Helix Inducers

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Comparative α-Helicity of Cyclic Pentapeptides in Water**

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Abstract: *Helix-constrained polypeptides have attracted great interest for modulating protein–protein interactions (PPI). It is not known which are the most effective helix-inducing strategies for designing PPI agonists/antagonists. Cyclization linkers (X_1–X_5) were compared here, using circular dichroism and 2D NMR spectroscopy, for α-helix induction in simple model pentapeptides, Ac-cyclo(1,5)-[X_1-Ala-Ala-Ala-X_5]-NH_2, in water. In this very stringent test of helix induction, a Lys1→Asp5 lactam linker conferred greatest α-helicity, hydrocarbon and triazole linkers induced a mix of α- and 3_{10}-helicity, while thio- and dithioether linkers produced less helicity. The lactam-linked cyclic pentapeptide was also the most effective α-helix nucleator attached to a 13-residue model peptide.*

Many biological processes are mediated by protein–protein interactions (PPIs), but discovering small drug-like molecules to target PPIs has been challenging due to the large polar interacting surface areas involved and only very shallow ligand-binding hydrophobic clefts.[1] PPIs often involve a protein α-helix[2] of 1–4 helical turns (4–15 amino acid residues), but corresponding synthetic peptides of these lengths do not tend to form thermodynamically stable α-helix structures in water.[3] This is because water competes with the polar amide CO-NH components of peptide backbones for hydrogen bonding, whereas three backbone CO···HN hydrogen bonds are needed to define each turn of an α-helical peptide (Figure 1 A). Thus, 7–10 helical turns are usually needed for a synthetic peptide to exhibit appreciable α-helicity in water away from a helix-stabilizing protein environment. Methods developed to stabilize synthetic peptides in α-helical structures include incorporating salt bridges, chelating metal ion clips or covalent linkages to cyclize peptide segments, or attaching helix-nucleating end groups.[2c,d] However, there is no consensus as to which is the most effective strategy for inducing α-helicity in short peptides in water and systematic comparisons are needed. The shortest native peptide sequence that can theoretically form three consecutive α-helix defining hydrogen bonds is a pentapeptide (Figure 1 A), with the terminal residues being on the same helix face for sidechain–sidechain connection to lock in an α-helical conformation. Here we use a cyclic pentapeptide scaffold, Ac-cyclo(1,5)-[X_1-Ala-Ala-Ala-X_5]-NH_2, to compare the relative effectiveness of six known cyclization linkers X_1–X_5 (Figure 1 B) reported to aid helicity in polypeptides. Despite their use in polypeptides, the central question as to which is the

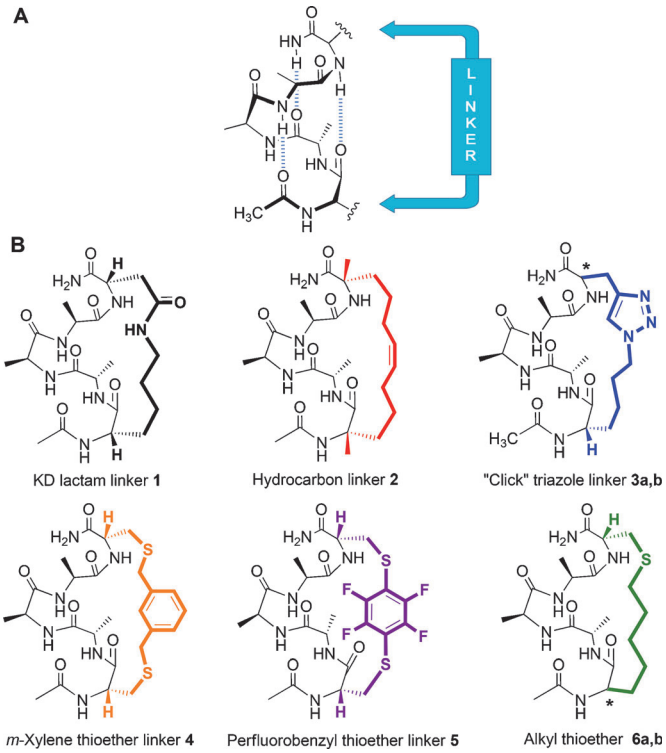

Figure 1. A) Three consecutive hydrogen bonds define an α-helix stabilized by linking sidechains at positions 1 and 5. B) Cyclic pentapeptides **1**–**6** (* denotes isomers: triazole **3a**: L-, **3b**: D- at X_5; thioether **6a**,**b**: L- or D- at X_1, see SI).

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most effective α-helix inducer in short peptides has not been answered. Only one α-helical turn is possible in pentapeptides **1**–**6**, which have no helicity in water when uncyclized, so this is a very demanding test of helix induction for these linkers.

Compounds **1**–**6** all have three alanines, known to favor α-helicity, between their different linkers at positions 1 and 5. Compound **1** incorporates a sidechain-to-sidechain lactam linker, which is a helix inducer in polypeptides,[3,4] hormones (PTH,[5] GLP-1,[6] nociceptin[7]), PPI inhibitors (HIV,[8] RSV[9] viral fusion) and others[10] and in short peptides in water.[11] The position of the amide bond in the linker of **1** is known to be crucial for optimal helicity, other lactam crosslinks showing partial or no α-helicity.[11b] Compound **2** was formed by a ring-closing metathesis cyclization[12] using α,α-disubstituted amino acids with olefin tethers.[13] Such a crosslinking strategy has been used[14] for example to design helices that promote Bcl2 apoptosis[14a] or inhibit HIV-1 capsid assembly[14b] or NOTCH transcription.[14c] The α-methyl groups in the linker in **2** reportedly assist helix stabilization, although may be not be essential.[14l] Compound **3** was made by Cu^{I}-mediated Huisgen 1,3-dipolar cycloaddition (click reaction)[15] of azido norleucine and L- or D-propargylglycine (Pra) at i and $i+4$ positions, and this has been applied to biological targets like PTH[15a] and β-catenin/Bcl9.[15c] Compounds **4** and **5** were cyclized by reacting cysteine side chains with dibromo-*m*-xylene[16] or perfluoroaryl crosslinkers, respectively (also independently reported elsewhere by others[17]). The thioether in **6**, not known as a helix constraint, was compared as it has no polar or ring linker atoms. Based on uses in polypeptides,[11,13,15] the linkers in Figure 1 B represent the best reported helix-inducing connectivity with optimized linker size (6-, 7-, 8-, 9-atom bridges), positioning of heteroatoms, rings or double bonds, and cycle-forming D/L-amino acids.

Linear peptide precursors to **1**–**6** were synthesized by standard Fmoc solid-phase peptide synthesis protocols and cyclized to **1**–**6** by reported procedures (see Supporting Information, SI). Circular dichroism spectra (Figure 2 A) recorded in phosphate buffer (pH 7.2, 298 K) are typically used[18a] to quantitate relative percentage helicity (Figure S1, SI), based on molar ellipticity at $\lambda = 222$ nm in polypeptides (but 215 nm in short peptides).[11] The Lys1–Asp5 lactam crosslinked peptide **1** showed strong α-helicity in water, with two symmetrical minima peaks at 207 and 215 nm (ratio 1.0:1.1) and a positive maximum at 190 nm. Relative to **1** (100% α-helicity), hydrocarbon **2** and triazole **3a** had reduced helicity (62%) and slightly shifted minima (203:217 nm; 1.0:0.8) consistent with less α-helical structure than **1**. The triazole linker afforded more helicity when formed from click cyclization of L-Pra at position 1 (**3a**, 62%) than D-Pra (**3b**, 48%). Thioether-bridged peptides **4**–**6** were much less helical and less structured, with weak ellipticity at $\lambda = 215$–220 nm, no maximum at 190 nm, and a negative minimum at 199 nm.

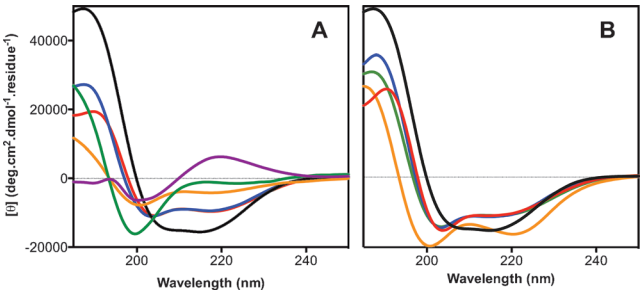

Figure 2. CD spectra of crosslinked pentapeptides: KD lactam **1** (black), hydrocarbon **2** (red), triazole **3a** (blue), *m*-xylene thioether **4** (orange), perfluorobenzyl **5** (violet) and alkyl thioether **6a** (green) at 298 K in: A) 10 mM phosphate buffer pH 7.2 or B) 50% TFE/10 mM phosphate buffer pH 7.2.

To identify any further capacity for helix induction in **1**–**6**, CD spectra were also measured after adding the helix-promoting solvent, 2,2,2-trifluoroethanol (TFE) (Figure 2 B). The CD spectrum for **1** did not change on adding TFE, indicating maximal helicity. However, α-helicity (based on $[\theta]_{215}$) increased for **2** and **3a** from 62% to 75% (50% TFE). Of thioether linkers **4**–**6**, **4** (35%) and **6a** (3%) seem to become more helical in 50% TFE.

Linkers in **1**–**6** are reportedly the best of their kinds for inducing helicity in polypeptides, but there are 7 atoms in the linker in **1** versus 8 atoms in **2** and **3a**. To investigate if shortening the linker in **2** or **3** to a 7-atom bridge might increase helix stabilization in water, we prepared analogues (Figure 3) with 7-membered hydrocarbon (**2a**,**b**) or triazole

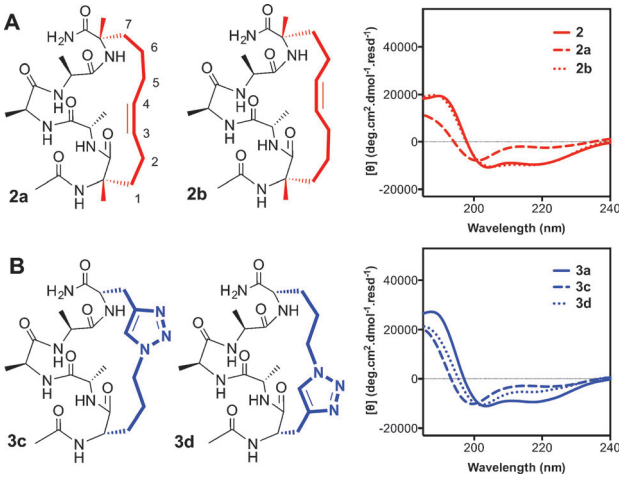

Figure 3. Cyclic pentapeptides with a 7-atom hydrocarbon (A) or triazole (B) linker and their CD spectra (10 mM phosphate buffer, pH 7.2, 298 K).

(**3c**,**d**) crosslinks. Compared to the 8-carbon linker in **2**, a 7-carbon linker induced similar helicity when the *cis*-alkene bond was at C4–C5 positions (**2b**, Figure 3 A), but no helicity when at C3–C4 (peptide **2a**) or C2–C3.[13a] Shortening the triazole linker in **3a** to a 7-atom bridge (**3c**, **3d**) was detrimental to helicity here (Figure 3 B), and in longer peptides reported.[15c] Thus, helicity was very sensitive to the location of the constraint in the linker, as also reported for lactam linked analogues of **1**.[11] This is likely due to some precision needed in aligning all 6 amides in **1**–**3** to form 3 H-bonds in an α-helix (Figure 1 A).

Using ^{1}H NMR spectroscopy, three-dimensional solution structures were determined for **1** and **3a** (90% H_2O:10%

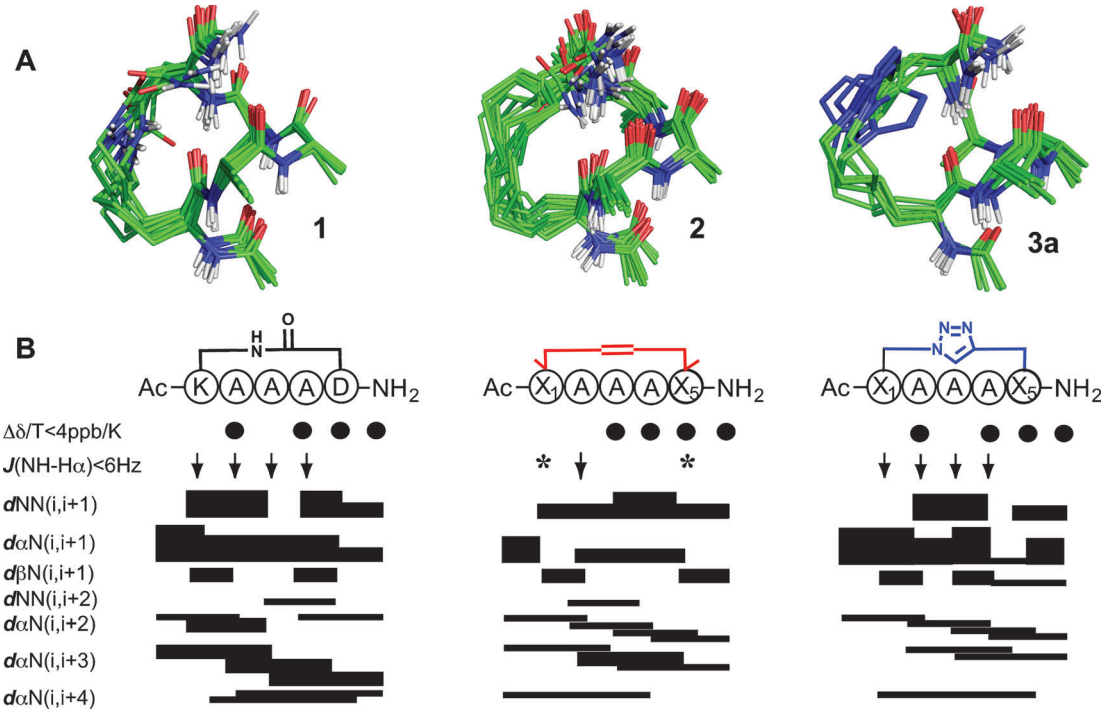

Figure 4. A) Superimposition of 20 lowest energy structures for cyclic pentapeptides **1**, **2** and **3a** calculated by NMR at 298 K. Average backbone RMSD for structure ensemble was 0.37, 0.49 and 0.32 Å, respectively. B) ROE summary diagram for **1**, **2** and **3a** showing distance restraints used to calculate the peptide structure. Bar thickness reflects the intensity of the ROE cross-peaks. Asterisk indicates absence of coupling constant due to presence of α-methyl group.

D_2O) and **2** (40% H_2O:10% D_2O, 50% CD_3CN). We have previously reported some structural data for **1** and close analogues.[10a,11] All the peptides **1**, **2** and **3a** showed some low amide coupling constants, $^3J_{NHC\alpha} < 6$ Hz (Figure 4; Table S1, SI) consistent with some helical structure.[18b] For **1** and **3a**, all but Asp5 (**1**) and X5 (**3a**) coupling constants were < 6 Hz, consistent with helicity. For **2**, Ala3 and Ala4 had $^3J_{NHC\alpha} >$ 6 Hz, indicating less helicity, consistent with CD spectra. Three consecutive low amide NH temperature coefficients (Figures S2–S4; Table S1, SI) for **1**, **2** and **3a** were consistent with three consecutive hydrogen bonds. In addition, the ROESY spectrum for **1**, **2** and **3a** showed some αN(*i*,*i*+2), αN(*i*,*i*+3) and αN(*i*,*i*+4) ROE signals indicative of helical structures (Figure 4). However, αN(*i*,*i*+3) and αN(*i*,*i*+4) ROE intensities were stronger and more numerous for peptide **1** than **2** and **3a**, which had more αN(*i*,*i*+2) than for **1**. This suggested more α-helical structure in **1** than **2** and **3a**, and some 3_{10}-helicity in **2** and **3a**. NMR-derived solution structures for **1** (Figure 4) showed a single α-helical turn with RSMD 3.360 Å versus an idealized α-helix, while **2** and **3a** had RMSD 3.375 Å and 3.365 Å, respectively. The Cα–Cα distance between first and fifth residues in **1**, **2** and **3a** (5.59 Å, 5.85 Å and 5.90 Å, respectively) was compared to the corresponding distance in an idealized α-helix (5.51 Å, $\phi = -57°$; $\phi = -47°$) and 3_{10}-helix (8.30 Å, $\phi = -50°$; $\psi = -28°$). This indicated slightly more elongated (mix with 3_{10}-) helical structures in **2** and **3a**, than the more compact α-helix in **1**, consistent with CD spectra. This is also supported by Ramachandran[18c] plots (Figure 5) obtained from the peptide structures (Figure 4). Only for lactam **1** did all (ϕ, ψ) angles occupy space corresponding to α-helicity in the plot, whereas peptides **2** and **3a** both had several angles located outside of the Ramachandran space that defines α-helicity.

The unique presence of an amide bond in the linker of **1** potentially allows additional H-bonding to the backbone, which might account for greater α-helicity in **1**. However, the temperature dependence of the chemical shift for the linker amide NH in **1** ($\Delta\delta/T = 9.3$ Hz) was much higher than is characteristic of a hydrogen bond ($\Delta\delta/T \leq 4$ Hz).[18d] Moreover, when the amide NH was replaced by a lactone O (**1a**) or amide NMe (**1c**) (Figure 6), the molar ellipticity (θ_{215}) was

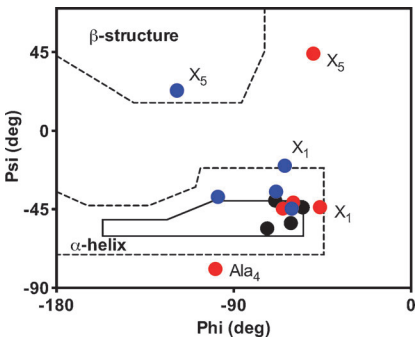

Figure 5. Ramachandran plots of (ϕ, ψ) angles derived from the average of the 20 lowest energy NMR-derived solution structures calculated for **1** (black), **2** (red), and **3** (blue). Only those for **1** are entirely in α-helix space. Solid line encloses a region allowed with full radii, dashed lines enclose regions allowed with smaller radii from hard-sphere calculations.[18c]

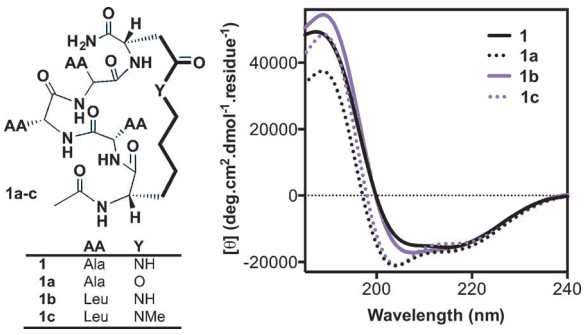

Figure 6. Lactone (**1a**) and lactam (**1b**, **1c**) linked cyclic peptide analogues of **1** and comparative CD spectra (10 mM phosphate buffer, pH 7.2, 298 K).

unchanged, indicating no effect of removing the amide NH on α-helicity. Although molar ellipticity was unchanged at θ_{215}, the CD spectrum for lactone **1a** differs from lactam **1** by an increase in the π–π* band at 204 nm and reduced intensity of the 190 nm band, which indicates a slight relaxation of the helical structure, consistent with more 3_{10}-helix in the mix. The molar ellipticity $[\theta]_{215}$ was similarly unchanged when the linker amide NH became NMe (**1b** vs **1c**, Figure 6), consistent with the linker NH not forming a hydrogen bond to the backbone. We used Ac-cyclo(1,5)-[KLLLD]-NH_2 (**1b**) to check the effect of N-methylation of the lysine ε-NH due to easier synthesis.

Having established the rank order of α-helix induction of these linkers in the shortest possible alanine-containing peptide helix, we tested the relative capacities of linkers to nucleate α-helicity in a longer peptide sequence. A series of 18 mer peptides, Ac-AARAARAARAARA-[X_{14}ARAX$_{18}$]-NH_2 (Ala and Arg residues used to aid peptide helicity and solubilization in water), was prepared and their CD spectra were examined in aqueous media (Figure 7). The cyclic pentapeptides **1**, **2** and **3a** substantially increased helicity (92%, 50%, and 51%, respectively) when attached to the linear sequence **7** (23% helicity).

In conclusion, the Lys1→Asp5 lactam bridge was the most effective crosslink in these cyclic pentapeptides at inducing α-helicity in water, producing the smallest and most compact helical structure for **1**. Hydrocarbon (in **2**) and triazole (in **3**) 1→5 crosslinks were also able to induce some helicity in water, but their structures were conformationally more flexible and less α-helical, as evidenced by CD and NMR spectroscopic studies. The linkers in **2** and **3** induced looser, slightly more elongated, helical structures in their conformational ensemble mix. This is not to say that **2** and **3** will be ineffective helix nucleators within longer peptides, but were assessed here under very demanding conditions in an otherwise non-helical 5-residue peptide and as helix nucleators attached to the end of a model 13-mer peptide with little helicity in water. In longer bioactive peptides already possessing some helicity in water, each of these linkers may be satisfactory for an intended use either because they aid helix formation or because they enable access to other non-α helical structures due to their inherent flexibility. However, in the very short peptides studied here, **1** was the most α-helical cyclic pentapeptide in water and also the most effective helix nucleator attached to a model 13-mer peptide. These findings may enable optimal selection of helix-constraining linkers in short peptides.

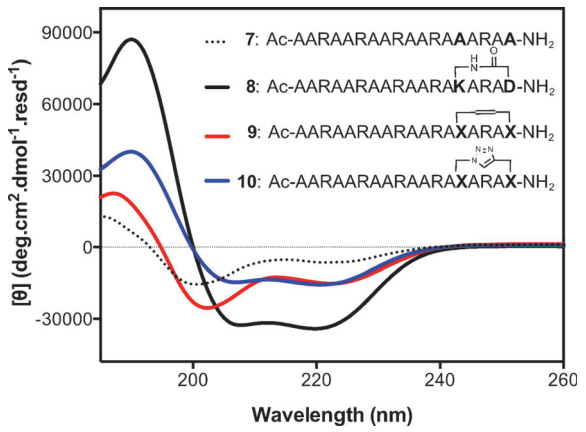

Figure 7. CD spectra of Ac-AARAARAARAARA (50 μM) attached to AARAA-NH_2 (**7**, dots), lactam **1** (**8**, black), hydrocarbon **2** (**9**, red), and triazole **3a** (**10**, blue) in 10 mM phosphate buffer (pH 7.2, 298 K).

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