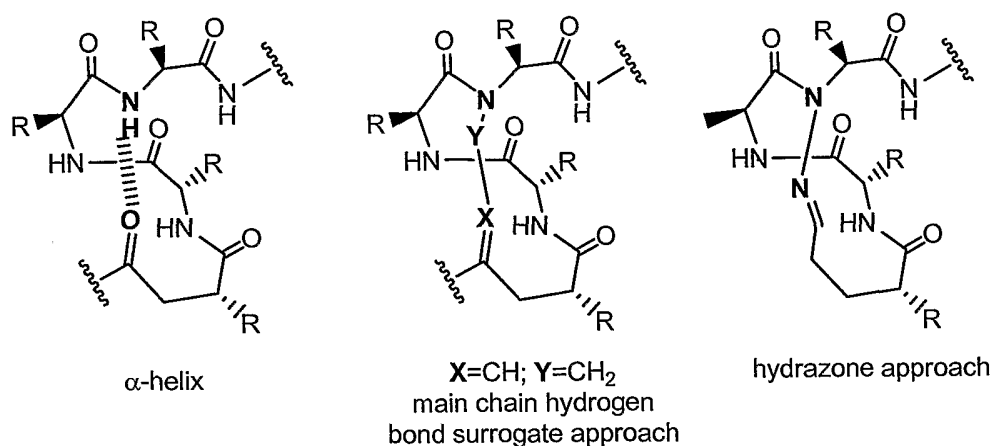
[0016] Yet another aspect of the present invention relates to a method for inhibiting HIV-1 entry into a cell. This method involves contacting the cell with one or more peptides having a stable, internally-constrained alpha-helical region under
15 conditions effective for the one or more peptides to inhibit HIV-1 entry into the cell. In this aspect of the present invention, one or more hydrogen bonds within the one or more peptides are replaced with a carbon-carbon bond.

[0017] Significantly, and advantageously, the methods of the present invention do not utilize side chain functionality, and the constraining element does not
20 block solvent-exposed surfaces of the molecule. In addition, the resulting conformationally-constrained peptides and peptide structures are more stable with regard to thermal stability and proteolytic degradation than those prepared with prior methods. Moreover, the methods of the present invention utilize irreversible crosslinks that can be generated for amino acid sequences.

25 [0018] Previously, Satterthwait and coworkers have explored the use of a hydrazone link to stabilize α -helices (Cabezas et al., "The Hydrogen Bond Mimic Approach: Solid-phase Synthesis of a Peptide Stabilized as an Alpha-helix with a Hydrazone Link," *J. Am. Chem. Soc.* 121:3862-3875 (1999), which is hereby incorporated by reference in its entirety). The methods of the present invention afford
30 a more stable and irreversible bond as compared to the hydrazone strategy and are

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applicable to a broader range of peptide sequences. Another advantage of the methods of the present invention is that they more closely mimic the single and double bond patterns of the helix, whereas in the hydrozone approach, a double bond replaces the hydrogen bond and a carbon-carbon single bond replaces the carbonyl.



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BRIEF DESCRIPTION OF THE DRAWINGS

[0019] Figures 1A-C are schematic diagrams of α -helices. Figure 1A shows an unconstrained α -helix. Figure 1B shows an α -helix constrained via the hydrogen bond surrogate approach of the present invention. Figure 1C shows an α -helix constrained via a side-chain crosslinking strategy.

[0020] Figure 2 is a reverse-phase HPLC plot of purified constrained α -helix 3. The HPLC conditions were as follows: C18 reverse phase column; 20-100% B in 15 mins; A: 0.1% aqueous TFA, B: acetonitrile; flow rate: 0.2 mL/min.

[0021] Figure 3 is circular dichroism spectra of (i) α -helix 3 in 30 mM phosphate buffer (pH 7.0); (ii) α -helix 3 in 20% TFE/phosphate buffer; and (iii) unconstrained peptide 1 in phosphate buffer. The spectra were recorded at 25°C.

[0022] Figure 4 is a graph illustrating the effect of temperature on helicity of α -helix 3.

[0023] Figure 5 is a graph illustrating the effect of temperature on stability of α -helix 3.

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[0024] Figure 6 is a graph illustrating the effect of GnHCl on stability of α -helix 3.

[0025] Figure 7 is an NOE ROESY correlation chart for α -helix 3. The alanine-4 residue does not contain an NH. Filled rectangles indicate relative intensity of the NOE crosspeaks. Empty rectangles indicate NOE that could not be unambiguously assigned due to overlapping signals.

[0026] Figure 8 is an image of the TOCSY spectrum of α -helix 3. The region between 3.80 and 5.00 ppm has suppressed peaks in the 1D ^1H NMR due to the water peak suppression-related effects.

10 [0027] Figure 9 is an image depicting the amide NH-NH region from the ROESY spectrum of α -helix 3.

[0028] Figure 10 is an image depicting the amide NH-C α region from the ROESY spectrum of α -helix 3. * indicates NOE crosspeaks (at 8.07 ppm) that result from peptide aggregation over time (as deduced from comparison of NMR experiments run at lower pH's and temperatures).

[0029] Figure 11 is a schematic diagram illustrating four common peptide secondary structures.

[0030] Figure 12 is a schematic diagram illustrating the hydrogen bonding patterns of α - and 3_{10} -helices.

20 [0031] Figure 13 is a graph illustrating the circular dichroism spectra of peptides **9a**, **10a** and **10b** in 20% TFE/PBS.

[0032] Figure 14 is a graph illustrating the results of the fluorescence polarization assay used to test the competitive inhibition of the fl-Bak/Bcl-xL complex with Bak peptides **9a**, **10a** and **10b**.

25 [0033] Figures 15A-C are graphs illustrating the results of degradation studies on Bak BH3 peptide **9a** and internally constrained helices **10a** and **10b**. These results demonstrate the metabolic stability of artificial α -helices. HPLC assay shows rapid

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proteolysis of the unconstrained peptide **9a** in the presence of trypsin (Figure 15A), while the HBS α -helix **10a** degrades at a 30-fold slower rate (Figure 15B).

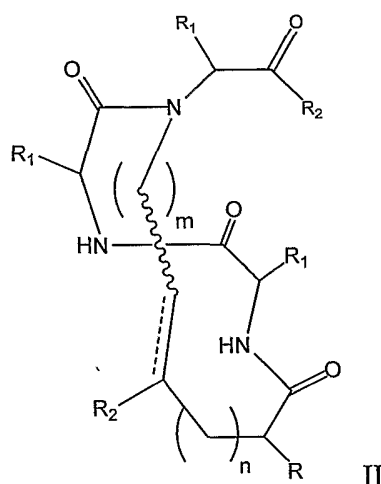
Tryptophan (500 μ M) was used as an internal control for the HPLC studies.

Comparison of the initial velocities for the proteolysis of **9a**, **10a**, and **10b** (Figure 5 15C) shows that **10b** is 60-fold more stable than **9a** toward proteolytic degradation.

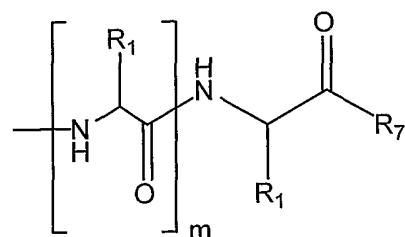
[0034] Figure 16 is a graph showing the saturation binding curve of fl-Bak **17** to Bcl-xL.

DETAILED DESCRIPTION OF THE INVENTION

[0035] The present invention relates to methods of preparing a compound of 10 Formula II:



where R is hydrogen, an amino acid side chain, an alkyl group, or an aryl group; R₁ is an amino acid side chain, an alkyl group, or an aryl group; R₂ is an amino acid, peptide, OR, CH₂NH₂, an alkyl group, an aryl group, or a group of formula



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where R₇ is an amino acid, peptide, OR, CH₂NH₂, an alkyl group, or an aryl group;

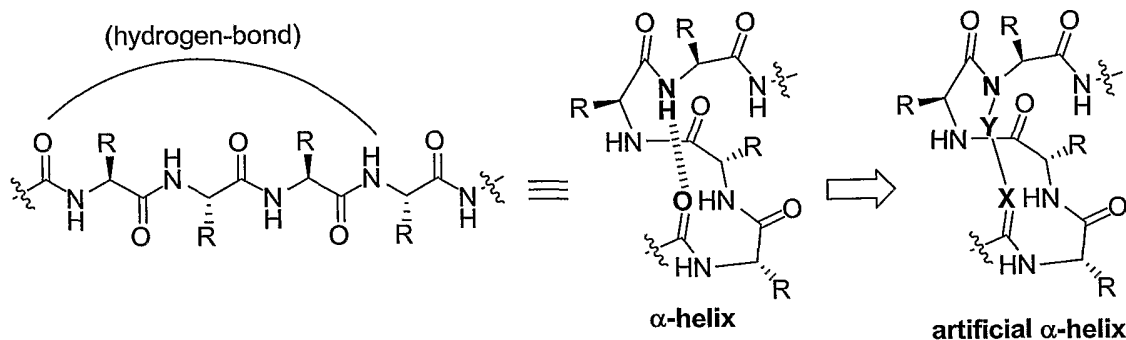
==== is a single or double carbon-carbon bond; ~~~~ is a single bond and is cis or trans when ===== is a double bond; n is 1 or 2; and m is any number.

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[0036] Protein secondary structures are defined by the hydrogen bonding patterns observed between the various main chain amide groups. Analyses of helix-coil transition in peptides emphasize the energetically demanding organization of three consecutive amino acids into the helical orientation as the slow step in helix formation (Qian & Schellman, *J. Chem. Phys.*, 96:3987-3994 (1992); Lifson & Roig, *J. Chem. Phys.*, 34:1963-1974 (1961); Zimm & Bragg, *J. Chem. Phys.*, 31:526-535 (1959), which are hereby incorporated by reference in their entirety). Preorganization of these amino acid residues is expected to overwhelm the intrinsic nucleation propensities and initiate helix formation (Austin et al., *J. Am. Chem. Soc.*, 119:6461-6472 (1997); Kemp et al., *J. Org. Chem.*, 56:6672-6682 (1991), which are hereby incorporated by reference in their entirety). In an α -helix, a hydrogen bond between the C=O of the i^{th} amino acid residue and the NH of the $i+4^{\text{th}}$ amino acid residue stabilizes and nucleates the helical structure (*see* Scheme 1 *infra*).

[0037] To mimic the C=O--H-N hydrogen bond as closely as possible, the main chain hydrogen bond surrogate strategies of this aspect of the present invention incorporate a covalent bond of the type C=X—Y-N, where X and Y correspond to part of the i and the $i+4$ residues, respectively, as shown in Scheme 1.

Scheme 1



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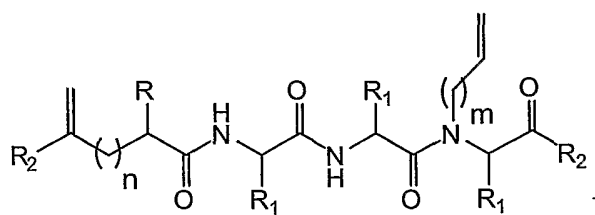
The internal placement of the crosslink allows the development of α -helices such that none of the exposed surfaces are blocked by the constraining element—i.e., placement of the crosslink on the inside of the helix does not alter side-chain functionality nor block solvent-exposed molecular recognition surfaces of the molecule, as shown in Figure 1 (*see* Sia et al., *Proc. Nat'l Acad. Sci. U.S.A.*, 99:14664-14669 (2002), which is hereby incorporated by reference in its entirety). Moreover, even very short

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peptides (i.e., peptides less than 10 amino acid residues) may be constrained into highly stable α -helices. The hydrogen-bond surrogate approach can also be used to develop sequence-specific ligands for RNA and DNA.

[0038] In one aspect of the present invention, preparing a compound of Formula II involves providing a compound of Formula I:



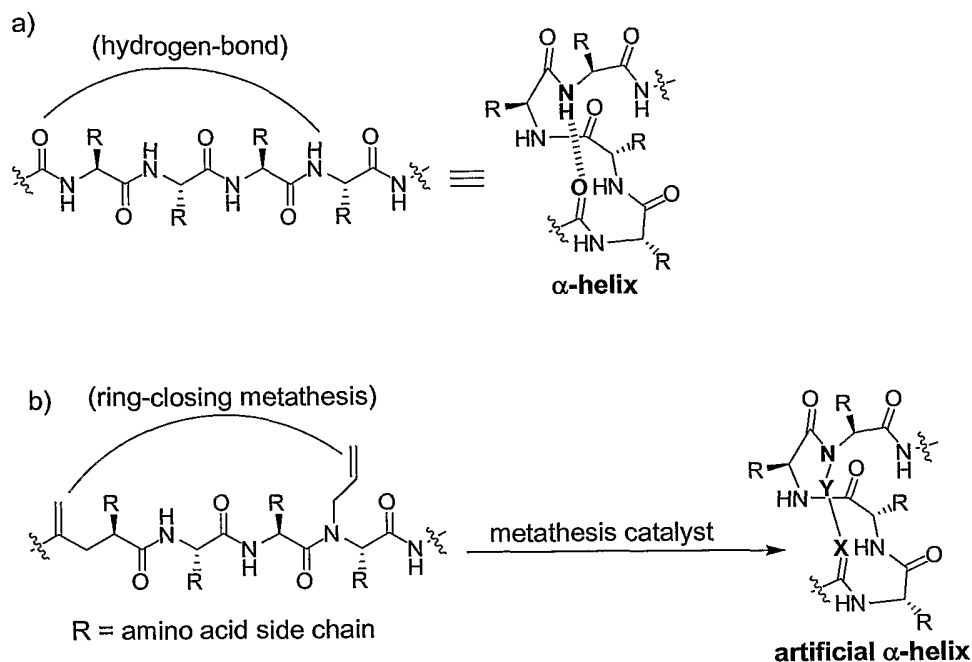
and reacting the compound of Formula I under conditions effective to produce a compound of Formula II.

[0039] To mimic the C=O--H-N hydrogen bond as closely as possible, the present invention incorporates a covalent bond of the type C=X—Y-N, where X and Y correspond to part of the i and the $i+4$ residues, respectively. 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).

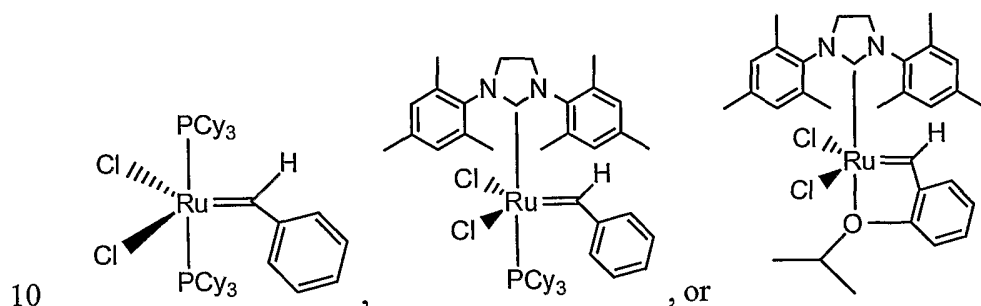
[0040] Scheme 2 illustrates a strategy for the stabilization of α -helices by replacement of an i and $i+4$ hydrogen bond (C=O—H-N) (Scheme 2A) with a covalent link (C=X—Y-N), for example a carbon-carbon bond through a ring-closing olefin metathesis reaction (Scheme 2B).

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Scheme 2

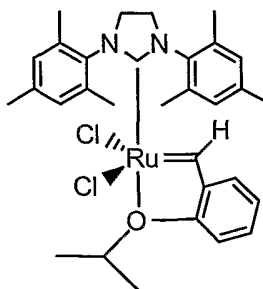


[0041] This aspect of the present invention may, preferably, involve a ring-closing olefin metathesis reaction. An olefin metathesis reaction couples two double bonds (olefins) to afford two new double bonds (one of which is typically ethylene gas). A ring-closing olefin metathesis utilizes an olefin metathesis reaction to form a macrocycle. In this reaction, two double bonds within a chain are connected. Preferably, the reaction is performed with a metathesis catalyst, for example of the formula



[0042] More preferably, the metathesis catalyst is of the formula

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[0043] 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.

5 [0044] The metathesis reaction may be performed with an organic solvent, such as dichloromethane, dichloroethane, trichloroethane, or toluene.

[0045] 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.

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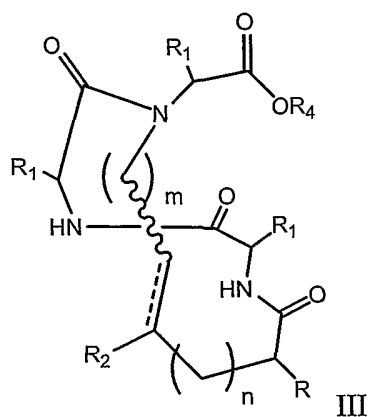
[0046] The metathesis reaction performed using a compound of Formula I will initially yield the compound of Formula II in which $\text{C}=\text{C}$ is a double bond. This double bond can be converted to a single bond by hydrogenation methods known in the art, after the peptide secondary structure is constrained.

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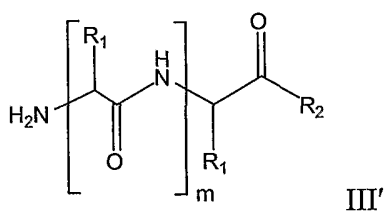
[0047] In another aspect of the present invention, illustrated in Scheme 3, preparing a compound of Formula II involves providing a compound of Formula III or a salt thereof

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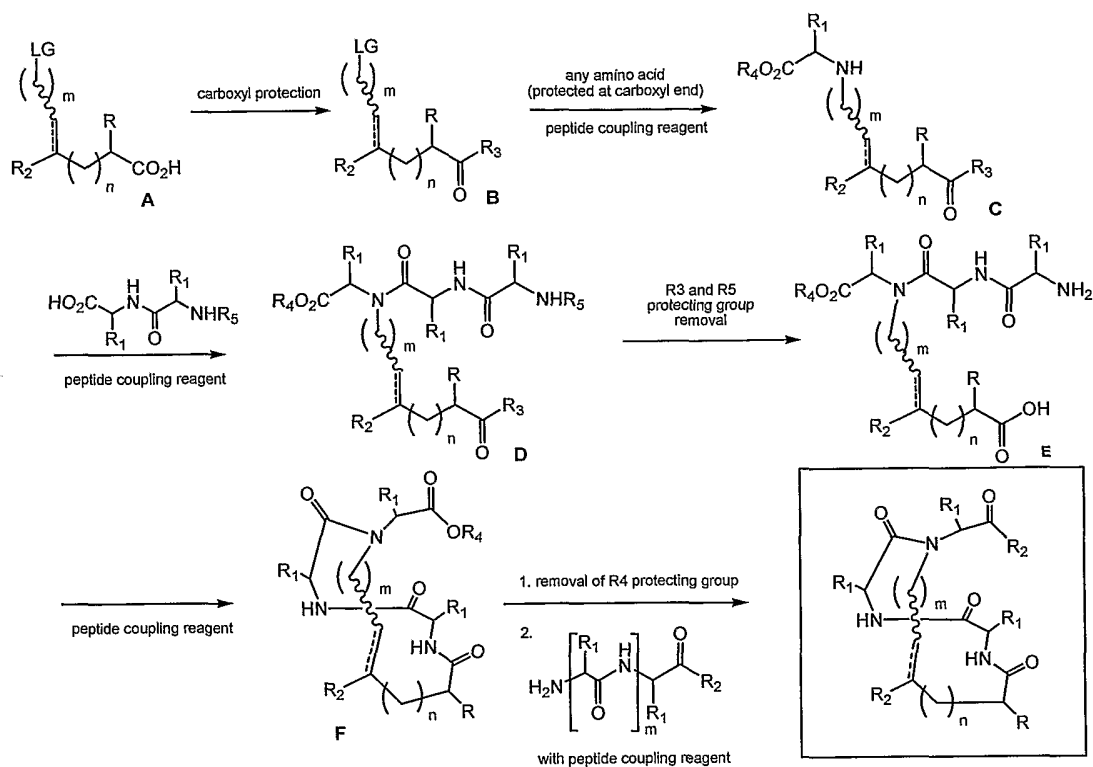
where R_4 is a carboxyl protecting group. According to this aspect of the present invention, the compound of Formula III or a salt thereof is reacted with a peptide coupling reagent of Formula III':



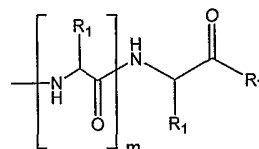
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under conditions effective to produce a compound of Formula II.

Scheme 3



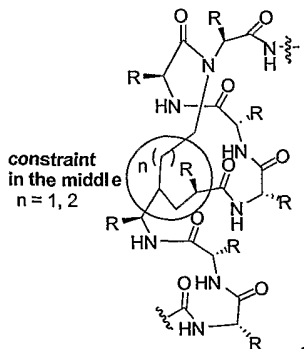
- 5 R = H, amino acid side chain, alkyl, or aryl
 R₁ = amino acid side chain, alkyl, or aryl
 R₂ = amino acid, peptide, OR, CH₂NH₂, alkyl, aryl, or compound of formula:
 R₃ = carboxyl protecting group
 R₄ = carboxyl protecting group that is not R₃
 R₅ = amine protecting group
 10 R₇ = amino acid, peptide, OR, CH₂NH₂, alkyl, or aryl
 n = 1 or 2
 m = any number



- [0048] The carboxylic acid **A** is protected as an ester (for example a methyl or
 15 ethyl ester), yielding compound **B**. This compound **B** is then condensed to
 commercially available amino acids, generating amine **C**. Amine **C** is then coupled to
 the dipeptide by standard coupling agents such as DCC/HOBT or 5%DIEA/HBTU,
 yielding compound **D**. The protecting groups R₃ and R₅ are removed to afford
 compound **E**, which is cyclized with peptide coupling agents to generate peptide **F**.
 20 Removal of the carboxylic acid protecting group R₄ exposes the carboxylic acid,
 which can be coupled to any amino acid to obtain the desired peptide.

[0049] 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, β -sheets/ β -turns, 3_{10} -helices, and π -helices. The

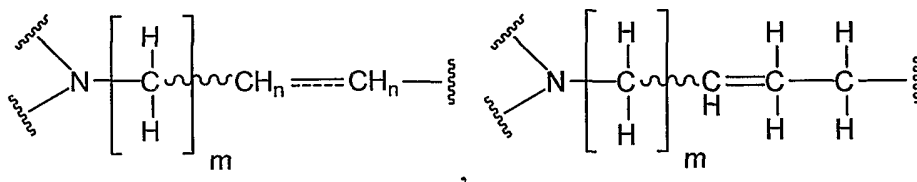
constraint may be placed anywhere within the peptide, not just at the N-terminus. For example, the compound of Formula II prepared according to the methods of the present invention may have the formula



5 [0050] 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.

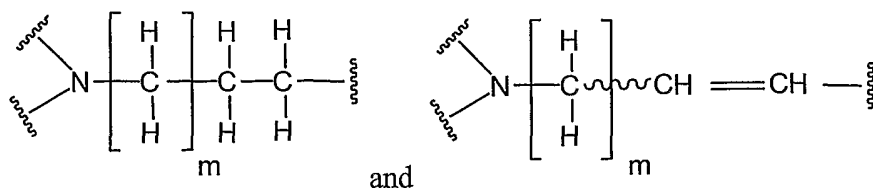
[0051] The present invention also relates to peptides having one or more stable, internally-constrained α -helices, β -sheets/ β -turns, 3_{10} -helices, or π -helices.

10 The one or more stable, internally-constrained secondary structures includes the following motifs:

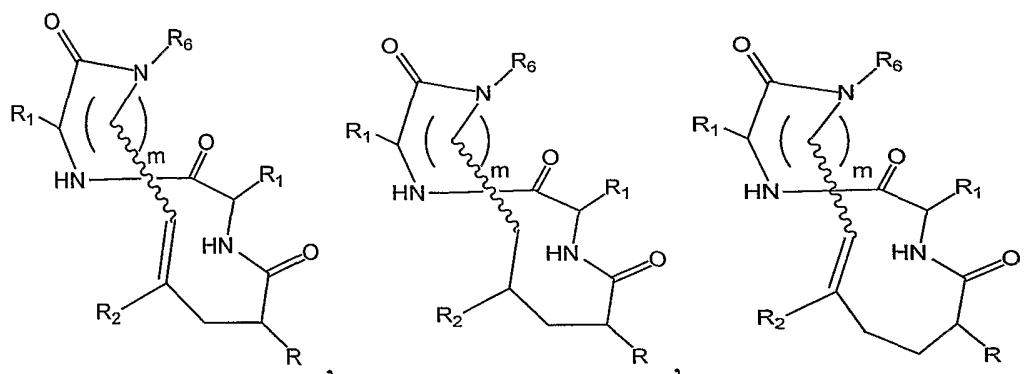


where ===== is a single or double bond, ~~~~~ is a single bond and is cis or trans

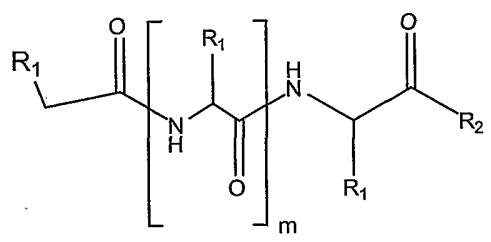
15 when ===== is a double bond; n is 1 or 2; and m is any number. Examples of such motifs include:



[0052] Exemplary peptides according to the present invention include peptides of the following formulae:



- 5 where R is hydrogen, an amino acid side chain, an alkyl group, or an aryl group; R₁ is an amino acid side chain, an alkyl group, or an aryl group; R₂ is an amino acid, peptide, OR, CH₂NH₂, an alkyl group, or an aryl group; and R₆ is (a) a chain of one or more amino acids; or (b) a side chain of formula:



- 10 ==== 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. More specific examples of such peptides are shown in Table 1.

Table 1. Exemplary Peptides

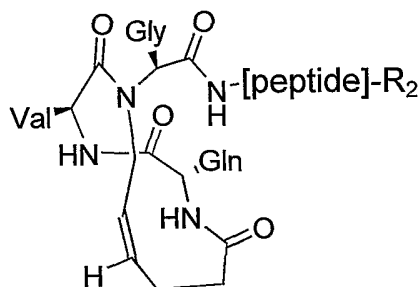
entry	peptide	target
1	GQVGRQLAIIGDDINR (SEQ ID NO: 1)	Bcl-xL/Bak BH3
2	GQVGRQLAI (SEQ ID NO: 2)	Bcl-xL/Bak BH3
3	GRQLAIIGDDINR (SEQ ID NO: 3)	Bcl-xL/Bak BH3
4	KETAAAKFEREHMDS (SEQ ID NO: 4)	RNase S protein/C-peptide
5	MKQLEDK (SEQ ID NO: 5)	GCN4 coiled-coil
6	VEELLSK (SEQ ID NO: 6)	GCN4 coiled-coil
7	QYHLEQE (SEQ ID NO: 7)	GCN4 coiled-coil
8	VARLKKL (SEQ ID NO: 8)	GCN4 coiled-coil
9	MKQLEDKVEELLSK (SEQ ID NO: 9)	GCN4 coiled-coil
10	VEELLSKQYHLEQE (SEQ ID NO: 10)	GCN4 coiled-coil
11	QYHLEQEVARLKKL (SEQ ID NO: 11)	GCN4 coiled-coil
12	mimics of Zinc finger recognition helix	DNA
13	EPGXLVR (X=N,H,A,E) (SEQ ID NO: 12)	DNA
14	TRQARRNRWRERQR (SEQ ID NO: 13)	RRE RNA
15	SQETFSDLWKLLENV (SEQ ID NO: 14)	p53/MDM2
16	QQLEEDLKGYLWITQ (SEQ ID NO: 15)	calcium ion channel (AID)
17	RIARLEEKVK (SEQ ID NO: 16)	Jun/Fos
18	hex-RIARLEEKVK (SEQ ID NO: 17)	Jun/Fos
19	ELASTANALRE (SEQ ID NO: 18)	Jun/Fos
20	QVAQLKQKVA (SEQ ID NO: 19)	Jun/Fos
21	ELASTANALREQVAQLKQKVAAY (SEQ ID NO: 20)	Jun/Fos
22	RIARLEEKVKTLKAQN (SEQ ID NO: 21)	Jun/Fos
23	EVAQLEDEKSALQ (SEQ ID NO: 22)	Jun/Fos
24	WAAWDREINNYT (SEQ ID NO: 23)	HIV gp41
25	WAAWDREIN (SEQ ID NO: 24)	HIV gp41
26	PRGTRGKGRIR (SEQ ID NO: 25)	HIV TAR RNA
27	VKKITVSIXXXISVTIKK (X= any amino acid) (SEQ ID NO: 26)	Met repressor
28	PQFNLRXXXTRLNFQP (X= any amino acid) (SEQ ID NO: 27)	Arc repressor

Although the peptides are disclosed in Table 1 in linear sequence form, they may have one or more stable, internally-constrained secondary structures (i.e., α -helices, β -sheets/ β -turns, 3_{10} -helices, or π -helices) at any location within the peptide, as will be apparent to one of skill the art. For example, Entry 1 (SEQ ID NO: 1) may have an artificial α -helix spanning residues 1–5, where G1 and R5 are i and $i+4$, respectively (see, e.g., Scheme 1 and accompanying text), constrained by methods described herein. Alternatively or additionally, for example, Entry 1 (SEQ ID NO: 1) may have an artificial α -helix spanning residues 12–16, where D12 and R16 are i and $i+4$, respectively. By way of another example, Entry 27 (SEQ ID NO: 26) may be an artificial internally-constrained beta-sheet with the constraint spanning residues 1–4, where X9 and X12 are i and $i+3$, respectively.

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[0053] Yet another aspect of the present invention relates to a method for promoting cell death. This method involves contacting a cell with one or more Bak BH3 peptides having a stable, internally-constrained alpha-helical region under conditions effective for the one or more Bak BH3 peptides to promote cell death. In this aspect of the present invention, one or more hydrogen bonds within the one or more Bak BH3 peptides are replaced with a carbon-carbon bond.

[0054] Suitable Bak BH3 peptides include:



where [peptide] is RQLAIIGDDINR (SEQ ID NO: 28) or RQLAIIGDK^{Ac}INR (SEQ ID NO: 29); and R₂ is (a) a peptide, (b) OR where R is an alkyl group, (c) CH₂NH₂, (d) an alkyl group, or (e) an aryl group.

[0055] The methods of the present invention relating to contacting a cell with one or more peptides may be carried out *in vitro* or *in vivo*.

[0056] When contacting is carried out *in vivo*, contacting may comprise administering to a subject a compound that includes one or more peptides of the present invention. The compounds of the present invention can be administered orally, parenterally, for example, subcutaneously, intravenously, intramuscularly, intraperitoneally, by intranasal instillation, or by application to mucous membranes, such as, that of the nose, throat, and bronchial tubes. They may be administered alone or with suitable pharmaceutical carriers, and can be in solid or liquid form such as, tablets, capsules, powders, solutions, suspensions, or emulsions.

[0057] The active compounds of the present invention 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

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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 active compound. The percentage of the compound 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 active compound in such therapeutically useful compositions is such that a suitable dosage will be obtained. Preferred compositions according to the present invention are prepared so that an oral dosage unit contains between about 1 and 250 mg of active compound.

[0058] 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, 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.

[0059] 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, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye, and flavoring such as cherry or orange flavor.

[0060] These active compounds may also be administered parenterally. Solutions or suspensions of these active compounds 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 solution, 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.

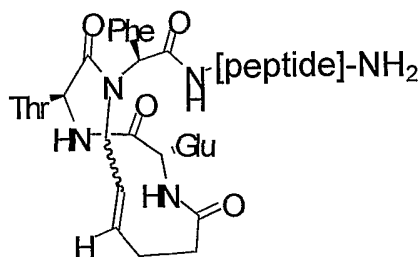
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[0061] The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile 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.


10 [0062] The compounds 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.

[0063] Yet another aspect of the present invention relates to a method for promoting cell death. This method involves contacting a cell with one or more peptides that inhibits p53/hDM2 interaction and has a stable, internally-constrained alpha-helical region, under conditions effective for the one or more peptides to promote cell death. In this aspect of the present invention, one or more hydrogen bonds within the one or more peptides are replaced with a carbon-carbon bond.

[0064] Suitable p53/hDM2 peptides include:



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where  is a cis or trans alkene and [peptide] is SDLWKLLP (SEQ ID NO: 30).

[0065] The p53/hDM2 interaction is known to stop apoptosis and lead to uncontrolled growth (a characteristic of cancer). The internally-constrained artificial p53/hDM2 shown above mimics a portion of p53 protein that binds to hDM2, and is expected to block p53/hDM2 interaction and induce apoptotic activity in cancer cells (Chene, P, "Inhibiting the p53-MDM2 Interaction: An Important Target For Cancer Therapy," *Nat. Rev. Cancer* 3:102-109 (2003); Chene et al., "Study of the Cytotoxic Effect of a Peptidic Inhibitor of the p53-HDN2 Interaction in Tumor Cells," *FEBS Lett.* 529:293-297 (2002); Garcia-Echeverria et al., "Discovery of Potent Antagonists of the Interaction between Human Double Mminute 2 and Tumor Suppressor p53," *J. Medicinal Chemistry* 43:3205-3208 (2000); Kritzer et al., "Helical α -peptide Inhibitors of the p53-hDM2 Interaction," *J. Am. Chem. Soc.* 126:9468-9469 (2004); Kussie et al., "Structure of the MDM2 Oncoprotein Bound to the p53 Tumor Suppressor Transactivation Domain," *Science* 274: 948-953 (1996); Vassilev et al. "In Vivo Activation of the p53 Pathway by Small-molecule Antagonists of MDM2," *Science* 303:844-848 (2004); Yin et al., "Terphenyl-based Helical Mimetics That Disrupt the p53/HDM2 Interaction," *Angew Chem. Int. Ed.* 44:2704-2707 (2005), which are hereby incorporated by reference in their entirety).

[0066] When using this method to treat a subject, the above-mentioned modes and forms of administering are used to contact the cell with the one or more peptides.

[0067] Yet another aspect of the present invention relates to a method for promoting cell death. This method involves contacting the cell with one or more peptides which inhibits Jun-Jun and/or Jun-Fos interactions and has a stable, internally-constrained alpha-helical region under conditions effective for the one or more peptides to promote cell death. In this aspect of the present invention, one or more hydrogen bonds within the one or more peptides are replaced with a carbon-carbon bond.

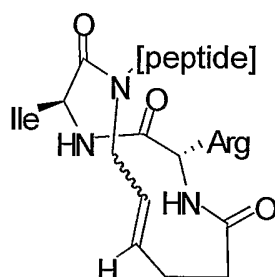
[0068] Jun and Fos belong to the basic leucine zipper (bZIP) DNA binding family of proteins, and regulate cell proliferation, apoptosis and transformation

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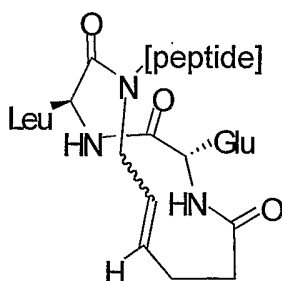
(Angel & Karin, "The Role of Jun, Fos and the AP-1 Complex in Cell-proliferation and Transformation," *Biochim. Biophys. Acta* 1072: 129-157 (1991); Chen et al., "Structure of the DNA-binding Domains from NFAT, Fos and Jun Bound Specifically to DNA," *Nature* 392:42-48 (1998); Daugherty & Gellman, "A Fluorescence Assay for Leucine Zipper Dimerization: Avoiding Unintended Consequences of Fluorophore Attachment," *J. Am. Chem. Soc.* 121:4325-4333 (1999); Glover & Harrison et al., "Crystal Structure of the Heterodimeric bZIP Transcription Factor c-Fos-c-Jun Bound to DNA," *Nature* 373:257-261 (1995); O'Shea et al., "Mechanism of Specificity in the Fos-Jun Oncoprotein Heterodimer," *Cell* 68:699-708 (1992), which are hereby

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incorporated by reference in their entirety). Internally-constrained artificial alpha-helices that target these coiled-coil proteins may be prepared in accordance with the methods of the present invention. Suitable peptides that inhibit Jun-Jun and/or Jun-Fos interactions include:



15 where \sim is a cis or trans alkene and [peptide] is ARLEEKVKTLLKAQNS (SEQ ID NO: 31) and



where [peptide] is STANALREQVAQLKQKV (SEQ ID NO: 32).

[0069] When using this method to treat a subject, the above-mentioned modes and forms of administering are used to contact the cell with the one or more peptides.

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[0070] Yet another aspect of the present invention relates to a method for inhibiting cell proliferation. This method involves contacting the cell with one or more peptides which inhibits Jun-Jun and/or Jun-Fos interactions and has a stable, internally-constrained alpha-helical region under conditions effective for the one or more peptides to inhibit cell proliferation. In this aspect of the present invention, one or more hydrogen bonds within the one or more peptides are replaced with a carbon-carbon bond.

[0071] Suitable peptides which inhibit Jun-Jun and/or Jun-Fos interactions include those mentioned above.

10 [0072] When using this method to treat a subject, the above-mentioned modes and forms of administering are used to contact the cell with the one or more peptides.

[0073] Yet another aspect of the present invention relates to a method for inhibiting cell transformation. This method involves contacting the cell with one or more peptides which inhibits Jun-Jun and/or Jun-Fos interactions and has a stable, internally-constrained alpha-helical region under conditions effective for the one or more peptides to inhibit cell transformation. In this aspect of the present invention, one or more hydrogen bonds within the one or more peptides are replaced with a carbon-carbon bond.

[0074] Suitable peptides which inhibit Jun-Jun and/or Jun-Fos interactions include those mentioned above.

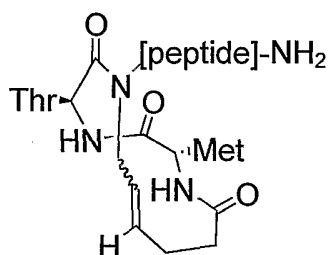
[0075] When using this method to treat a subject, the above-mentioned modes and forms of administering are used to contact the cell with the one or more peptides.


[0076] Yet another aspect of the present invention relates to a method for inhibiting HIV-1 entry into a cell. This method involves contacting the cell with one or more peptides having a stable, internally-constrained alpha-helical region under conditions effective for the one or more peptides to inhibit HIV-1 entry into the cell. In this aspect of the present invention, one or more hydrogen bonds within the one or more peptides are replaced with a carbon-carbon bond.

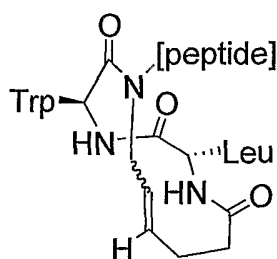
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[0077] The role of coiled-coil domains in the fusion of HIV-1 to the host cell membrane has been the subject of intense research activity. Viral fusion is mediated by glycoproteins on the virus surface that contain an α -helical coiled-coil domain. α -Helical peptides that mimic portions (C-terminal heptad repeat of gp41) of this coiled-coil complex can disrupt coiled-coil formation and have been shown to be potent inhibitors of HIV-1 infection *in vitro* and *in vivo*. It has been postulated that stabilized α -helices corresponding to this C-terminal heptad repeat sequence (C-peptides) may be more potent inhibitors than the unconstrained peptide, which adopts a random coil conformation in solution. (Eckert et al., "Mechanisms of Viral Membrane Fusion and its Inhibition," *Annu. Rev. Biochem.* 70:777-810 (2001); Kilby et al., "Potent Suppression of HIV-1 Replication in Humans by T-20, a Peptide Inhibitor of gp41-Mediated Virus Entry," *Nat. Med.* 4:1302-1307 (1998); Sia et al., "Short Constrained Peptides that Inhibit HIV-1 Entry," *Proc. Nat'l Acad. Sci. USA* 99:14664-14669 (2002); Wild et al., "Peptides Corresponding to a Predictive Alpha-helical Domain of Human Immunodeficiency Virus Type 1 gp41 are Potent Inhibitors of Virus Infection," *Proc. Nat'l Acad. Sci. USA* 91:9770-9774 (1994), which are hereby incorporated by reference in their entirety.)

[0078] Suitable peptides include:



20 wherein  is a cis or trans alkene and [peptide] is WMEWDREINNYT (SEQ ID NO: 33), and



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where [peptide] is NWFNI (SEQ ID NO: 34). These peptides are expected to target gp41 and block its interactions with CD4.

[0079] When using this method to treat a subject, the above-mentioned modes and forms of administering are used to contact the cell with the one or more peptides.

5 [0080] The present invention may be further illustrated by reference to the following examples.

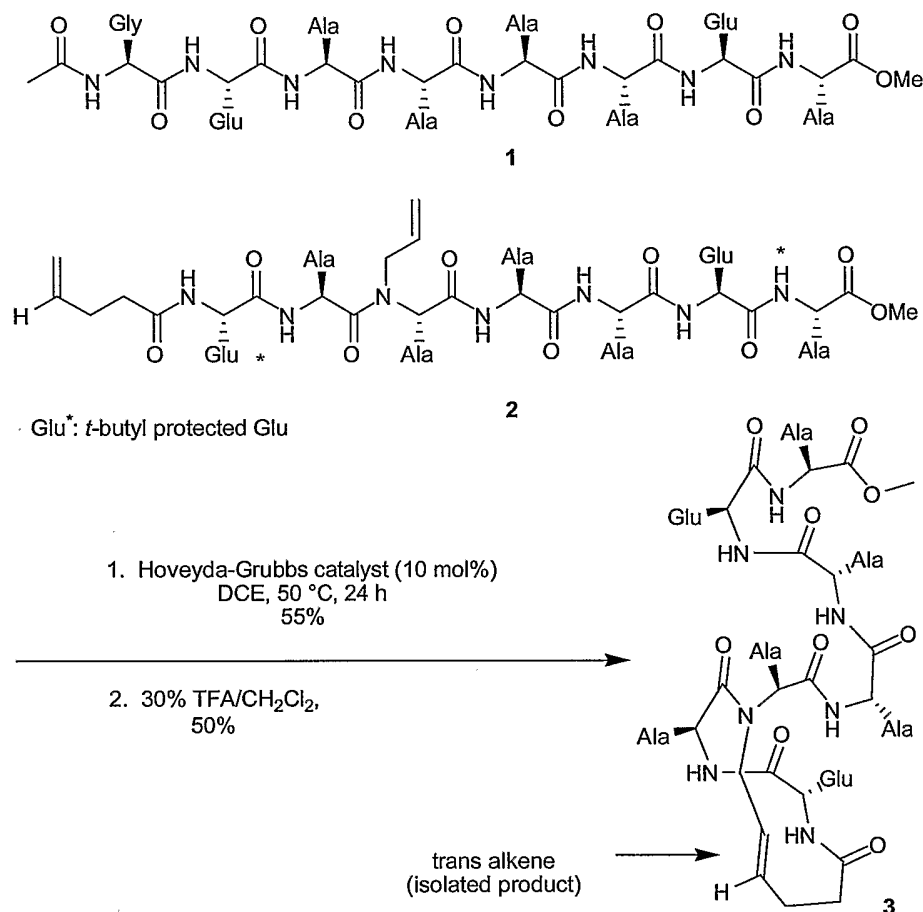
EXAMPLES

Example 1 -- Synthesis of an Internally-constrained α -Helix

[0081] To test the stabilization properties of the metathesis-derived internal
10 crosslinks, an 8-mer constrained peptide **3** bearing a crosslink at the *N*-terminus was synthesized as shown in Scheme 4. This particular peptide was chosen because the control peptide **1** (AcGEAAAAEA-OMe (SEQ ID NO: 35)) does not display any α -helicity, thus allowing for an observation of an increase in α -helical content following the modification. Two glutamic acid residues were incorporated in this alanine-rich
15 peptide at different faces of the putative helix to increase the solubility of the constrained peptide in aqueous buffers. The metathesis precursor peptide **2** underwent a ring-closing metathesis reaction with Hoveyda-Grubbs metathesis catalyst (Hoveyda et al., "Ru Complexes Bearing Bidentate Carbenes: From Innocent Curiosity to Uniquely Effective Catalysts for Olefin Metathesis," *Org. Biomolec.*
20 *Chem.* 2:8-23 (2004), which is hereby incorporated by reference in its entirety) to afford the trans-alkene constrained peptide **3** after the removal of the *t*-butyl esters.

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Scheme 4

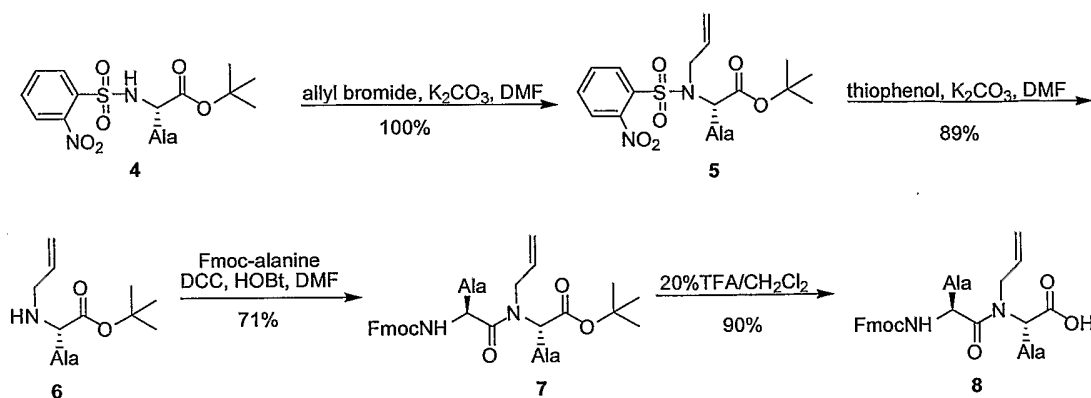


Peptide Synthesis

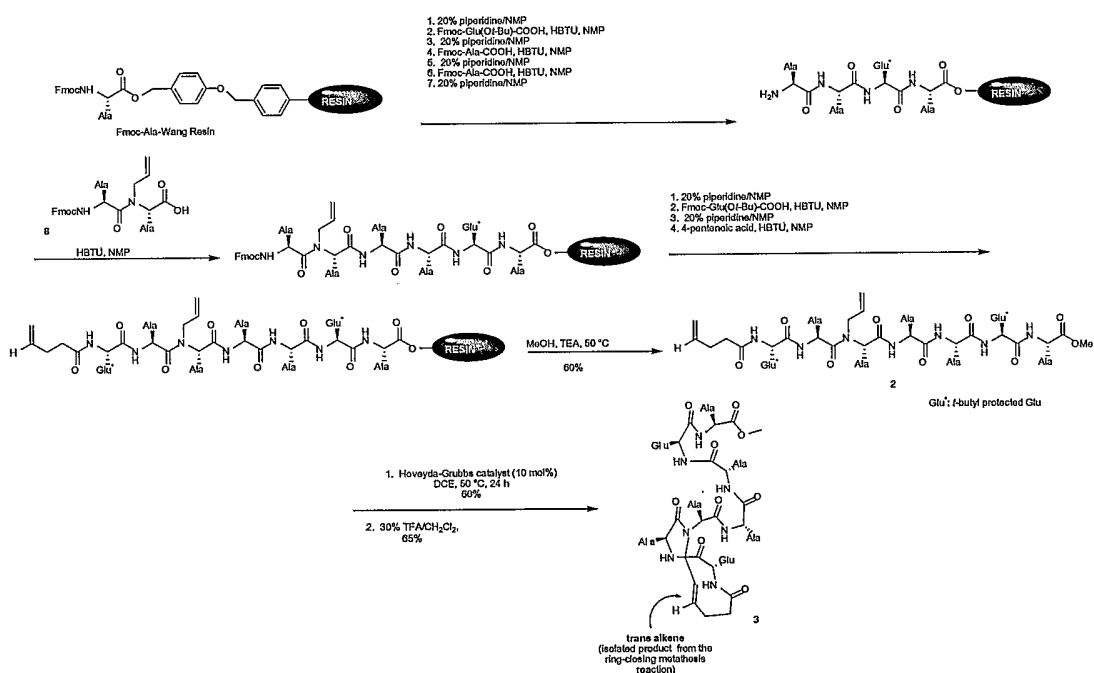
[0082] As illustrated in Schemes 5 and 6, peptides were synthesized using standard Fmoc solid-phase methodology on Wang resin. The Fukuyama alkylation method has been used extensively to prepare allyl-bearing peptides on resin (Miller et al., "A New Method for Solid-phase Peptide Synthesis," *J. Am. Chem. Soc.* 120:2690-2691 (1998), which is hereby incorporated by reference in its entirety). As shown in Scheme 5, automated solid-phase synthesis was carried out with Fmoc-dipeptide **8**, because prior preparation of the alkylated amino acid reduces the synthetic procedure to high yielding reactions optimized for solid phase peptide synthesis. Dimer **8** was synthesized from *t*-butyl alanine in five steps with the Fukayama alkylation as the key step.

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Scheme 5



Scheme 6



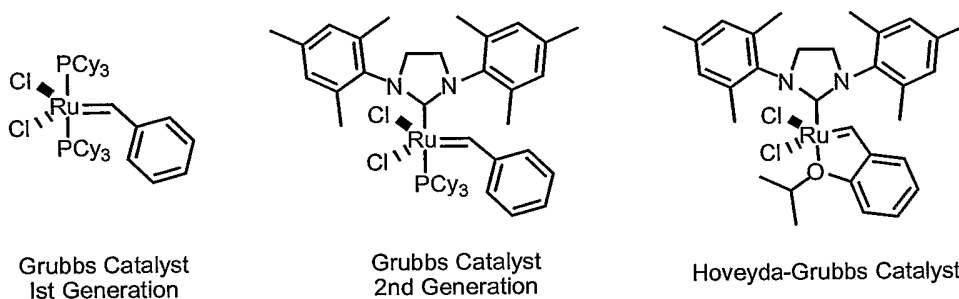
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[0083] The last step of the peptide synthesis included coupling with 4-pentenoic acid, which serves as an alkene-bearing glycine mimic. The peptide was cleaved from the resin as a C-terminal methyl ester with methanol and triethylamine, as shown in Scheme 6. These cleavage conditions were used to obtain peptide 2 with the two glutamic acid side chains protected as the *t*-butyl esters. The protecting groups were kept to increase the solubility of the peptide in organic solvents for the metathesis step and because carboxylic acids are known to react with the metathesis

catalyst (Trnka et al., "The Development of L₂X₂Ru = CHR Olefin Metathesis Catalysts: An Organometallic Success Story," *Accounts Chem. Res.* 34:18-29 (2001), which is hereby incorporated by reference in its entirety).

Ring-Closing Metathesis Reaction

- 5 [0084] The ring-closing metathesis ("RCM") step was found to be challenging, and different RCM catalysts (including, for example, Grubbs 1st generation, Grubbs 2nd generation, and Hoveyda-Grubbs),



- various reaction temperatures (including, for example, 25°C, 40°C, 50°C, 80°C, and 10 110°C), and solvent systems (including, for example, dichloromethane, dichloroethane, trichloroethane, and toluene) were used to optimize the metathesis reaction (Trnka et al., "The Development of L₂X₂Ru = CHR Olefin Metathesis Catalysts: An Organometallic Success Story," *Accounts Chem. Res.* 34:18-29 (2001); Hoveyda et al., "Ru Complexes Bearing Bidentate Carbenes: From Innocent Curiosity to Uniquely Effective Catalysts for Olefin Metathesis," *Org. Biomolec. Chem.* 2:8-23 15 (2004), which are hereby incorporated by reference in their entirety). The Hoveyda-Grubbs catalyst was found to afford the highest yield for the metathesis step (40-65% after HPLC purification). The Grubbs second generation catalyst afforded the metathesis product in 5-20% yields.

- 20 [0085] Typical RCM reactions yield a mixture of *cis* and *trans* alkene isomers, favoring *trans* for large macrocycles. RCM studies performed on a closely related 13-membered macrocyclization system have been reported to yield a *trans* to *cis* ratio of ≥4:1 (Banerji et al., "Synthesis of Cyclic Beta-turn Mimics from L-Pro-Phe/Phe-L-Pro Derived Di- and Tripeptides Via Ring Closing Metathesis: The Role of Chirality of the Phe Residue During Cyclization," *Tetrahedron Lett.* 43:6473-6477 (2002), 25 which is hereby incorporated by reference in its entirety). HPLC analysis of the crude

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mixture showed a new peak corresponding to the *trans* isomer as indicated by ^1H NMR spectroscopy and analysis of the alkene proton coupling constraints (16.2 Hz). The *cis* isomer was likely formed in small amounts, but its presence was not detected by HPLC. The macrocyclized peptide was purified by prep-HPLC and treated with TFA to remove the side chain protecting groups. The fully deprotected and constrained peptide was purified again by HPLC to obtain constrained peptide 3 in an overall yield of 50% from the linear peptide 2.

Materials and Methods

[0086] Commercial-grade reagents and solvents were used without further purification except as indicated. CH_2Cl_2 and DMF were dried prior to use by percolation through anhydrous Al_2O_3 as described by Grubbs and coworkers (Pangborn et al., *Organometallics*, 15:1518-1520 (1996), which is hereby incorporated by reference in its entirety). All reactions were stirred magnetically; moisture-sensitive reactions were performed under nitrogen in flame-dried glassware. Thin-layer chromatography (TLC), usually using either ethyl acetate/hexane or methanol/ CH_2Cl_2 as the solvent system, was used to monitor reactions. Visualization was accomplished by either ultraviolet light or by immersing the plate in a 1% aqueous solution of potassium permanganate and heating. Flash chromatography with silica gel was performed following the conditions described by Still and coworkers (Burfield & Smithers, *J. Org. Chem.*, 43:3966-3968 (1978), which is hereby incorporated by reference in its entirety). Solvents were removed by rotary evaporation under reduced pressure; where appropriate, the residue was further dried using a vacuum pump. Reverse-phase HPLC experiments were conducted with 4.6 x 150 mm (analytical scale) or 21.4 x 150 mm (preparative scale) Waters C₁₈ reverse phase columns using a Beckman Coulter HPLC equipped with a System Gold 168 Diode array detector. The typical flow rates for analytical and preparative HPLC were 1 mL/min and 8 mL/min, respectively. In all cases, 0.1% aqueous TFA and acetonitrile buffers were used. Proton NMR spectra were obtained on a Bruker AV-400 (400 MHz), Bruker AV-500 (500 MHz), or Varian-200 (200 MHz) spectrometer. Carbon NMR spectra were obtained on a Bruker (100.5 MHz) spectrometer. Proton chemical shifts are reported as δ values relative to tetramethylsilane (0.00 ppm) or to

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the particular solvent used in the experiment (CDCl₃: 7.26 ppm or D₂O: 4.80 ppm). Carbon chemical shifts are reported as δ values relative to the particular solvent used in the experiment (CDCl₃: 77.0 ppm). Data is reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet, br = broad), coupling constant, and integration. Infrared (IR) spectra were obtained with a Thermo Nicolet Avatar 360 FTIR. High-resolution mass spectra (HRMS) were obtained by fast atom bombardment (FAB) of samples in *m*-nitrobenzyl alcohol with Cs⁺ ions. FAB experiments were performed by MSU-NIH Mass Spectrometry Facility, East Lansing, MI. LCMS data was obtained on an Agilent 1100 series LC/MSD (XCT) electrospray trap.

Synthesis of Compound 5

[0087] To synthesize *o*-NBS-N(Allyl)-Ala-OtBu **5**, allyl bromide (0.650 mL, 7.60 mmol) was added to a solution of *o*-NBS-Ala-OtBu **4** (Turner et al., *Tetrahedron Lett.*, 40:7039-7042 (1999), which is hereby incorporated by reference in its entirety) (1.36 g, 4.12 mmol) and K₂CO₃ (1.15 g, 8.34 mmol) in dry DMF (20 mL). The mixture was stirred overnight, and then poured into water (20 mL). The aqueous layer was extracted with ether (3 x 30 mL) and the combined organic layers were dried (MgSO₄), filtered, and concentrated *in vacuo* to afford 1.53 g of compound **5** (100%) as a pale yellow solid (¹H NMR (400 MHz, CDCl₃) δ 8.10-8.05 (m, 1H), 7.69-7.66 (m, 2H), 7.62-7.59 (m, 1H), 5.85 (ddt, *J* = 17.2, 10.2, 6.74 Hz, 1H), 5.20 (dd, *J* = 10.2, 1.3 Hz, 1H), 5.08 (dd, *J* = 17.2 Hz, *J* = 1.2, 1H), 4.69 (q, *J* = 7.3 Hz, 1H), 4.18 (dd, AB pattern, *J* = 16.8, 6.2 Hz, 1H), 3.84 (dd, AB pattern, *J* = 16.9, 6.2 Hz, 1H), 1.48 (d, *J* = 7.3 Hz, 3H), 1.35 (s, 9H); ¹³C NMR (100 MHz) δ 170.26, 148.03, 135.11, 133.99, 133.37, 131.60, 131.27, 124.06, 117.86, 82.07, 56.64, 48.52, 27.80, 16.86; IR (film) 1733 cm⁻¹; HRMS (FAB) *m/z* for C₁₆H₂₃N₂O₆S [M+H]⁺, calcd 371.1229, found 371.1277).

Synthesis of Compound 6

[0088] To synthesize HN(Allyl)-Ala-OtBu **6**, thiophenol (0.135 mL, 1.32 mmol) was added to a solution of *o*-NBS-N(Allyl)-Ala-OtBu **5** (0.400 g, 1.09 mmol) and K₂CO₃ (0.450 g, 3.25 mmol) in DMF (3 mL). After 2 hours, the reaction mixture was poured into water (3 mL). The aqueous layer was extracted with ether (3 x 30

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mL), and the combined organic layers were extracted with 1 M HCl (3 x 25 mL). The pH of the combined aqueous layers was adjusted with saturated aqueous NaHCO₃ to pH 7.5. The aqueous layer was then extracted with ether (3 x 30 mL), dried (MgSO₄), filtered, and concentrated to yield 0.18 g of compound **6** (89%) as a yellow oil (¹H NMR (400 MHz, CDCl₃) δ 5.88 (ddt, *J* = 17.1, 10.2, 6.1 Hz, 1H), 5.19 (dd, *J* = 17.1, 1.5 Hz, 1H), 5.09 (dd, *J* = 10.2, 1.5 Hz, 1H), 3.29-3.11 (m, 3H), 1.47 (s, 9H), 1.26 (d, *J* = 7.0 Hz, 3H); ¹³C NMR (100 MHz) δ 175.06, 136.41, 116.11, 80.77, 56.47, 50.47, 28.44, 19.07; IR (film) 3299, 1723 cm⁻¹; HRMS (FAB) *m/z* for C₁₀H₂₀NO₂ [M+H]⁺, calcd 186.1448, found 186.1494).

10 *Synthesis of Compound 7*

[0089] To synthesize Fmoc-Ala-N(Allyl)-Ala-OtBu **7**, *N,N*-Dicyclohexylcarbodiimide (0.250 g, 1.19 mmol) was added to a solution containing Fmoc-Ala-OH (0.370 g, 1.19 mmol) and 1-hydroxybenzotriazole (0.160 g, 1.19 mmol) in DMF (10 mL). The mixture was stirred for 10 minutes and then compound **6** (0.202 g, 1.09 mmol) was added. After 18 hours, the reaction mixture was filtered, poured into 10 mL water, and extracted with ether (3 x 30 mL). The organic layer was dried (MgSO₄), filtered, and concentrated. The crude product was purified by flash chromatography (5:1 hexane-ethyl acetate) to afford 0.37 g of compound **7** (71 %) as a colorless oil (¹H NMR (400 MHz, CDCl₃) δ 7.75 (d, *J* = 7.5 Hz, 2H), 7.59 (d, *J* = 7.3 Hz, 2H), 7.38 (t, *J* = 7.4 Hz, 2H), 7.29 (t, *J* = 7.4 Hz, 2H), 5.90 (ddt, *J* = 16.9, 10.1, 8.1 Hz, 1H), 5.73 (d, *J* = 8.1 Hz, 1H) 5.23-5.31 (m, 2H), 4.72-4.79 (m, 1H), 4.63 (quintet, *J* = 7.1 Hz, 1H), 4.31-4.44 (m, 2H), 4.21 (t, *J* = 7.1 Hz, 1H), 4.05 (dd, AB pattern, *J* = 17.2, *J* = 4.4, 1H), 3.87 (dd, AB pattern *J* = 17.5, 5.0 Hz, 1H), 1.44 (s, 9H), 1.39 (d, *J* = 3.0 Hz, 3H), 1.37 (d, *J* = 2.3 Hz, 3H); ¹³C NMR (100 MHz) δ 173.15, 170.58, 155.44, 143.96, 141.29, 133.86, 127.68, 127.05, 125.18, 119.96, 117.63, 81.59, 66.92, 54.33, 48.32, 47.41, 47.17, 27.99, 19.41, 14.62; IR (film) 3299, 1730 cm⁻¹; HRMS (FAB) *m/z* for C₂₈H₃₅N₂O₅ [M+H]⁺ calcd 479.2501, found 479.2546).

Synthesis of Dipeptide 8

[0090] To synthesize Fmoc-Ala-N(Allyl)-Ala-OH **8**, Fmoc-Ala-N(Allyl)-Ala-OtBu **7** (0.270 g, 0.650 mmol) was dissolved in a solution of trifluoroacetic acid (2

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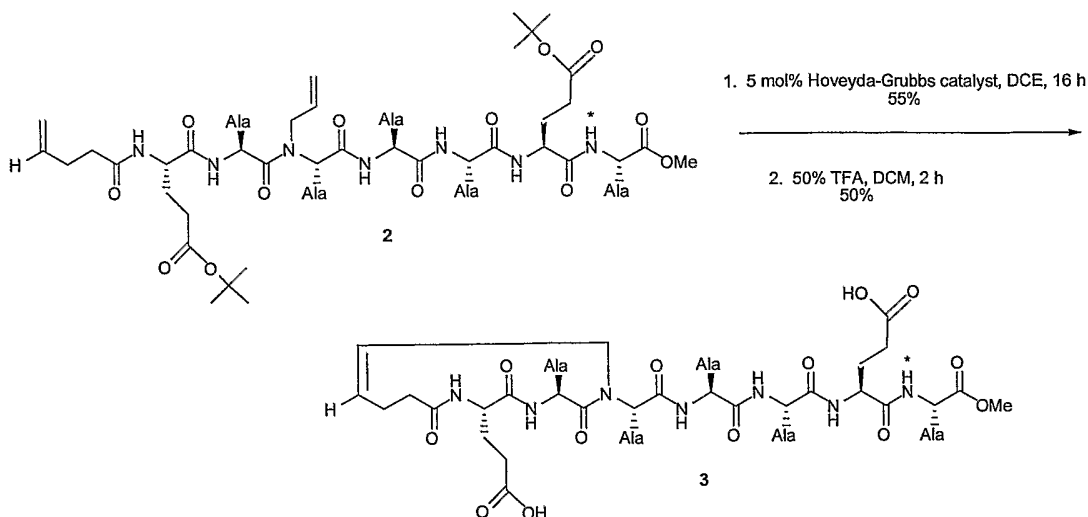
mL) and CH₂Cl₂ (10 mL). The mixture was stirred at room temperature and the reaction progress monitored by TLC. After 2 hours, the mixture was concentrated and the residue was redissolved in 10 mL of ethyl acetate and washed with water (2 x 10 mL). The organic layer was extracted with saturated aqueous NaHCO₃ (3 x 10 mL), and the combined aqueous layers were washed with EtOAc (2 x 10 mL). The aqueous fraction was then acidified to pH 2 with concentrated aqueous HCl and extracted with ethyl acetate (3 x 10 mL). The combined organic layers were dried (MgSO₄), filtered, and concentrated to afford 0.250 g (90%) of dipeptide **8** as a white solid (¹H NMR (400 MHz, CDCl₃) δ 7.75 (d, *J* = 7.5 Hz, 2H), 7.48-7.59 (m, 2H), 7.39 (t, *J* = 7.4 Hz, 2H), 7.30 (t, *J* = 7.4, 2H), 5.97-5.84 (m, 1H), 5.83 (d, *J* = 8.3 Hz, 1H), 5.26-5.33 (m, 2H), 5.32-5.21 (m, 2H), 4.63-4.72 (m, 1H), 4.66-4.58 (m, 1H), 4.35 (d, *J* = 7.3 Hz, 2H), 4.18-4.22 (m, 1H), 4.08 (dd, AB pattern, *J* = 11.8, 5.4 Hz, 1 H), 4.97 (dd, AB pattern, *J* = 12.8, 4.7 Hz, 1 H), 1.48 (d, *J* = 7.3 Hz, 3H), 1.39 (d, *J* = 6.8 Hz, 3H); ¹³C NMR (100 MHz) δ 175.61, 173.94, 155.66, 143.93, 143.81, 141.28, 133.27, 127.69, 127.05, 125.22, 119.9, 118.93, 67.04, 54.06, 48.97, 47.41, 47.11, 27.59, 19.05, 15.87, 14.37; IR (film) 3400, 3297, 1719 cm⁻¹; HRMS (FAB) *m/z* for C₂₄H₂₇N₂O₅ [M+H]⁺ calcd 423.1874, found 423.1920).

Synthesis of Peptide 3

[0091] Constrained peptide **3** was synthesized as shown in Scheme 7. The modified octapeptide **2** was prepared by conventional Fmoc solid phase chemistry. The pre-loaded Fmoc-Ala Wang resin was purchased from NovaBiochem. The Fmoc group was removed from Fmoc-Ala (0.3mmol, 441.18mg) by treatment with 20% piperidine in NMP (2 x 20 min), followed by washings with DMF (3x), DCM (3x), MeOH (3x) and ether (3x). The resulting amine was treated with activated Fmoc-Glu(OtBu)-OH (0.750 mmol, 0.319 g), HBTU (0.675 mmol, 0.256 g) and 5% DIEA/NMP for 45 minutes, followed by washings with DMF (3x), DCM (3x), MeOH (3x) and ether (3x). This procedure was repeated for the introduction of the remaining amino acids, dipeptide **8**, and pentenoic acid residues. Treatment of the resin with TEA:MeOH (1:10) at 50 °C for 16 hours afforded crude peptide **2**. Column chromatography (gradient:1% MeOH/DCM to 4% MeOH/DCM) afforded 171 mg (64.8%) of peptide **2** as a white solid (ESIMS for C₄₂H₇₀N₇O₁₃ [M + H]⁺ calcd 880.5, found 880.4).

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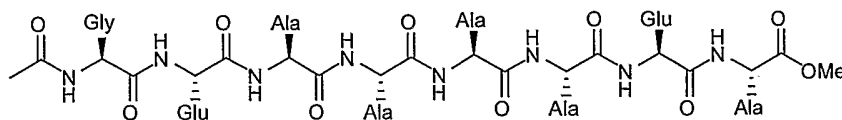
Scheme 7



[0092] To a solution of bis olefin peptide **2** (60.0 mg, 68.0 μmol) in 2 mL of anhydrous dichloroethane at 50 °C was added a solution of Hoveyda-Grubbs catalyst (2.14 mg, 3.40 μmol) in 0.270 mL anhydrous dichloroethane. After 16 hours, the reaction mixture was concentrated under vacuum and purified by HPLC to afford 31.8 mg (55%) of the *t*-butyl ester protected constrained peptide as a white solid (ESIMS for $\text{C}_{40}\text{H}_{66}\text{N}_7\text{O}_{13}$ $[\text{M} + \text{H}]^+$ calcd 852.5, found 852.5).

[0093] The protected peptide (20.0 mg, 23.5 μmol) was dissolved in TFA (2.5 mL) and CH_2Cl_2 (2.5 mL), and the reaction progress was monitored by TLC. After 3 hours, the reaction mixture was concentrated and purified by HPLC (*see* Figure 2 and accompanying text) to yield the cyclized deprotected peptide **3** as a white solid in 8.7 mg (50.2%) (ESIMS for $\text{C}_{32}\text{H}_{50}\text{N}_7\text{O}_{13}$ $[\text{M} + \text{H}]^+$ calcd 740.3, found 740.2).

[0094] The control peptide **1** (AcGEAAAEA (SEQ ID NO:35)) was prepared by conventional Fmoc solid phase chemistry on Wang resin and was cleaved as a methyl ester with methanol and triethylamine as described above for peptide **2** (ESIMS for $\text{C}_{30}\text{H}_{49}\text{N}_8\text{O}_{14}$ $[\text{M} + \text{H}]^+$ calcd 745.3, found 745.7).



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Analysis Of Constrained Peptide 3

Circular Dichroism

[0095] The structure of constrained peptide 3 was studied with circular dichroism ("CD"). CD studies on the constrained peptide 3 and the control peptide 1 in 30 mM phosphate buffer pH 7.0 were performed to obtain a quantitative measure of the helical content. The results are shown in Figure 3. As can be seen in Figure 3, the CD spectrum of peptide 3 displays a double minimum at 209 nm and 222 nm, characteristic of α -helices (Marqusee et al., "Helix Stabilization by $\text{Glu}^- \cdots \text{Lys}^+$ Salt Bridges in Short Peptides of *de novo* Design," *Proc. Nat'l Acad. Sci. U.S.A.* 84:8898-8902 (1987), which is hereby incorporated by reference in its entirety). The relative percent helicity of peptides can theoretically be measured by the mean residue ellipticity at 222 nm (Lyu et al., "Alpha-helix Stabilization by Natural and Unnatural Amino Acids with Alkyl Side Chains," *Proc. Nat'l Acad. Sci.* 88:5317-5320 (1991), which is hereby incorporated by reference in its entirety), although these estimates are not typically accurate for short helices.

[0096] As seen in Figure 3, the observed mean residue ellipticity ($[\theta]_{\text{obsd}}$) for peptide 3 is -31000 in 20% trifluoroethanol ("TFE") and phosphate buffer (ii) and phosphate buffer (i), calculated per mole of amide groups present. The calculated maximal mean residue ellipticity value ($[\theta]_{\text{max}}$) for the constrained peptide is -25000 and -275000 $\text{deg cm}^2 \text{ dmol}^{-1}$ based on Yang's method (Chen et al., "Determination of the Secondary Structures of Proteins by Circular Dichroism and Optical Rotatory Dispersion," *Biochemistry* 11:4120-4131 (1972), which is hereby incorporated by reference in its entirety) and Baldwin's (Chin et al., "Circular Dichroism Spectra of Short, Fixed-nucleus Alanine Helices," *Proc. Nat'l, Acad. Sci. U.S.A.* 99:15416-15421 (2002), which is hereby incorporated by reference in its entirety) correction for short peptides. Accordingly, this constrained peptide was estimated to be 100% helical. As expected, the unconstrained peptide 1 showed a featureless spectrum in aqueous buffer (Figure 3 (iii)) and TFE solutions.

[0097] CD spectra were recorded on an AVIV 202SF CD spectrometer equipped with a temperature controller using 1mm path length cells. The scan speed was set to 5 nm/min and spectra were averaged over 5 scans. Spectral baselines were

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obtained under analogous conditions as that for the samples. All spectra are baseline subtracted, converted to a uniform scale of molar ellipticity, and replotted. The helix content of each peptide was determined from the mean residue CD at 222 nm, $[\theta]_{222}$ ($\text{deg cm}^2 \text{ dmol}^{-1}$) corrected for the number of amide bonds (Chin et al., *Proc. Nat'l Acad. Sci. U.S.A.*, 99:15416-15421 (2002); Chen et al., *Biochemistry*, 11:4120-4131 (1972), which are hereby incorporated by reference in their entirety). CD measurements were performed at a peptide concentration of 50 μM in 30 mM phosphate buffer (pH 7.0) at 25 °C unless indicated otherwise. Varied temperature CDs were allowed to equilibrate at the given temperature for 30 minutes before each run. Figure 4 shows the CD spectra of peptide 3 at various temperatures between 5 °C and 95 °C. The intensity of 222 nm band remains essentially constant while that of the 202 nm band changes slightly.

Denaturation Studies

[0098] Thermal and guanidinium chloride ("GnHCl") denaturation studies were performed to determine the stability of artificial α -helix 3. Referring now to Figure 5, it is shown that the constrained peptide remains helical when heated from 5°C to 95°C, indicating that the peptide is structurally robust. The results of GnHCl titration experiments are shown in Figure 6. The intensity of the $[\theta]_{222}$ transition for the constrained peptide remained essentially unchanged (85% helical) up to a concentration of 4M GnHCl. The peptide started to unravel between 6-8M GnHCl. These CD studies illustrate that peptide 3 clearly adopts a highly stable constrained α -helix structure whose stability compares favorably to highly stable α -helical coiled-coil motifs (Litowski, et al., "Designing Heterodimeric Two-stranded Alpha-helical Coiled-coils: Effects of Hydrophobicity and Alpha-helical Propensity on Protein Folding, Stability, and Specificity," *J. Biol. Chem.* 277:37272-37279 (2002), which is hereby incorporated by reference in its entirety), and to a previously reported constrained α -helix with three side-chain lactam bridges (Osapay & Taylor, *J. Am. Chem. Soc.*, 114:6966-6973 (1992), which is hereby incorporated by reference in its entirety).

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NMR Analysis

[0099] The α -helix structure of peptide 3 was further confirmed by 1D and 2D NMR spectroscopy. A combination of 2D total correlation spectroscopy ("TOCSY") and rotating frame overhauser enhancement spectroscopy ("ROESY") spectra was used to assign ^1H NMR resonances for the constrained peptide. Sequential NN (i and $i+1$) ROESY crosspeaks, which provide a strong evidence for α -helical structure, were observed for the entire sequence, as shown in Figure 7.

[0100] The ROESY spectrum also reveals several medium range NOEs, e.g. $d_{\alpha\text{N}}(i, i+3)$, that provide unequivocal evidence for the helical structure. The fact that NOEs involving the last residue (alanine-8) can be detected indicates that the helix has not started fraying at the ends.

[0101] The ROESY and TOCSY experiments were performed at 40°C in slightly acidic phosphate buffer (pH 6.2), which was determined to be the optimum pH for the ROESY experiments. This unusually high temperature was used, because aggregation (as detected by ^1H NMR) of this very hydrophobic peptide was observed at lower temperatures for samples at NMR concentration (1 mM) in the acidic buffer. It is a further indication of the stability of this helix that strong ROESY crosspeaks can be observed at 40°C.

[0102] These results demonstrate that the replacement of a hydrogen bond between the i and $i+4$ residues at the N -terminus of a short peptide with a carbon-carbon bond results in a highly stable constrained α -helix at physiological conditions as indicated by CD and NMR spectroscopies. A significant advantage of this strategy is that it allows access to short α -helices with strict preservation of molecular recognition surfaces required for biomolecular interactions.

[0103] NMR experiments were carried out in a solution of 10% D_2O in 30 mM phosphate buffer, pH 6.2 at 40°C. These conditions were determined to be optimum for this peptide for the ROESY studies, as aggregation of the peptide was observed at lower temperatures. All experiments were performed at a peptide concentration of 1 mM. The proton resonances for the constrained peptide were numbered according to amino acid residues $\text{X}_1\text{E}_2\text{A}_3\text{A}_4^*\text{A}_5\text{A}_6\text{E}_7\text{A}_8$, where X refers to

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the pentenoic acid residue and A* to the *N*-allylalanine residue. These resonances were assigned by means of TOCSY and ROESY experiments.

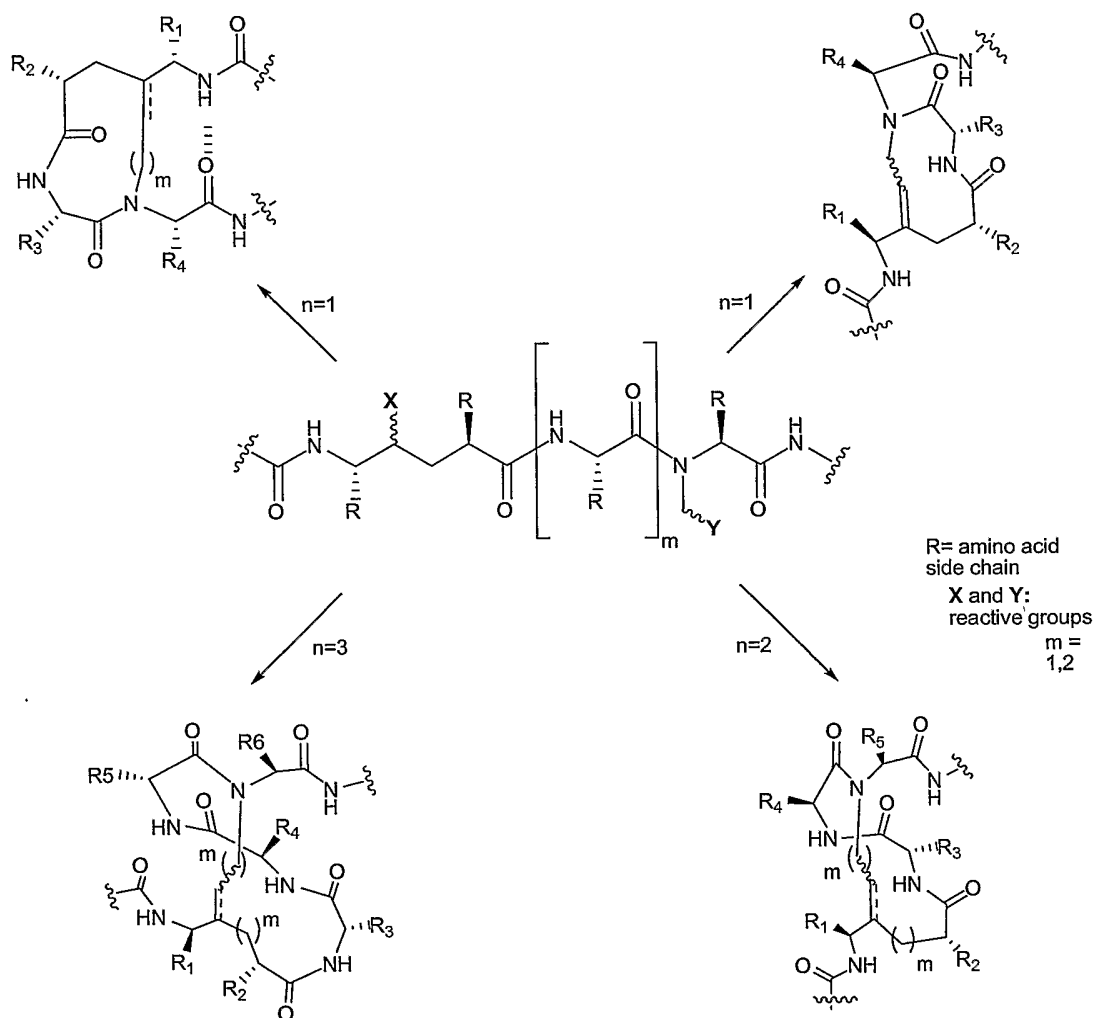
[0104] ¹H NMR TOCSY studies on the constrained helix **3** were performed on Bruker Avance 500 spectrometer. TOCSY spectra were recorded at 313 K with a
5 mixing time of 70 minutes. An 8.1 μsec 90° pulse was employed to collect 2k points in *f2* domain and 256 points in *f1* domain. The data were processed using Bruker XWINNMR software on a HP Workstation X1100. A 90° sine-squared window function was applied in both directions. The data were zero-filled to give a final matrix of 1k by 1k real points. The TOCSY spectrum is shown in Figure 8.

10 [0105] ¹H NMR pfg-ROESY studies on **3** were performed on Bruker Avance 500 spectrometer. The ROESY spectra were recorded at 313 K with a mixing time of 200 minutes using the pfg-ROESY pulse sequence. A ROESY continuous wave spin-lock of 1.5 KHz was used to collect 2k points in the *f2* domain and 256 points in the
15 *f1* domain. The data were processed using Bruker XWINNMR software on an HP Workstation X1100. A 90° sine-squared window function was applied in both directions. The data were zero-filled once in *f1* domain to give a final matrix of 1k by 1k real points. Important ROESY cross-peaks are shown in Figures 9 and 10.

Example 2 -- Preparation of Other Peptidomimetics

[0106] Figure 11 shows common peptide secondary structures. These
20 secondary structures are defined by hydrogen-bonding patterns, as demonstrated in Figure 12. As shown in Scheme 8, the peptide modification methodology of the present invention not only allows preparation of artificial α-helices, but also of β-sheets/β-turns, 3₁₀-helices, and π-helices. These secondary structures may be prepared with the RCM method or the alkylation/macrocyclization strategy as
25 discussed previously for internally-constrained α-helices.

Scheme 8



Example 3 -- Stabilization of BAK BH3 α -Helical Region

- 5 [0107] Example 1 demonstrates that the hydrogen-bond surrogate approach of the present invention affords highly stable α -helices from alanine-rich peptide sequences. This Example demonstrates the use of the hydrogen bond-surrogate approach of the present invention for the stabilization of biologically-relevant α -helices. This method can effectively stabilize α -helical conformations in non-alanine
- 10 rich sequences. The resulting molecules resist proteolytic degradation as compared to their unconstrained analogs and bind their protein targets with high affinity.

Bak BH3

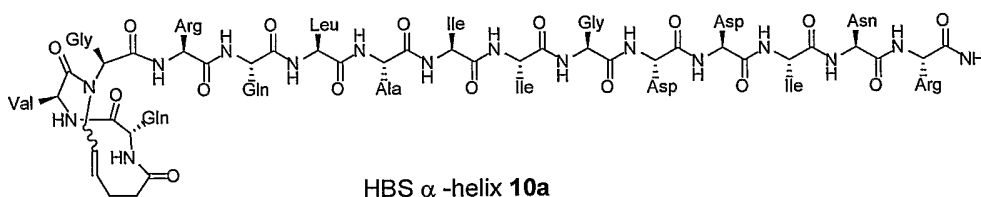
- [0108] Bcl-xL is an extensively studied α -helix binding protein (Sattler et al., *Science* 275:983-986 (1997), which is hereby incorporated by reference in its entirety). Bcl-xL is an anti-apoptotic protein that regulates cell death by binding the
5 α -helical BH3 domain of a family of pro-apoptotic proteins (such as Bak, Bad, Bid, and Bax) (Cory et al., *Oncogene*, 22:8590-8607 (2003); Letai et al., *Cancer Cell*, 2:183-192 (2002); Rutledge et al., *Curr. Opin. Chem. Biol.* 6:479-485 (2002), which are hereby incorporated by reference in their entirety). NMR studies by Fesik and coworkers have shown that a 16mer peptide **9a** derived from the Bak BH3 domain
10 adopts an α -helical conformation on binding to Bcl-xL (Sattler et al., *Science*, 275:983-986 (1997), which is hereby incorporated by reference in its entirety). Circular dichroism (CD) studies demonstrate that this peptide is not structured at physiological conditions in the absence of the protein partner and is only slightly helical in 30% trifluoroethanol (TFE), a helix promoting solvent (Petros et al., *Protein*
15 *Sci.* 9:2528-2534 (2000), which is hereby incorporated by reference in its entirety).
- [0109] Several methods that afford stabilized α -helices or helix mimetics have already been used to target Bcl-xL, allowing the direct evaluation of the performance of the internally-constrained artificial α -helices of the present invention (Kutzki et al.,
20 "Development of a Potent Bcl-x(L) Antagonist Based on Alpha-helix Mimicry," *J. Am. Chem. Soc.* 124:11838-11839 (2002); Chin & Schepartz, *Angew. Chem. Int. Ed. Engl.*, 40:3806-3809 (2001); Degtarev et al., *Nature Cell Biol.*, 3:173-182 (2001), which are hereby incorporated by reference in their entirety). Significantly, it was reported that Bak BH3 α -helices stabilized by a lactam-based side chain cross-linking strategy were unable to bind Bcl-xL (Yang et al., *Bioorg. Med. Chem. Lett.* 14:1403-
25 1406 (2004), which is hereby incorporated by reference in its entirety). In this report, Huang and coworkers speculated that the lack of binding might be due to steric clashes between the cross-link and the narrow binding pocket of Bcl-xL. In a related study, Verdine, Korsmayer and coworkers reported that side chain bridged α -helices corresponding to the BH3 domain of a different pro-apoptotic protein, Bid, can target
30 Bcl-xL and suppress the growth of leukemia cells in mice (Walensky et al., *Science* 305:1466-1470 (2004), which is hereby incorporated by reference in its entirety).

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Their report highlights the potential of constrained α -helices as tools for the control of protein-protein interactions *in vivo*. However, taken together these two protein binding studies from the Huang and Verdine laboratories illustrate potential problems with the side chain bridging strategy. It is believed that an important advantage of the hydrogen-bond surrogate approach of the present invention over the side chain bridging strategy for preparation of stabilized helices is that the helix surfaces are not encumbered by the constraining element. Judicious placement of the side-chain constraints requires prior knowledge of the protein-ligand complex; otherwise, multiple randomly constrained helices must be prepared and tested. The hydrogen-bond surrogate approach is expected to greatly simplify the helix design process. Artificial α -helices mimicking Bak BH3 were prepared according to the hydrogen-bond surrogate approach of the present invention, and their effectiveness for targeting Bcl-xL was examined. This Example addresses whether the HBS-derived Bak α -helix can bind Bcl-xL although the side-chain constrained (lactum bridge) Bak helix was unable to bind to this same target protein receptor.

Synthesis of Artificial Bak BH3

[0110] The artificial Bak BH3 α -helix **10a** has the following structure:

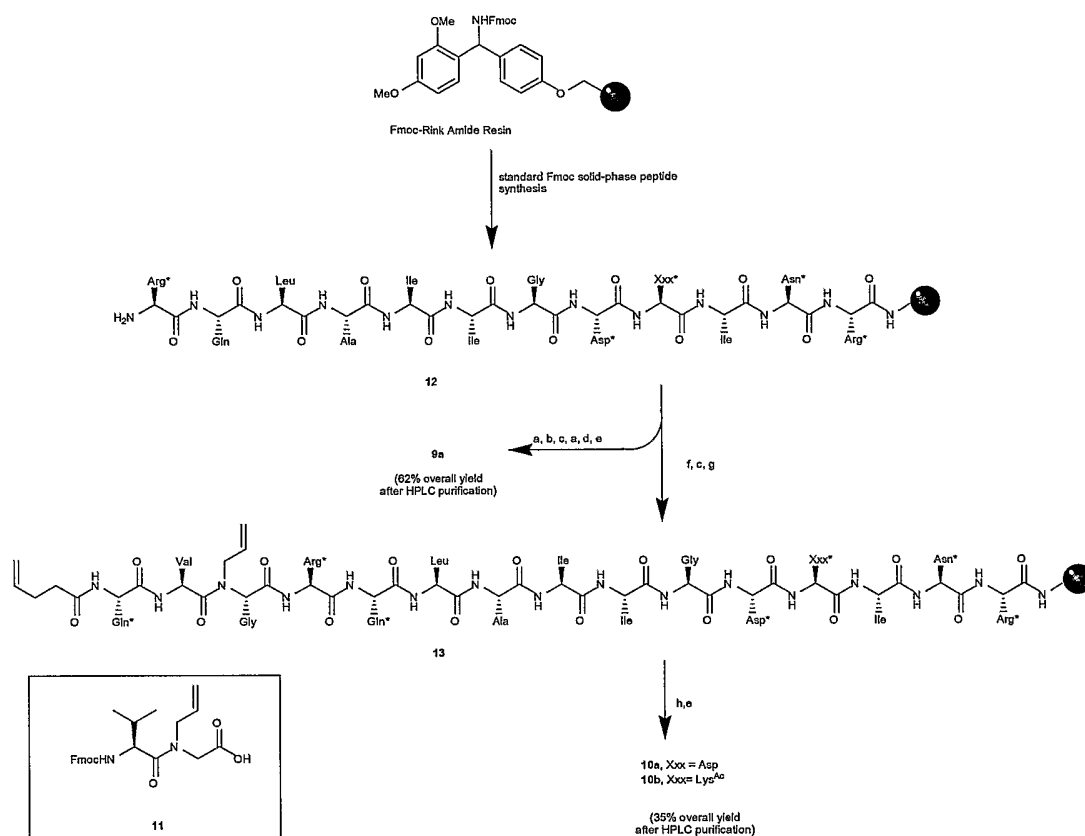


It was synthesized on Wang resin by the ring closing metathesis reaction as shown in Scheme 9. Hydrogen bond surrogate α -helices can be synthesized from commercially available amino acids or simple amino acid derivatives and do not require preparation of enantiomerically pure amino acid analogs. In the present case, standard solid phase peptide synthesis utilizing appropriate Fmoc amino acids, dipeptide **11**, and pentenoic acid afforded the fully-protected resin-bound bis-olefin peptide **12**, which was subjected to the Hoveyda-Grubbs ring-closing metathesis catalyst to afford the peptide macrocycle (Chapman et al., *J. Am. Chem. Soc.*, 126:12252-12253 (2004); Hoveyda et al., *Org. Biomolec. Chem.*, 2:8-23 (2004), which are hereby incorporated

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by reference in their entirety). The metathesized peptide was cleaved from the resin with trifluoroacetic acid to obtain the constrained peptide **10a** as a mixture of the *cis* and *trans* alkene isomers. The isomers were not able to be separated by HPLC.

Scheme 9



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Scheme 9. Solid phase synthesis of HBS α -helices **10a-b**: (a) (i) Fmoc Gly-OH (3.2 equiv), HBTU (2.88 equiv), *i*Pr₂NEt, NMP; (ii) piperidine, NMP; (b) (i) Fmoc Val-OH (3.2 equiv) HBTU (2.88 equiv), *i*Pr₂NEt, NMP; (ii) piperidine, NMP; (c) (i) Fmoc Gln*-OH (3.2 equiv), HBTU (2.88 equiv), *i*Pr₂NEt, NMP; (ii) piperidine, NMP; (d) acetic anhydride, *i*Pr₂NEt, DMF; (e) CF₃CO₂H/H₂O/triisopropylsilane (95:2.5:2.5), 1.5 hours; (f) (i) **11** (3.2 equiv), HBTU (2.88 equiv), *i*Pr₂NEt, NMP; (ii) piperidine, NMP; (g) pentenoic acid (3.2 equiv), HBTU (2.88 equiv), *i*Pr₂NEt, NMP; (h) 20 mol% Hoveyda-Grubbs Catalyst, dichloroethane, 50 °C, 24 hours; Arg*: Pbf-protected Arg; Asn*: trityl-protected Asn; Asp*: *t*-butyl-protected Asp; Gln*: trityl protected Gln; Xxx*: Asp* or Lys(Ac); HBTU = *O*-(1H-benzotriazol-1-yl)-N,N,N'-tetramethyluronium hexafluorophosphate; NMP = *N*-methyl pyrrolidinone; Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl.

Analysis of Artificial Bak BH3

Circular Dichroism Studies

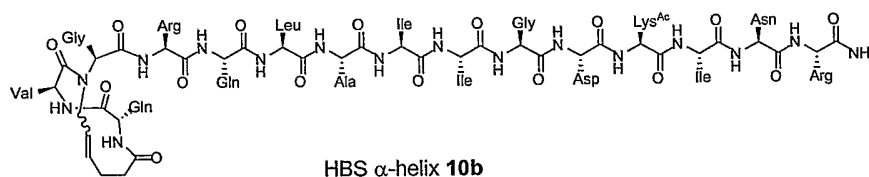
[0111] The α -helical conformation of constrained peptide **10a** was determined by circular dichroism spectroscopy. CD studies on the constrained peptide **10a** and the control peptide **9a** were performed in 20% TFE in PBS buffer to obtain a

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quantitative measure of their helical content, as shown in Figure 13. The CD spectra of the artificial α -helix **10a** displays double minima at 206 and 222 nm, and maximum at 189 nm consistent with those observed for canonical α -helices (the CD spectra of canonical α -helices typically contain double minima at 208 and 222 nm and maxima at 193 nm). The HBS α -helix **10a** is roughly 46% helical as measured by Yang's method (Chen et al., *Biochemistry*, 11:4120-4131 (1972), which is hereby incorporated by reference in its entirety). Consistent with previous studies (Petros et al., *Protein Sci.*, 9:2528-2534 (2000), which is hereby incorporated by reference in its entirety), the unconstrained Bak peptide was found to be significantly less helical (~26%). It is presumed that the GDD tripeptide residue in the middle of the Bak peptide sequence may be limiting the propagation of the helix and lowering the overall helical content of **10a**, as glycine is known to be a potent "helix breaker" and the aspartic acid residue has been implicated as a helix stop signal (Chakrabarty et al., *Protein Sci.*, 3:843-852 (1994); O'Neil & DeGrado, *Science*, 250:646-651 (1990); Nelson & Kallenbach, *Biochemistry*, 28:5256-5261 (1989), which are hereby incorporated by reference in their entirety). Fesik and coworkers have previously shown that the Gly-82 and Asp-83 residues cannot be substituted with alanine without sacrificing binding affinities for the protein. However, Asp-84 may be replaced without any deleterious effects (Sattler et al., *Science* 275:983 (1997), which is hereby incorporated by reference in its entirety). To test the effect of replacing Asp-84 on the helicity of Bak peptide, HBS α -helix **10b** in which Asp-84 is substituted with side chain acetylated-lysine (Lys^{Ac}) was prepared. This particular substitution was made because Bak BH3 peptide with a capped-lysine residue has been shown to bind Bcl-xL with high affinity (Zhang et al., *Anal. Biochem.* 307:70 (2002), which is hereby incorporated by reference in its entirety). It was found that this single substitution provided a significant boost in α -helicity, as demonstrated in Figure 13. HBS helix **10b** is roughly 65% helical—an increase in helicity of 140% over **10a**. As expected, a similar increase in helicity for the unconstrained Bak BH3 was observed. Importantly, this set of experiments shows that the hydrogen-bond surrogate approach can successfully stabilize α -helical conformations in biologically relevant sequence. HBS helix **10b** has the following structure:

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Binding Affinity Assays

[0112] The binding affinities of the unconstrained Bak peptide **9a** and artificial Bak α -helices **10a** and **10b** for Bcl-xL were assessed by a previously described fluorescence polarization assay using fluoresceine-labeled 16mer Bak peptide (fl-Bak). The binding affinity of the fl-Bak for Bcl-xL was determined to be 264 ± 23 nM, which is consistent with the previously reported values. Competitive inhibition of the fl-Bak/Bcl-xL complex with the Bak peptides **9a** and **10a-b** results in a decrease of fluorescence polarization. Regression analysis (Roehrl et al., *Biochemistry* 43:16056 (2004), which is hereby incorporated by reference in its entirety) provided a K_d of 154 ± 23 nM for the unconstrained Bak peptide **9a**, which is within range of the previously reported values, as shown in Figure 14. Under the same assay conditions, HBS helices **10a** and **10b** bound Bcl-xL with K_d 's of 325 ± 51 nM and 69 ± 16 nM, respectively, as shown in Figure 14. These binding results illustrate the degree of difficulty involved in forcing an artificial helix into this pocket as the unconstrained Bak BH₃ peptide **9a** targets this protein with two-fold higher affinity than the constrained peptide **10a**. However, these results validate the helix design principles of the present invention, as it is found that the internally constrained Bak α -helices of the present invention can bind to a deep hydrophobic cleft whereas the Bak α -helices prepared by the side chain bridging method showed no affinity for the same target. Moreover, it is shown that very high affinity binder (**10b**) for Bcl-xL can be developed by increasing the helicity of the constrained peptide through rational substitutions. HBS Bak α -helices designed to be more helical and to be a higher affinity binder than **10b** are being prepared. It remains to be determined if these HBS α -helices show selectivity for Bcl-xL over other closely related members of the Bcl-2 family (and over other helix-binding proteins) (Chin & Schepartz, *Curr. Op. Chem. Bio.* 6:479 (2002); Gemperli et al., *J. Am. Chem. Soc.* 127:1596 (2005); Yin et al.,

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Agnew Chem. Int. Ed. Engl. 44:2704 (2005), which are hereby incorporated by reference in their entirety).

[0113] Proteolytic cleavage is one of the principal reasons limiting the *in vivo* efficacy of peptides. Proteases are known to bind their substrates in linear or beta strand conformations (Tyndall et al., *Chem. Rev.* 105:973 (2005), which is hereby incorporated by reference in its entirety), and peptides locked into helical conformations have been shown to resist proteolytic degradation (Schafmeister & Verdine, *J. Am. Chem. Soc.* 122:5891 (2005); Shepherd et al., *J. Am. Chem. Soc.* 127:2974 (2005), which are hereby incorporated by reference in their entirety). The proteolytic stability of HBS Bak helices **10a-b** as compared to Bak peptide **9a** in the presence of trypsin, which was expected to cleave the peptide at the arginine residue (Arg-76) positioned two residues away from the macrocycle in helices **10a-b**, was determined. This was done to find out how this residue, which lies outside the constraint yet is close enough to be in a highly helical conformation, responds to the protease. By comparing the initial velocities of cleavage by trypsin, it was found that the HBS α -helix **10a** is proteolyzed roughly 30-fold slower than the unconstrained Bak peptide analog **9a**, as shown in Figures 15A–C. As expected, an increase in the helicity of the constrained peptide results in a further decrease in the initial velocity of trypsin cleavage. Thus, the HBS helix **10b** is roughly two-fold more stable than **10a** and 60-fold more stable than **9a** toward proteolysis by trypsin. The proteolytic stabilities observed for **10a-b** are similar to that reported for a side-chain crosslinked α -helix (Schafmeister & Verdine, *J. Am. Chem. Soc.* 122:5891 (2005), which is hereby incorporated by reference in its entirety).

[0114] This Example demonstrates that artificial α -helices prepared by the replacement of a hydrogen bond between the *i* and *i*+4 residues at the N-terminus of a short peptide with a carbon-carbon bond can stabilize biologically relevant peptides in helical conformations. These HBS α -helices can bind their expected protein receptor with high affinity, and resist proteolytic degradation. Analogs of the Bak peptide are being developed to further increase the helicity and affinity of the HBS-derived Bak helices for the target protein.

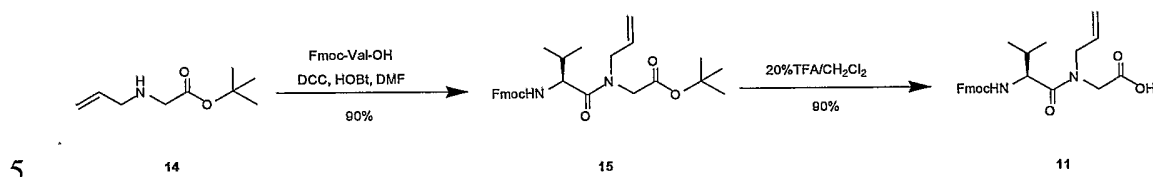
Materials and Methods

[0115] Commercial-grade reagents and solvents were used without further purification except as indicated. CH₂Cl₂, THF, and DMF were dried prior to use by percolation through anhydrous Al₂O₃ as described by Grubbs and coworkers (Pangborn et al., *Organometallics*, 15:1518-1520 (1996), which is hereby incorporated by reference in its entirety). All reactions were stirred magnetically; moisture-sensitive reactions were performed under argon in flame-dried glassware. Thin-layer chromatography (TLC), usually using either ethyl acetate/hexane or ethyl acetate/CH₂Cl₂ as the solvent system, was used to monitor reactions. Visualization was accomplished by either ultraviolet light or by immersing the plate in a 1% aqueous solution of potassium permanganate and heating. Flash chromatography with silica gel was performed following the conditions described by Still and coworkers (Still et al., *J. Org. Chem.*, 43:2923-2925 (1978), which is hereby incorporated by reference in its entirety). Solvents were removed by rotary evaporation under reduced pressure; where appropriate, the residue was further dried using a vacuum pump. Reverse-phase HPLC experiments were conducted with 4.6 x 150 mm (analytical scale) or 21.4 x 150 mm (preparative scale) Waters C₁₈ reverse phase columns using a Beckman Coulter HPLC equipped with a System Gold 168 Diode array detector. The typical flow rates for analytical and preparative HPLC were 1 mL/min and 8 mL/min, respectively. In all cases, 0.1% aqueous trifluoroacetic acid and acetonitrile buffers were used. Proton NMR spectra were obtained on a Bruker AV-400 (400 MHz). Carbon NMR spectra were obtained on a Bruker (100.5 MHz) spectrometer. Proton chemical shifts are reported as δ values relative to tetramethylsilane (0.00 ppm) or to the particular solvent used in the experiment (CDCl₃:7.26 ppm). Carbon chemical shifts are reported as δ values relative to the particular solvent used in the experiment (CDCl₃:77.0 ppm). Data is reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet, br = broad), coupling constant, and integration. Infrared (IR) spectra were obtained with a Thermo Nicolet Avatar 360 FTIR. High-resolution mass spectra (HRMS) were obtained on a LC/MSD TOF (Agilent Technologies). LCMS data was obtained on an Agilent 1100 series LC/MSD (XCT) electrospray trap.

Synthesis of Peptide 11

[0116] Fmoc-Val-*N*(allyl)-Gly-OH **11** was synthesized as shown in Scheme 10.

Scheme 10



[0117] A solution of *N,N*-dicyclohexylcarbodiimide (DCC, 2.62 g, 12.7 mmol), 1-hydroxybenzotriazole (HOBt, 1.72 g, 12.7 mmol), FmocVal-OH (4.32 g, 12.7 mmol) and 40 mL of DMF was stirred for 15 minutes. *N*-Allyl-glycine-*t*-butyl ester **14** (1.67g, 9.78 mmol) was then added to the flask and the reaction mixture was stirred at room temperature. After 13 hours, the reaction mixture was poured into 40 mL of water and extracted with ether (3 × 40 mL). The combined ether layers were washed with water (3 × 40 mL) and dried with anhydrous magnesium sulfate, filtered and concentrated by rotary evaporation. The residue was purified with flash chromatography (95:5, dichloromethane: ethyl acetate) to afford 4.33 grams of the dipeptide *t*-butyl ester **15** (90%) as a colorless oil (¹H NMR (400 MHz, CDCl₃) δ 7.75 (d, *J* = 7.5 Hz, 2H), 7.60 (d, *J* = 7.4 Hz, 2H), 7.39 (t, *J* = 7.4 Hz, 2H), 7.30 (tt, *J* = 7.4 Hz, 1.0 Hz, 2H), 5.88-5.70 (m, 1H), 5.60 (d, *J* = 9.2 Hz, 1H), 5.26-5.13 (m, 2H), 4.56 (dd, *J* = 11.0, 6.2 Hz, 1H), 4.42-4.36 (m, 1H), 4.35-4.31 (m, 1H), 4.29-4.18 (m, 2H), 4.14-4.08 (m, 1H), 4.04-3.97 (m, 1H), 3.67 (d, *J* = 17.0 Hz, 1H), 2.12-1.99 (m, 1H), 1.45 (s, 9H), 1.05 (d, *J* = 6.7 Hz, 3H), 0.95 (d, *J* = 6.9 Hz, 3H); ¹³C NMR (100 MHz) δ 172.34, 167.98, 156.28, 143.92, 141.26, 132.40, 127.65, 127.05, 125.18, 119.93, 118.45, 81.81, 66.98, 55.50, 51.35, 47.88, 47.18, 31.72, 28.02, 19.68, 17.32; IR (film) 2974, 1734, 1717, 1652, 1647 cm⁻¹; HRMS *m/z* for C₂₉H₃₇N₂O₅ [M+H]⁺ calcd 493.2702, found 493.2699).

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[0118] A solution of dipeptide *t*-butyl ester **15** (2.37g, 4.81mmol), 80 mL of dichloromethane, 20 mL of trifluoroacetic acid (TFA) was stirred for 4 hours, and then concentrated under vacuum. The residue was redissolved in 80 mL of dichloromethane and washed with water (3 × 40 mL). The organic layer was dried

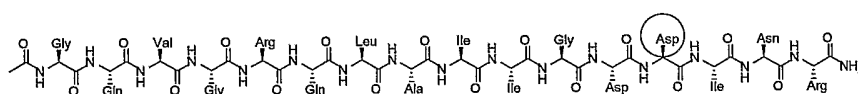
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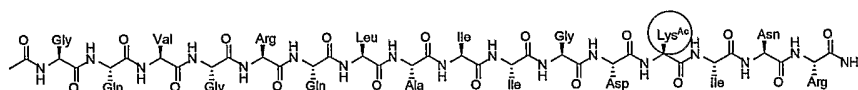
over anhydrous magnesium sulfate, filtered, and concentrated. The residue was purified by flash chromatography (1:1, hexane: ethyl acetate) to afford 1.89 grams of peptide **11** (90%) as a white foam (^1H NMR (400 MHz, CDCl_3) δ 7.68 (d, $J = 7.4$ Hz, 2H), 7.52 (d, $J = 7.5$ Hz, 2H), 7.32 (t, $J = 7.4$ Hz, 2H), 7.24 (t, $J = 7.4$ Hz, 2H), 5.80-5.71 (m, 1H), 5.60 (d, $J = 8.8$ Hz, 1H), 5.21-5.08 (m, 2H), 4.48 (dd, $J = 9.0$ Hz, 6.5 Hz, 1H), 4.35-4.22 (m, 4H), 4.16-4.09 (m, 2H), 4.06-3.97 (m, 2H), 3.79 (d, $J = 17.3$ Hz, 1H), 2.02-1.91 (m, 1H), 0.93 (d, $J = 6.7$ Hz, 3H), 0.87 (d, $J = 6.4$ Hz, 3H); ^{13}C NMR (100 MHz) δ 173.30, 172.57, 156.46, 143.80, 141.27, 132.00, 127.72, 127.06, 125.17, 119.96, 119.01, 67.11, 55.66, 51.64, 49.47, 47.14, 31.59, 19.48, 17.36; IR (film) 1722, 1711, 1657, 1642 cm^{-1} ; HRMS m/z for $\text{C}_{25}\text{H}_{29}\text{N}_2\text{O}_5$ $[\text{M}+\text{H}]^+$ calcd 437.2076, found 437.2067).

Synthesis of Bak BH3₇₂₋₈₇ Peptide **9a** and **9b**

[0119] Bak BH3₇₂₋₈₇ peptide **9a** and **9b** have the following structures:



BakBH3₇₂₋₈₇ peptide **9a**

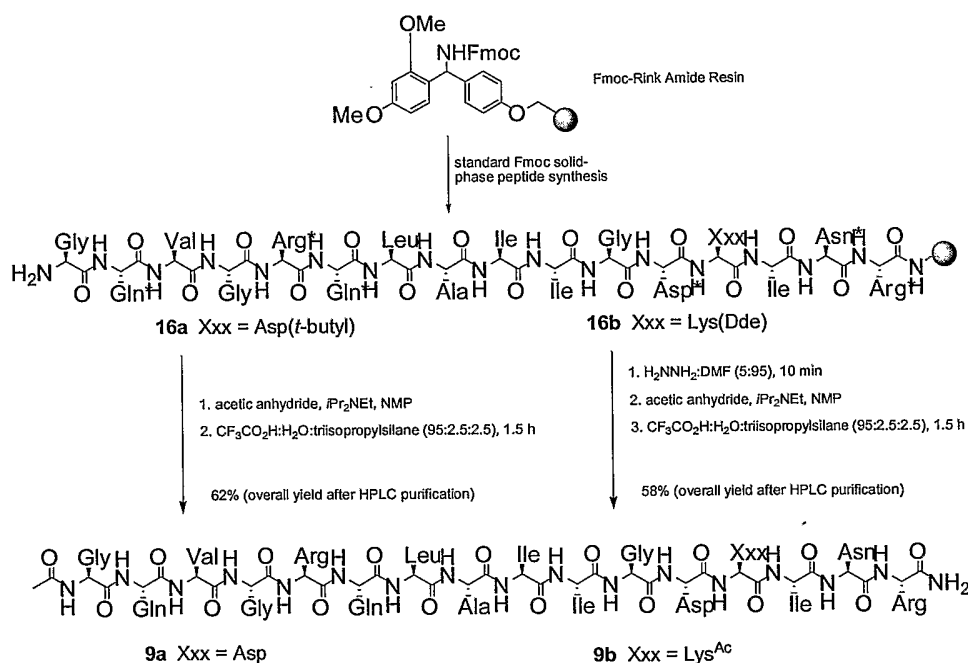


BakBH3₇₂₋₈₇ peptide **9b**

15 [0120] They were synthesized as shown in Scheme 11.

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Scheme 11



Arg*: Pbf protected Arg; Gln*: trityl protected Gln; Asn*: *t*-butyl protected Asn; Asp*: *t*-butyl protected Asp; Dde: *N*- α -1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl group; NMP = *N*-methylpyrrolidinone; Pbf = 2,2,4,6,7-pentamethylidihydrobenzofuran-5-sulfonyl.

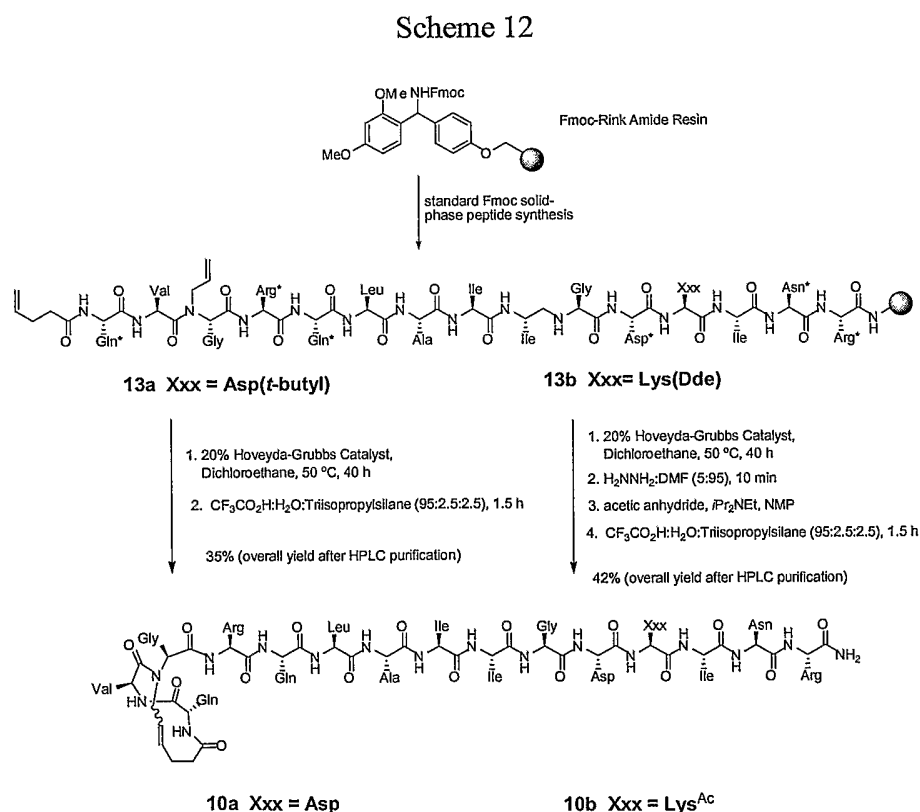
[0121] The Bak BH3 peptide **9a** was synthesized by conventional Fmoc solid phase chemistry on Rink amide resin (NovaBiochem). The Fmoc group was removed from Rink amide resin (125 mg, 0.08 mmol) by treatment with 1.0 mL of 20% piperidine in NMP (2 \times 20 min). The resin was then washed with DMF (2 \times), dichloromethane (2 \times) and MeOH (3 \times). The free amine was treated with preactivated Fmoc-Asn (Trt)-OBt, which was prepared from Fmoc-Arg (Pfp)-OH (166 mg, 0.256 mmol), HBTU (88 mg, 0.230 mmol) and 1.2 mL of 5% DIPEA/NMP. After 45 minutes of shaking, the resin was washed with DMF (2 \times), dichloromethane (2 \times) and MeOH (3 \times). This procedure was repeated for the introduction of the remaining amino acids to afford resin-bound peptide **16a**, which was acetylated with acetic anhydride (0.4 mL) in DMF (3.2 mL). The resin was treated with the cleavage cocktail (CF₃CO₂H:H₂O:triisopropylsilane, 95:2.5:2.5) for 1.5 hours and then concentrated by rotary evaporation. The residue was purified by reversed-phase HPLC to yield **1** (88 mg, 62%) as a white solid after lyophilization (ESIMS *m/z* for C₇₄H₁₂₈N₂₆O₂₄ [M+H]⁺ calcd 1766.0, found 1766.8).

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[0122] The mutated Bak BH3 peptide **9b** was synthesized on Rink amide resin similar to **9a** with appropriate substitution with Fmoc-Lys(Dde)-OH. The N- α -1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) protecting group in lysine residue was removed by treatment with 5% hydrazine DMF solution, and the lysine side chain was acetylated with acetic anhydride. Peptide **9b** was then cleaved from resin by treatment with the cleavage cocktail (TFA:H₂O:triisopropylsilane, 95:2.5:2.5) in 58% yield (ESIMS *m/z* for C₇₈H₁₃₈N₂₇O₂₃ [M+H]⁺ calcd 1821.0, found 1821.7).

Synthesis of α -Helices **10a** and **10b**

[0123] The HBS constrained α -helices **10a** and **10b** were synthesized on the solid phase as shown in Scheme 12.



Arg*: Pbf protected Arg; Gln*: trityl protected Gln; Asn*: *t*-butyl protected Asn; Asp*: *t*-butyl protected Asp; Dde: N- α -1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl group; NMP =N-methylpyrrolidinone; Pbf = 2,2,4,6,7-pentamethylidihydrobenzofuran-5-sulfonyl.

[0124] Bis-olefin resin bound peptide **13a** was synthesized by conventional Fmoc solid phase chemistry (as described earlier for the synthesis of peptide **9a**) on Rink amide resin (250 mg, 0.16 mmol) with appropriate substitutions with dipeptide

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11 and pentenoic acid. The resin bound peptide **13a** was dried under vacuum overnight and then swelled in 1.5 mL of anhydrous 1, 2-dichloroethane (DCE) in a dried flask under an argon atmosphere for 30 minutes. The Hoveyda-Grubbs catalyst (20 mg, 0.032 mmol) in 1.5 mL of DCE was added to the flask and the mixture was stirred at 50 °C. After 40 hours, the resin was washed with dichloromethane (3×), 10% 1,3 - bis (diphenylphosphino) propane in dichloromethane solution (1×), dichloromethane (3×) and methanol (3×). The washed peptide was cleaved from the resin by treatment with 30 mL of the cleavage cocktail (CF₃CO₂H:H₂O:triisopropylsilane, 95:2.5:2.5) for 1.5 hours. The reaction mixture was concentrated and purified by reversed-phase HPLC to yield peptide **10a** (99 mg, 35% overall yield) (ESIMS for C₇₆H₁₂₉N₂₅O₂₃ [M+H]⁺, calcd 1761.0, found 1762.0).

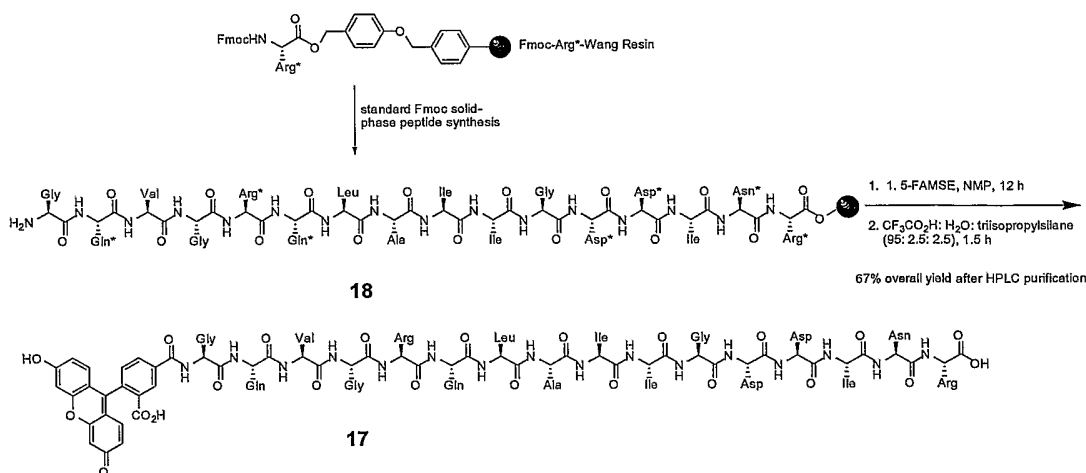
[0125] The HBS α-helix **10b** was synthesized from Rink amide resin similar to HBS α-helix **10a** with appropriate substitution with Fmoc-Lys(Dde)-OH. The Dde protecting group in lysine residue was removed by treatment with 5% hydrazine DMF solution, and the lysine side chain was consequently acetylated by acetic anhydride. Peptide **10b** was then cleaved from the resin by treatment with the cleavage cocktail (TFA:H₂O:triisopropylsilane, 95:2.5:2.5) in 42% yield (ESIMS *m/z* for C₈₀H₁₃₉N₂₆O₂₂ [M+H]⁺ calcd 1816.0, found 1816.5).

Synthesis of Bak BH3₇₂₋₈₇ peptide fl-Bak peptide 17

[0126] The fluoresceine-labeled Bak (fl-Bak) peptide **17** was synthesized from the Wang resin-bound peptide **18** by treatment with 5-carboxylfluorescein succinimidyl ester (5-FAMSE, Molecular Probes), followed by cleavage (TFA:H₂O:triisopropylsilane, 95:2.5:2.5) from the Wang resin as a carboxylic acid in 67% yield (ESIMS *m/z* for C₉₃H₁₃₆N₂₅O₃₀ [M+H]⁺ calcd 2084.2, found 2084.0), as shown in Scheme 13.

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Scheme 13



Arg*: Pbf protected Arg; Asn*: trityl protected Asn; Asp*: *t*-butyl protected Asp; Gln*: trityl protected Gln; Pbf = 2,2,4,6,7-pentamethylidihydrobenzofuran-5-sulfonyl.

Circular Dichroism Spectra

[0127] CD spectra were recorded on AVIV 202SF CD spectrometer equipped with a temperature controller using 1 mm length cells and a scan speed of 5 nm/min at 25 °C. The spectra were averaged over 10 scans with the baseline subtracted from analogous conditions as that for the samples. The samples were prepared in 10 mM phosphate buffered saline containing 20% trifluoroethanol, with the final peptide concentration of 50 μM. The helix content of each peptide was determined from the mean residue CD at 222 nm, $[\theta]_{222}$ (deg cm² dmol⁻¹) corrected for the number of amide bonds (Chen et al., *Biochemistry* 11:4120–4131 (1972), which is hereby incorporated by reference in its entirety). Percent helicity was calculated from ratio $[\theta]_{222}/[\theta]_{\max}$, where $[\theta]_{\max} = -39500 \times [1 - (2.57/n)]$. The $[\theta]_{\max}$ for HBS α-helices is calculated to be -33155 for n = 16 (number of amide bonds in the peptide).

15 Bcl-xL Binding Assay

[0128] The relative affinity of each peptide for His₆-tagged human Bcl-xL (obtained from ProteinX Lab, San Diego, CA) was determined using fluorescence polarization-based competitive binding assay with fluoresceine-labeled Bak peptide (fl-Bak) **17**. The anisotropy experiments were performed with a DTX 880 Multimode Detector (Beckman) at 4 °C, with excitation and emission wavelengths of 485 and 525

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nm, respectively. All the titration samples were prepared in a 96 well plate in the presence of 4% DMSO and 0.1% pluronic acid. The binding affinity (K_D or IC_{50}) values reported 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. The concentration of the Bcl-xL protein stock solution used in these experiments was determined with the BCA protein assay kit (Pierce).

[0129] Prior to the competition experiments, the affinity of fl-Bak 17 for Bcl-xL was determined by monitoring polarization of the fluorescent probe upon addition of Bcl-xL. Addition of an increasing concentration (0 nM to 4 μ M) of the Bcl-xL protein to a 15 nM solution of fl-Bak 17 in PBS buffer at 4 °C afforded a saturation binding curve, shown in Figure 16 (Zhang, *Anal. Biochem.* 307:70–75 (2002), which is hereby incorporated by reference in its entirety). The IC_{50} value gotten from this binding curve was fit into the following equation (a) to calculate the dissociation constant (K_{D1}) for the fl-Bak 17 and Bcl-xL complex (Roehrl et al., *Biochemistry*, 43:16056-16066 (2004); Roehrl et al., *Biochemistry* 43:16067-16075 (2004), which are hereby incorporated by reference in their entirety).

$$\mathbf{a:} \quad K_{D1} = (R_T * (1 - F_{BS}) + L_{ST} * F_{SB}^2) / F_{BS} - L_{ST}$$

K_{D1} : K_D of fluoresceine probe 17
 R_T : Total concentration of Bcl-xL;
 L_{ST} : Total concentration of fl-Bak;
 F_{SB} : Fraction of bound fl-Bak.

[0130] This K_{D1} value was used to calculate the optimum concentrations of the probe (15 nM fl-Bak) and the protein (500 nM Bcl-xL) needed for the competition binding anisotropy assays. These concentrations were chosen to develop a highly sensitive fluorescence polarization assay. The sensitivity and usability of a polarization assay largely depends on two important considerations: (a) concentration of the complex should be chosen so that the polarized probe affords an observable signal (beyond experimental error) over background, and (b) the concentration of the protein should be lower than that needed for saturation of the probe because excess protein concentrations would lead to inaccurately high IC_{50} values. Based on the K_{D1} of fl-Bak and Bcl-xL complex, at 500 nM Bcl-xL and 15 nM fl-Bak, 82% of the

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fluorescent probe should be bound to the protein while a measurable change in polarization signal (~90-100 mP) is expected upon inhibition of the fl-Bak/Bcl-xL complex by the antagonist (**9a**, **9b**, **10a** or **10b**).

Competition Polarization Assay

5 [0131] Appropriate concentrations (10 nM to 10 μM) of the antagonists (**9a**, **9b**, **10a** or **10b**) were added to a solution of 500 nM Bcl-xL and 15 nM fl-Bak **3**, and the resulting mixtures were incubated at 4 °C. After 1 hour, the value of the dissociated fluorescent probe **17** was determined by the fluorescence polarizer. The dissociated constant (IC₅₀) was fit into the following equation (**b**) to calculate the K_{D2} value of the antagonist (**9a**, **9b**, **10a** or **10b**) (Roehrl et al., *Biochemistry*, 43:16056-16066 (2004); Roehrl et al., *Biochemistry* 43:16067-16075 (2004), which are hereby incorporated by reference in their entirety).

$$\text{b: } K_{D2} = K_{D1} * F_{SB} * \left(\frac{L_T}{L_{ST} * F_{SB}^2 - (K_{D1} + L_{ST} + R_T) * F_{SB} + R_T} - \frac{1}{1 - F_{SB}} \right)$$

15 K_{D1}: K_D of floresceine probe **17**
 K_{D2}: K_D of antagonist peptide (**9a**, **9b**, **10a** or **10b**);
 R_T: Total concentration of Bcl-xL;
 L_{ST}: Total concentration of fl-Bak;
 F_{SB}: Fraction of bound fl-Bak.
 L_T: Total concentration of antagonist (**9a**, **9b**, **10a** or **10b**)

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Trypsin Cleavage Assay

[0132] A solution containing 500 μM of tryptophan, 1 ng/μL of trypsin and 0.5 mM of peptide (**9a**, **9b**, **10a** or **10b**) in phosphate buffer saline (PBS) was incubated at 4 °C. At different time intervals, 100 μL of the above solution was taken out, quenched with 100 μL of 2% TFA aqueous solution, and then injected into reversed-phase HPLC to analyze the change in the area of the peptide peak compared to the area of internal control (tryptophan).

[0133] Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions, and the like can be made without departing

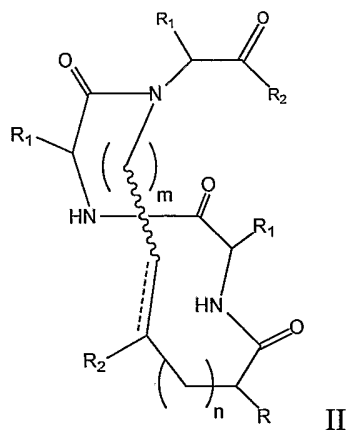
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from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.

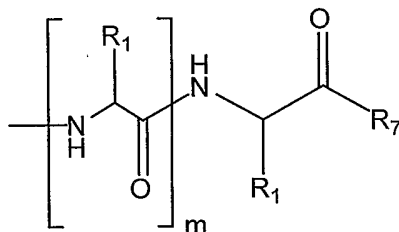
WHAT IS CLAIMED:

1. A method of preparing a compound of Formula II:



wherein

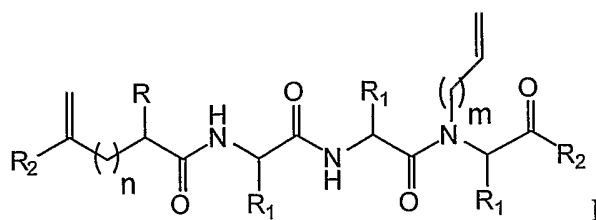
- 5 R is hydrogen, an amino acid side chain, an alkyl group, or an aryl group;
 R₁ is an amino acid side chain, an alkyl group, or an aryl group;
 R₂ is an amino acid, peptide, OR, CH₂NH₂, an alkyl group, an aryl group, or a group of formula:



- 10 wherein R₇ is an amino acid, peptide, OR, CH₂NH₂, an alkyl group, or an aryl group;

==== 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; and m is any number, said method comprising:

- 15 providing a compound of Formula I:



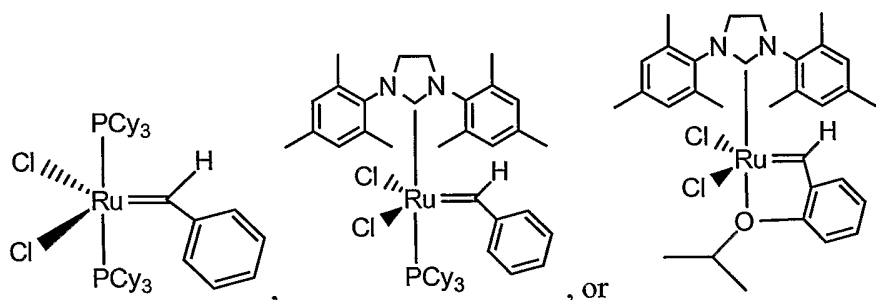
and

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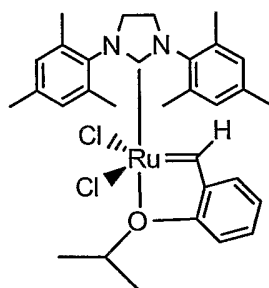
reacting said compound of Formula I under conditions effective to produce a compound of Formula II.

2. The method according to claim 1, wherein said reacting comprises a ring-closing metathesis reaction.

5 3. The method according to claim 2, wherein said metathesis reaction is performed with a metathesis catalyst of the formula:



4. The method according to claim 3, wherein said metathesis catalyst is of
10 the formula:



5. The method according to claim 2, wherein said metathesis reaction is performed at a temperature between about 25°C and 110°C.

6. The method according to claim 2, wherein said metathesis reaction is
15 performed with an organic solvent.

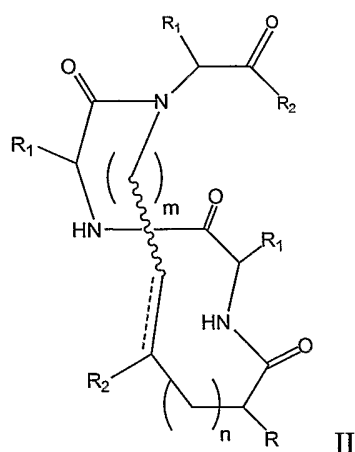
7. The method according to claim 6, wherein said organic solvent is selected from the group consisting of dichloromethane, dichloroethane, trichloroethane, and toluene.

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8. The method according to claim 7, wherein said organic solvent comprises dichloroethane.

9. The method according to claim 1, wherein said reacting is carried out on a solid support.

5 10. A method of preparing a compound of Formula II:

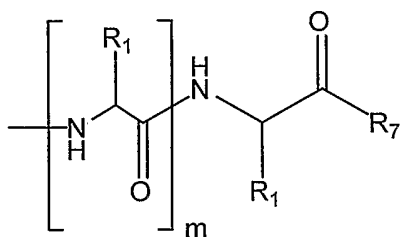


wherein

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

R₁ is an amino acid side chain, an alkyl group, or an aryl group;

10 R₂ is an amino acid, peptide, OR, CH₂NH₂, an alkyl group, an aryl group, or a group of Formula:

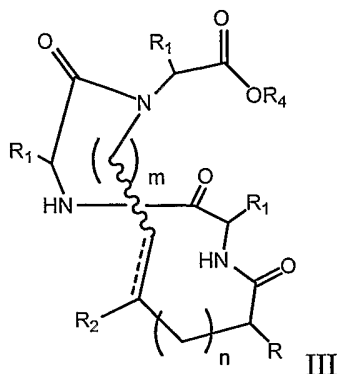


wherein R₇ is an amino acid, peptide, OR, CH₂NH₂, an alkyl group, or an aryl group;

15 ----- 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; and m is any number, said method comprising:

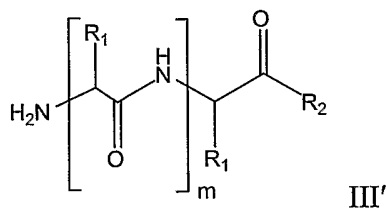
providing a compound of Formula III or a salt thereof:

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wherein R₄ is a carboxyl protecting group and

reacting said compound of Formula III or a salt thereof with a peptide coupling reagent of Formula III':

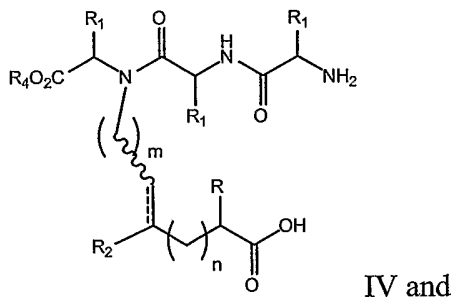


5

under conditions effective to produce a compound of Formula II.

11. The method according to claim 10, wherein said providing a compound of Formula III or salt thereof comprises:

providing a compound of Formula IV:



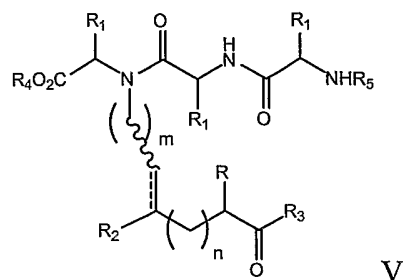
10

reacting said compound of Formula IV under conditions effective to produce a compound of Formula III or salt thereof.

12. The method according to claim 11, wherein said providing a compound of Formula IV comprises:

15 providing a compound of Formula V:

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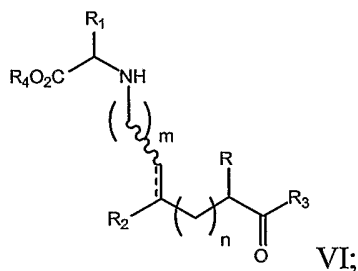


wherein R_3 is a carboxyl protecting group that is different from R_4 and R_5 is an amine protecting group and

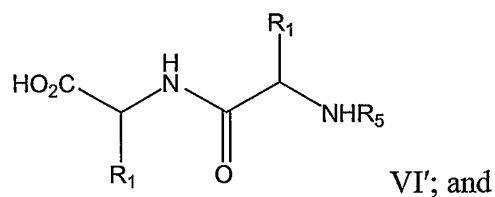
5 reacting said compound of Formula V under conditions effective to produce a compound of Formula IV.

13. The method according to claim 12, wherein said providing a compound of Formula V comprises:

providing a compound of Formula VI:



10 providing a compound of Formula VI':

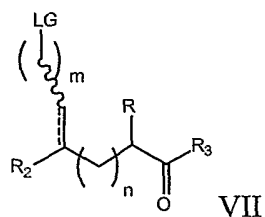


reacting said compound of Formula VI with said compound of Formula VI' under conditions effective to produce a compound of Formula V.

14. The method according to claim 13, wherein said providing a compound of Formula VI comprises:

15 providing a compound of Formula VII:

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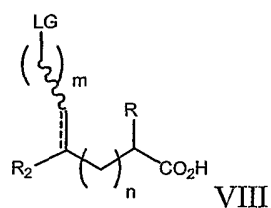


wherein LG is a leaving group and

reacting said compound of Formula VII with an amino acid protected at a carboxyl end of the amino acid under conditions effective to produce a compound of
 5 Formula VI.

15. The method according to claim 14, wherein said providing a compound of Formula VII comprises:

providing a compound of Formula VIII:



10

and

reacting said compound of Formula VIII with a carboxyl protecting agent under conditions effective to produce a compound of Formula VII.

16. The method according to claim 10, wherein said reacting is carried out on a solid support.

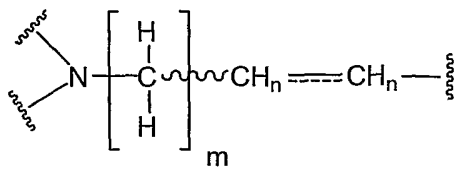
15

17. A compound prepared according to the method of claim 1.

18. A compound prepared according to the method of claim 10.

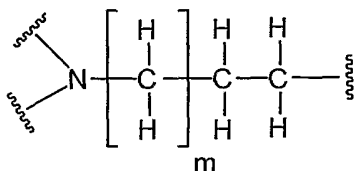
19. A peptide having one or more stable, internally-constrained alpha-helical, beta-sheet/beta-turn, 3_{10} -helical, or pi-helical regions.

20. The peptide according to claim 19, wherein the one or more stable, internally-constrained regions comprises the following motif:

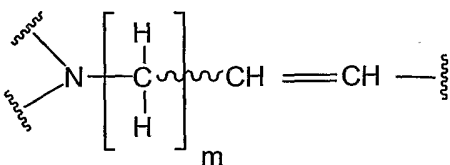


wherein ==== is a single or double bond, ~~~~ is a single bond and is cis or trans when ==== is a double bond; n is 1 or 2; and m is any number.

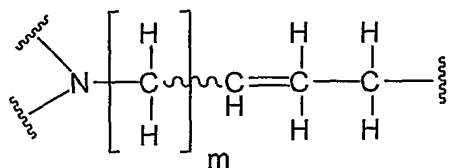
21. The peptide according to claim 20, wherein the one or more stable, internally-constrained regions comprises the following motif:



22. The peptide according to claim 20, wherein the one or more stable, internally-constrained regions comprises the following motif:



23. The peptide according to claim 19, wherein the one or more stable, internally-constrained regions comprises the following motif:



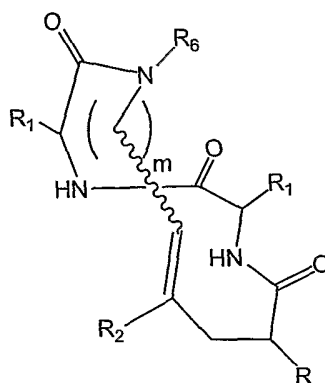
24. The peptide according to claim 19, wherein said one or more regions comprises a pi helix.

25. The peptide according to claim 19, wherein said one or more regions comprises a beta sheet/beta turn.

26. The peptide according to claim 19, wherein said one or more regions comprises a 3_{10} helix.

27. The peptide according to claim 19, wherein said one or more regions comprises an alpha-helix.

5 28. The peptide according to claim 27, wherein the peptide comprises the formula:



wherein

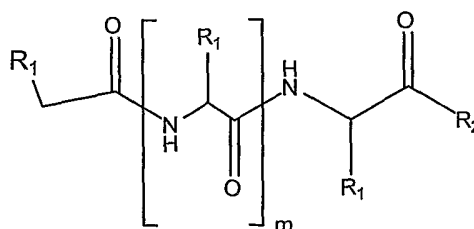
R is hydrogen, an amino acid side chain, an alkyl group, or an aryl group;

10 R₁ is an amino acid side chain, an alkyl group, or an aryl group;

R₂ is an amino acid, peptide, OR, CH₂NH₂, an alkyl group, or an aryl group;

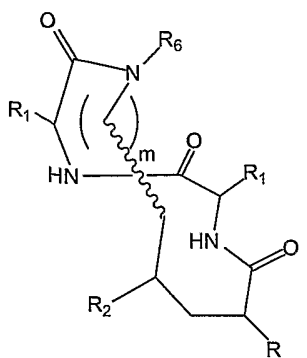
R₆ is (a) a chain of one or more amino acids; or

(b) a side chain of formula:

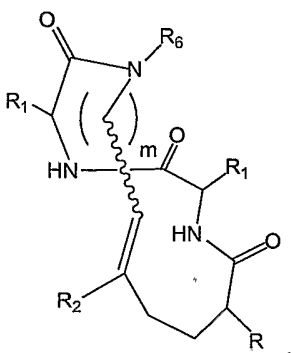


15 29. The peptide according to claim 27, wherein the peptide comprises the formula:

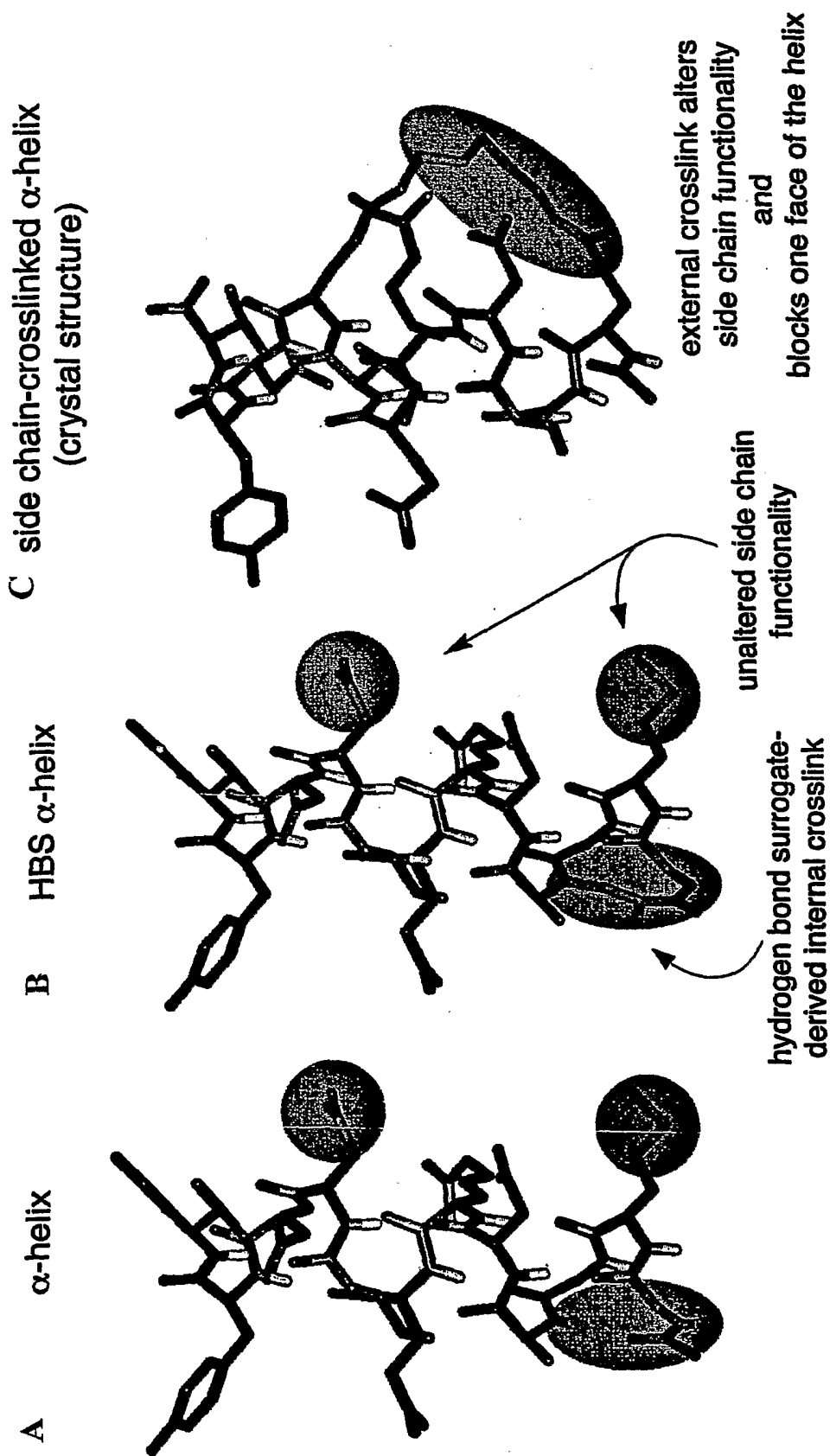
- 64 -



30. The peptide according to claim 27, wherein the peptide comprises the formula:



Comparison of the HBS and Side Chain Constraining Strategies



Figures 1A-C

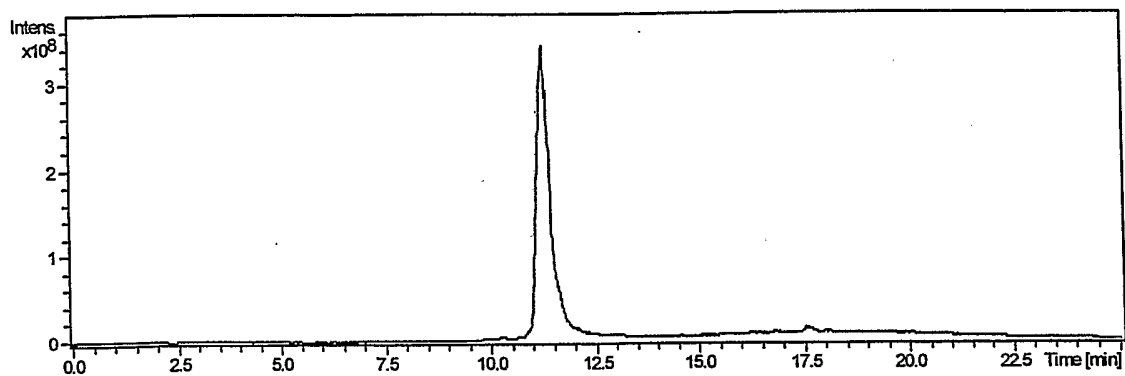


Figure 2

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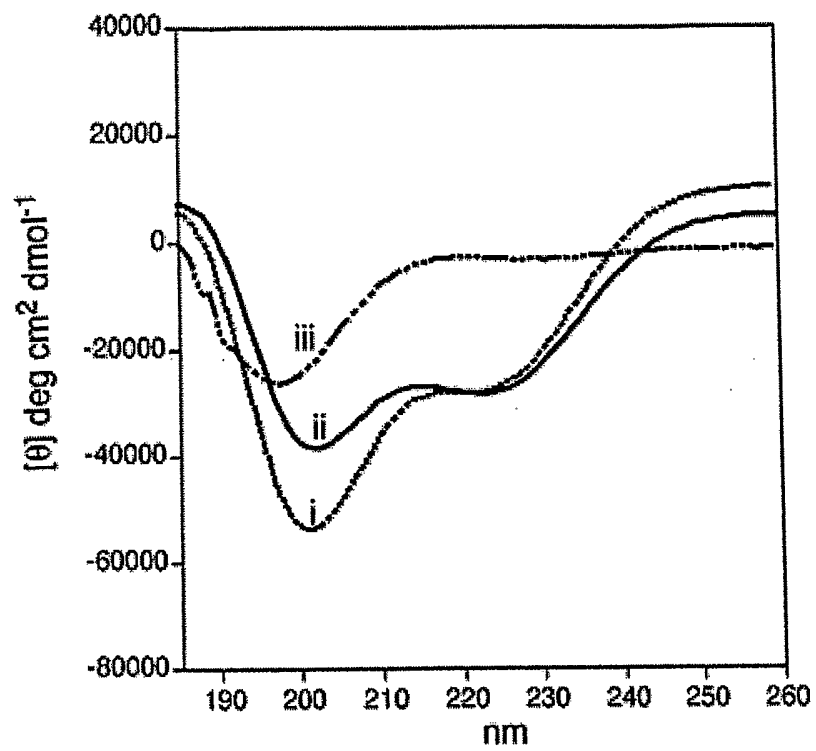


Figure 3

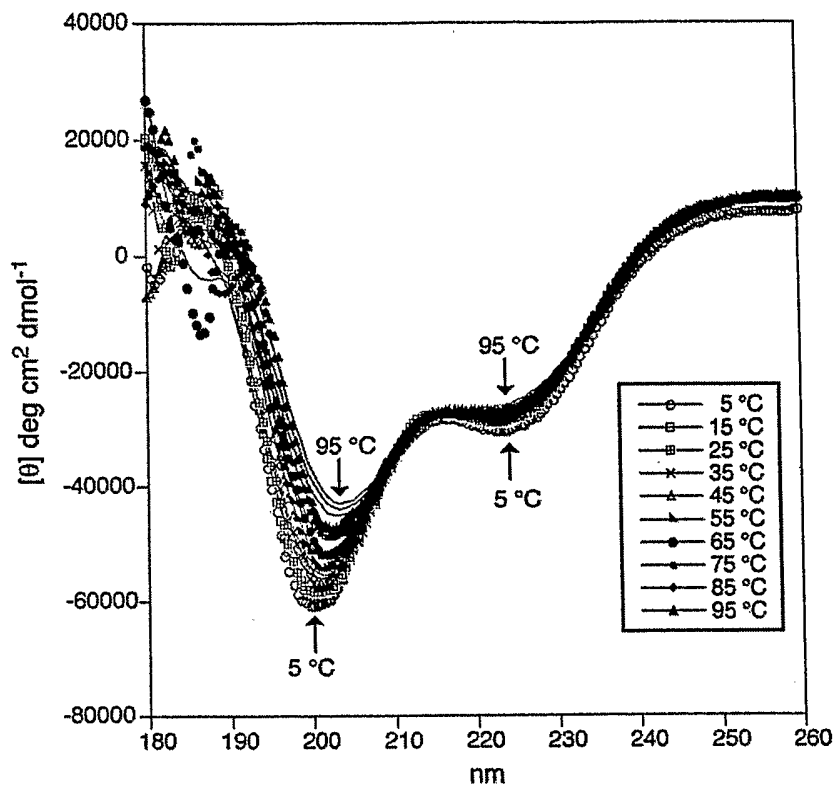


Figure 4

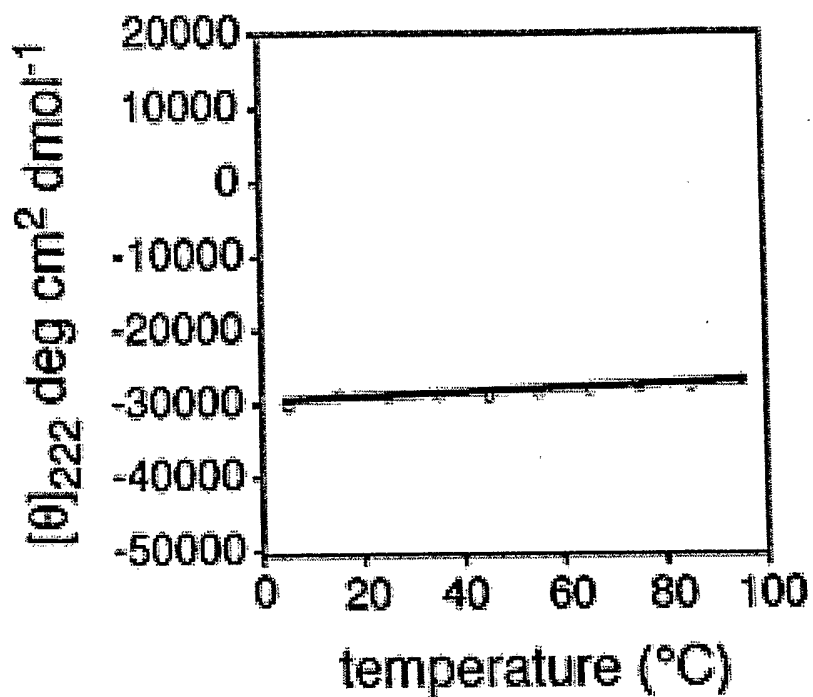


Figure 5

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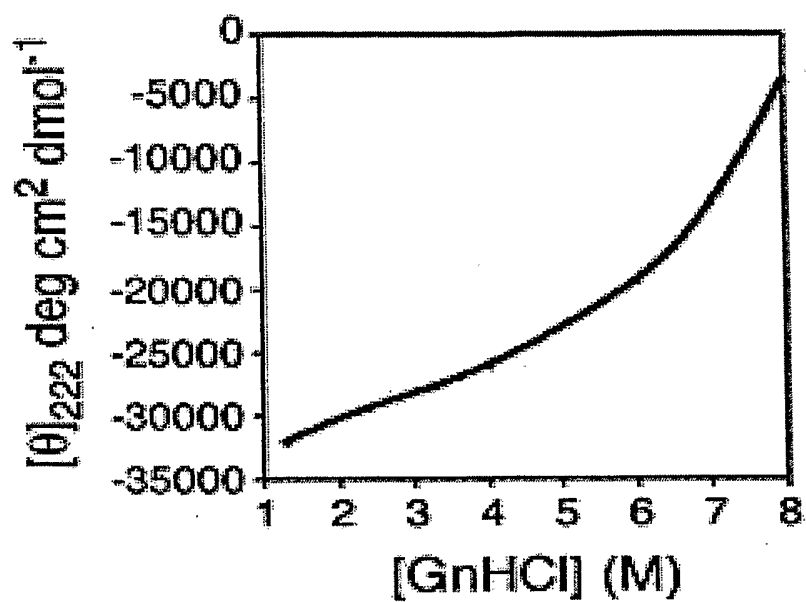


Figure 6

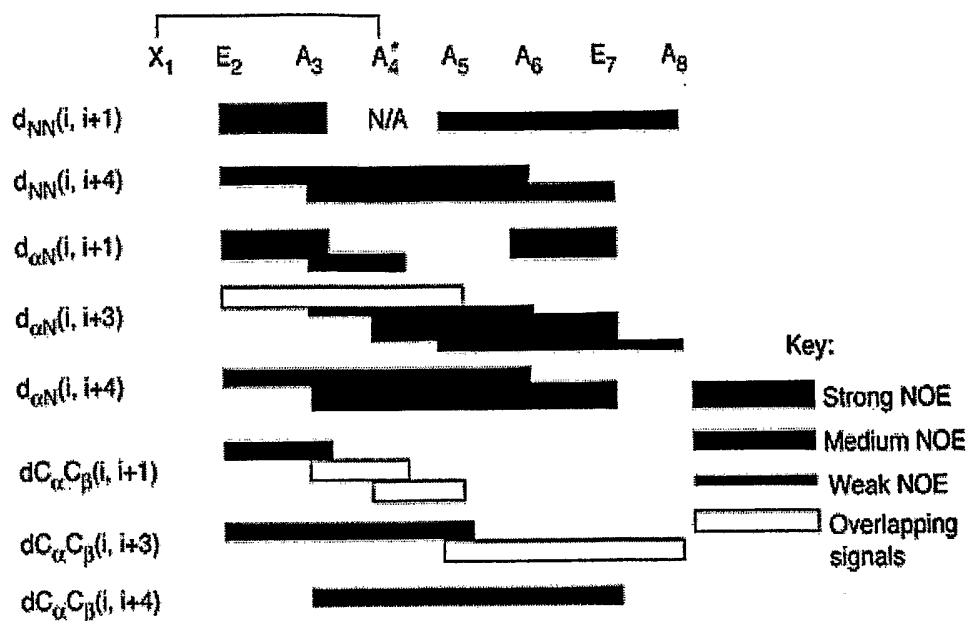


Figure 7

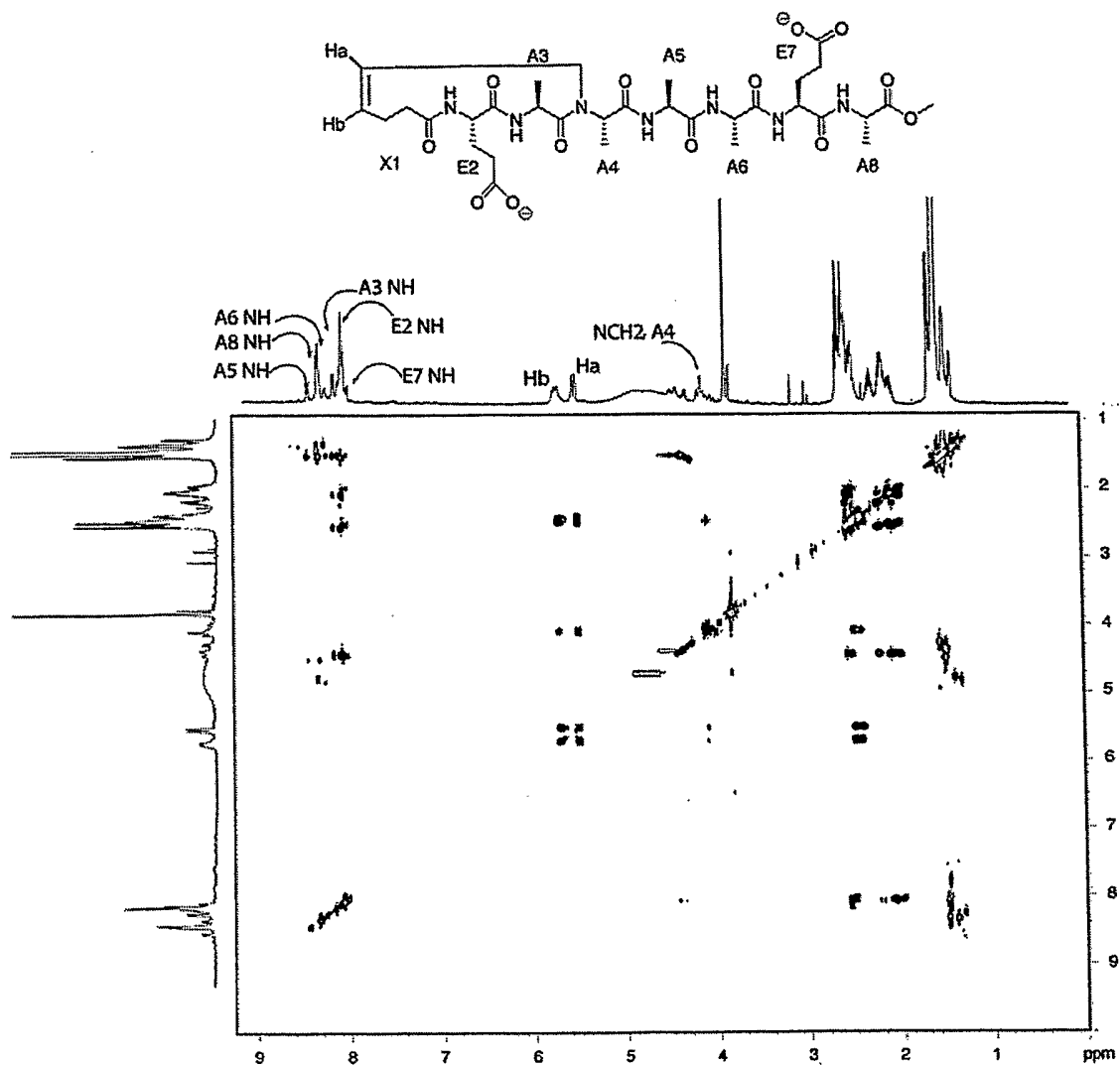


Figure 8

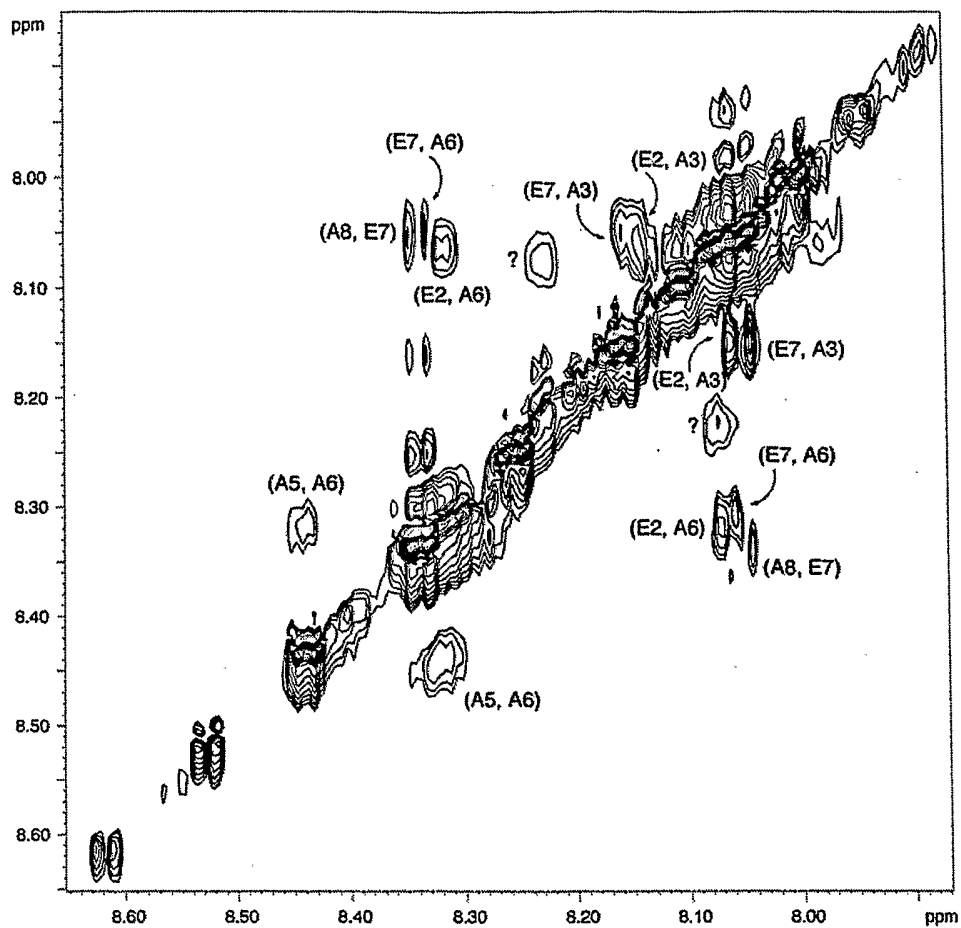


Figure 9

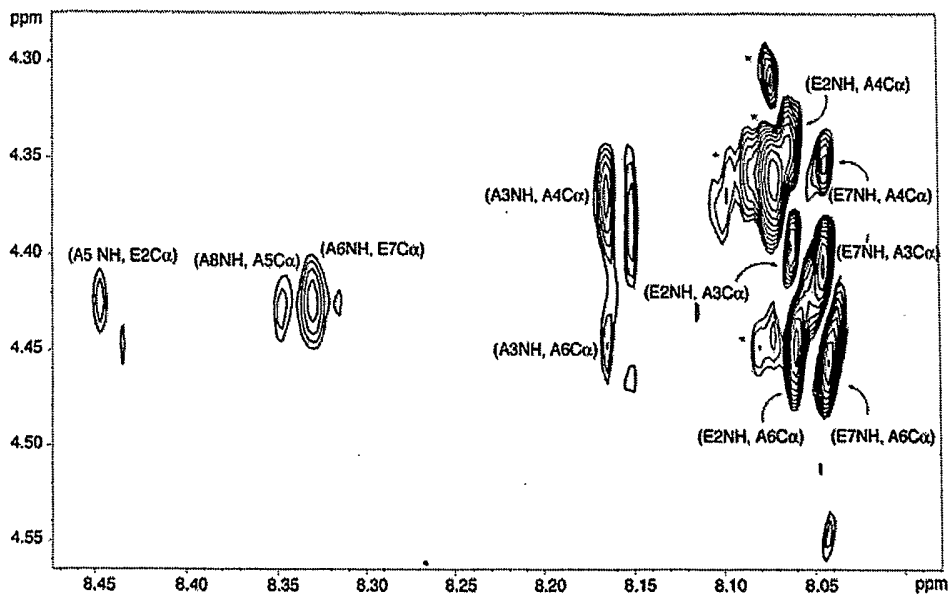


Figure 10

Common Peptide Secondary Structures

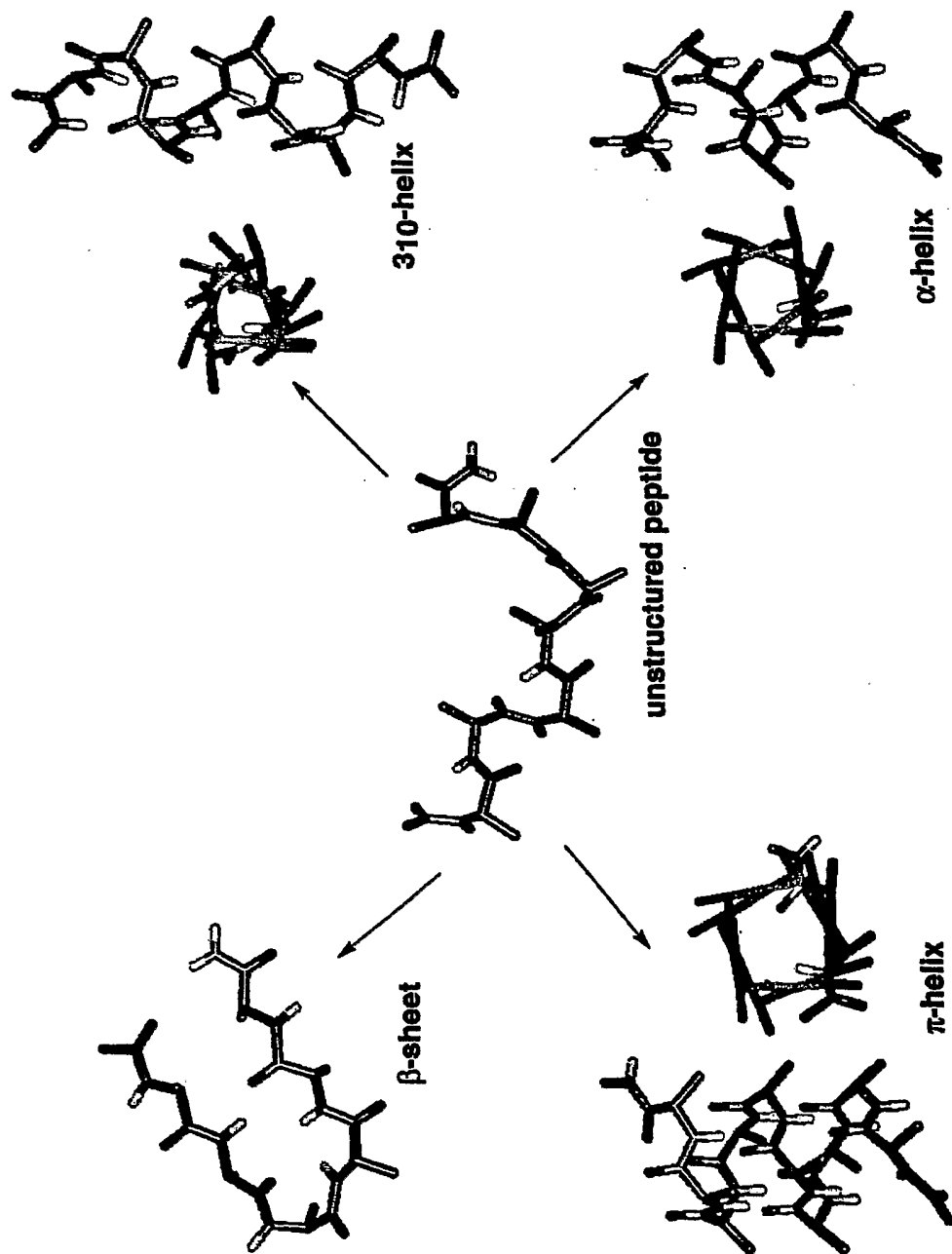


Figure 11

Hydrogen-Bonding Patterns Define Peptide Secondary Structures

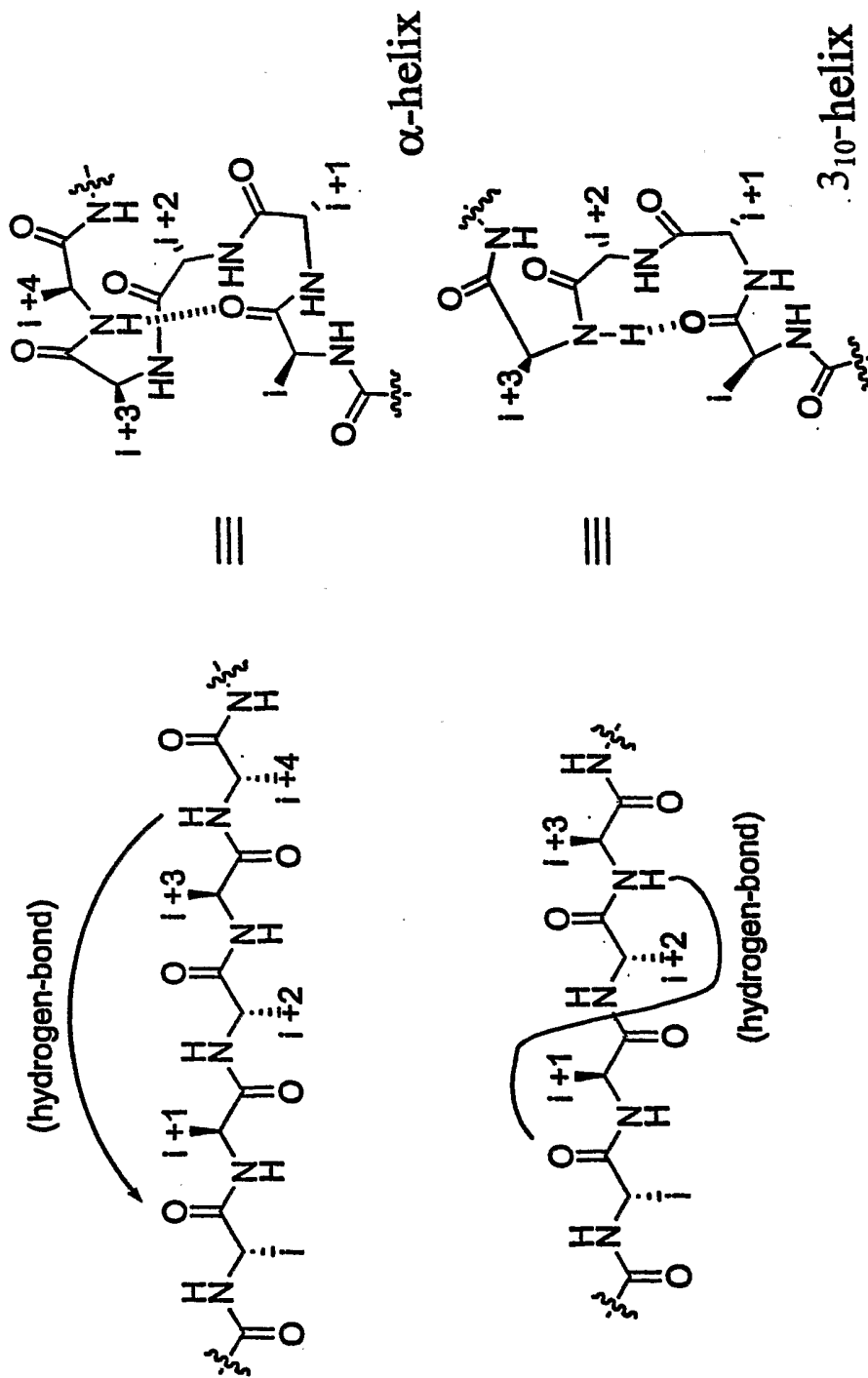


Figure 12

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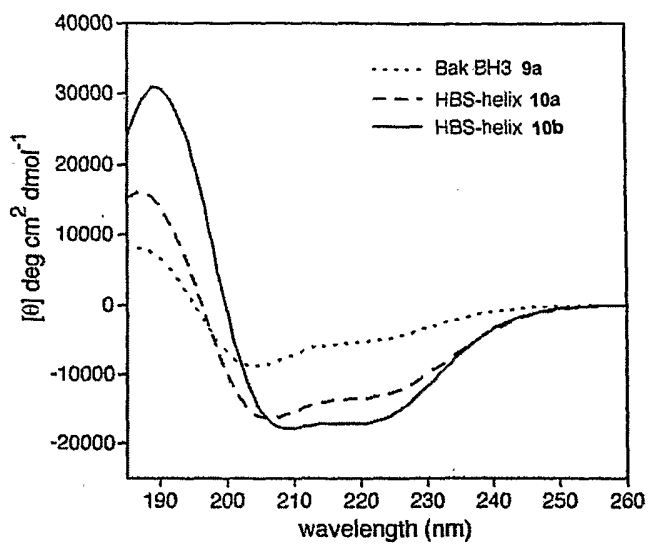


Figure 13

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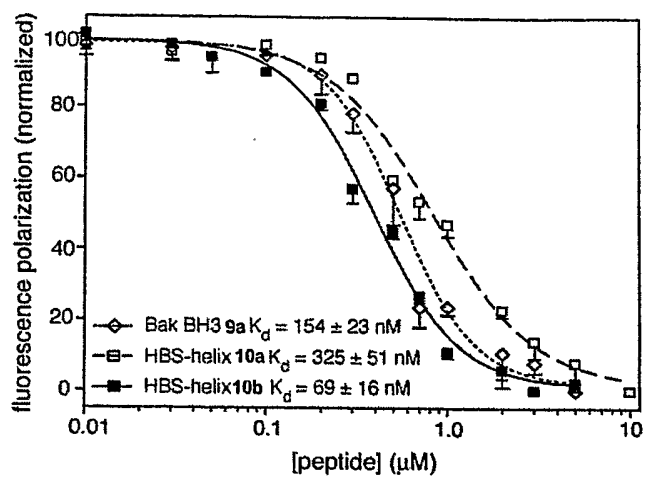
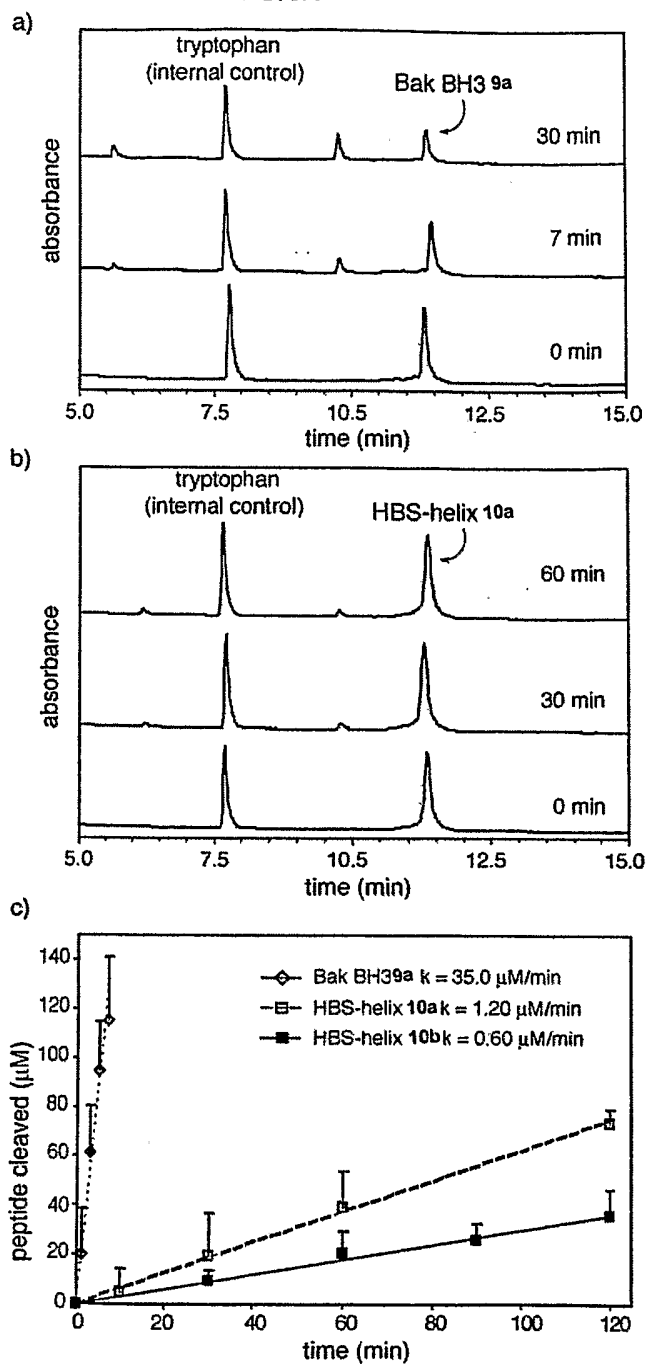


Figure 14

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Figures 15A-C

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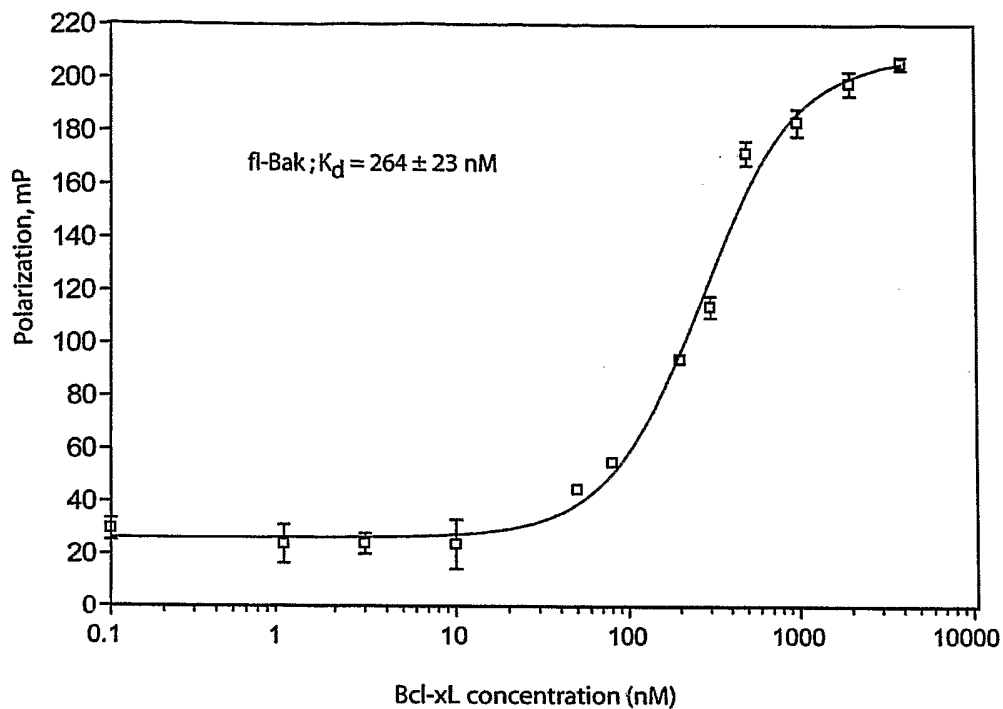


Figure 16