Helical Structures

Consecutive Cyclic Pentapeptide Modules Form Short α-Helices that are Very Stable to Water and Denaturants**

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The α-helix accounts for approximately 30% of protein structures. Often only a few α-helical turns of exposed protein surfaces are recognized by other proteins, DNA, or RNA.[1] Such helical segments in isolation could be valuable biological probes and drug leads, however, the corresponding short peptides ($\leq$15 residues) do not form thermodynamically stable α-helices in water.[2] Helicity can be stabilized to some extent in longer peptides by using helix-nucleating templates,[3] metal-ion clips,[4] unnatural amino acids,[5] or noncovalent[6] and covalent[7–10] side chain constraints (disulfide,[7] hydrazone,[8] lactam,[9] aliphatic[10]). Small molecules that stabilize or mimic an α-turn have proven elusive, although α-helix side chains have been mounted on non-peptidic scaffolds.[11] Here we describe a promising modular strategy for mimicking short α-helices by using consecutive sequences of cyclic pentapeptide modules to form short α-helices that are remarkably stable in water, resistant to protein denaturants, likely tolerant of amino acid substitution, easy to synthesize, and with promising utility for biological applications.

Lactam bridges ($i\rightarrow i+3$, $i\rightarrow i+4$, $i\rightarrow i+7$) have previously been reported to increase α-helicity in longer peptides to some extent.[9] However, consecutive lactam bridges have not previously been reported in short peptides. In principle, cyclic pentapeptides with an $i\rightarrow i+4$ lactam bridge (for example, cyclo(1→5)-[KARAD], **1**) are α-turn modules that could be directly linked together through amide bonds. Thus, a dimer would have positions i, $i+4$, $i+5$, and $i+9$ occupied by lactam bridges, while exposed positions $i+1, 2, 3, 6, 7, 8$ could in principle be occupied by any peptide side chain (Figure 1). This modular approach to mimicking α-helices is exemplified for the first time below for cyclo(1→5,6→10)-Ac-[KARADKARAD]-NH_2 (**2**) and cyclo(1→5,6→10,11→15)-Ac-[KARADKARADKARAD]-NH_2 (**3**). These compounds are shown to be remarkably stable α-helices in water and maintain their extremely high helicity even under strong denaturing conditions.

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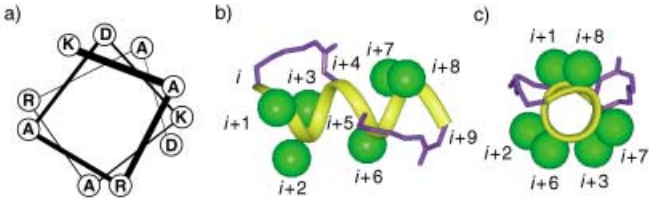

Figure 1. a) Helical wheel structure for dimer **2** (cyclo(1→5,6→10)-Ac-[KARADKARAD]-NH_2) showing the distribution of the side chains; b) side view of **2** showing its helical backbone (yellow), bridging lactam restraints (purple), and exposed side chains (green spheres); and c) **2** viewed end on.

Conventional solid-phase synthesis of **2** and **3** was first attempted using allyl/alloc (alloc = allyloxycarbonyl) orthogonal protection of Asp/Lys residues followed by deprotection and cyclization. However, successive additions of amino acids to the resin-bound cycle **1** were accompanied by extensive aspartimide formation. Consequently, dimer **2** and trimer **3** were instead prepared by solution-phase coupling of cyclo(1→5)-Ac-[KARAD]-OH and cyclo(1→5)-H-[KARAD]-NH_2 (Figure 2). These macrocycles were respectively obtained by standard Fmoc protocols on chlorotrityl (TCP) and rink amide MBHA (RAM) resins using orthogonally protected Fmoc-Asp(OAll)-OH and Boc-Lys(Fmoc)-OH, followed by deprotection, cyclization, and cleavage from the resin.

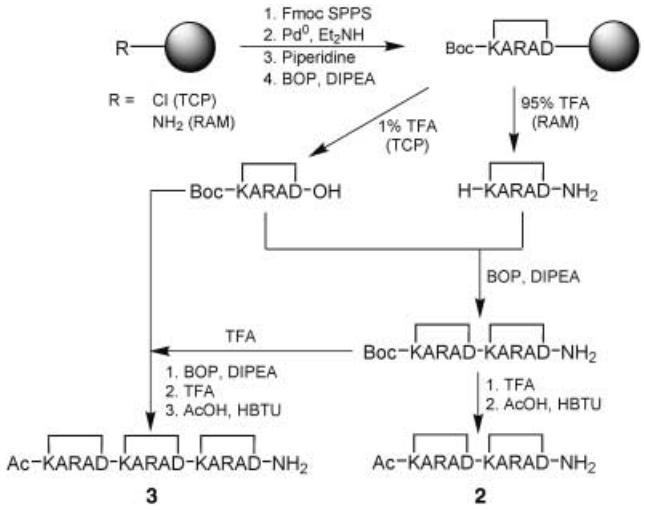

Figure 2. Synthetic strategy for the synthesis of bicyclic **2** and tricyclic **3**. Boc = *tert*-butoxycarbonyl, BOP = 1-benzotriazolyloxytris(dimethylamino)phosphonium hexafluorophosphate, DIPEA = *N*,*N*-diisopropylethylamine, Fmoc = 9-fluorenylmethoxycarbonyl, HBTU = *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, SPPS = solid-phase peptide synthesis, TFA = trifluoroacetic acid.

The circular dichroism spectra recorded in water (Figure 3) reveal the strongly α-helical nature of **2** and **3** (99% and 88%)[12] compared with their acyclic analogues **4** Ac-$(KARAD)_2$-NH_2 (3%) and **5** Ac-$(KARAD)_3$-NH_2 (7%), respectively. The addition of the helix-inducing diluent trifluoroethanol (TFE) failed to increase the helicity in **2** and **3** (85 and 83%, respectively, Figure 3b), which is consistent with high inherent helicity. Compounds **2** and **3** had almost identical molar elipticity θ_{222}, θ_{208}, $\theta_{192} = -32$, -24.5, $102\ \mathrm{mdeg\,cm^2\,dmol^{-1}\,residue^{-1}}$, respectively (Figure 3a), and a high $\theta_{222}/\theta_{208}$ ratio of approximately 1.3:1.[13] Calculations of the percentage helicity are derived from equations formulated for longer peptides, and can underestimate the α-helicity of short peptides, especially those containing Ala,[14a] charged residues,[14b] or in the presence of significant amounts of TFE.[14c]

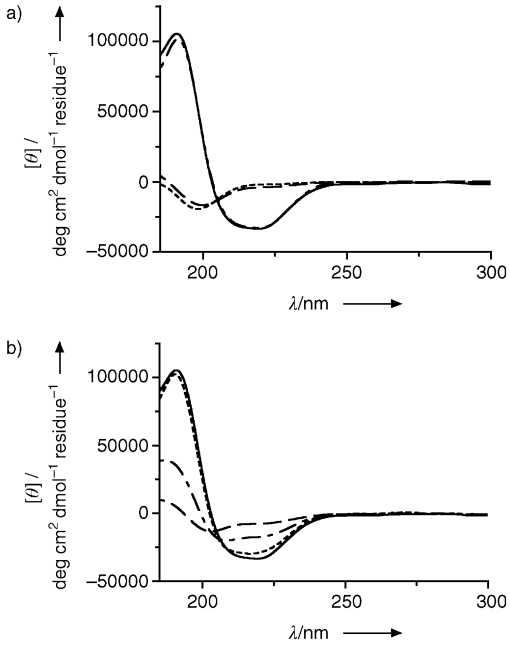

Figure 3. CD spectra obtained in 10 mM phosphate buffer, pH 7.4, 25 °C for 32–44 μM solutions of a) **2** (——), **3** (–•–•), and acyclic analogues **4** (– – –) and **5** (----); b) **2** (——) versus **3** (----), **4** (– – –), and **5** (–•–•) in 50% TFE.

To confirm this compelling CD evidence of high α-helical structure, NMR spectra were obtained for **2** in H_2O:D_2O (9:1). Features (Figure 4a) characteristic of α-helicity included coupling constants $^3J_{NHC\alpha} \leq 6$ Hz[15] for all the amide NH protons (2.2–5.2 Hz) except D_{10}, a small temperature dependence of the chemical shifts ($\Delta\delta/T < 4$ ppb per deg) for seven of the amide NH protons,[16] which is consistent with all the expected helix-defining H-bonds except for $K_1 \rightarrow D_5$, and nonsequential medium-range NOE interactions $\delta_{\alpha N}(i, i+3)$, $\delta_{\alpha N}(i, i+4)$, and $\delta_{\alpha\beta}(i, i+3)$ in the NOESY spectra.[17] In particular, prominent $\delta_{\alpha N}(i, i+4)$ versus weak $\delta_{\alpha N}(i, i+2)$ NOE interactions establish α- rather than 3_{10}-helicity and indicate there is only a small number of β- or γ-turns in the conformational mix.[18]

Three-dimensional structures were calculated for **2** in water, initially using torsional angle dynamic simulated annealing using the program DYANA,[19] followed by dynamic simulated annealing and energy minimization in Xplor (3.851)[20] from 122 NOE (32 sequential, 25 medium range, 65 intra-residue) distance restraints, 9 ϕ angle restraints ($^3J_{NHC\alpha}$, $\phi = -65 \pm 30°$), and 2 χ_1 angle restraints ($^3J_{NHC\alpha}$, $\chi_1 = -60 \pm 30°$). No explicit H-bond restraints were

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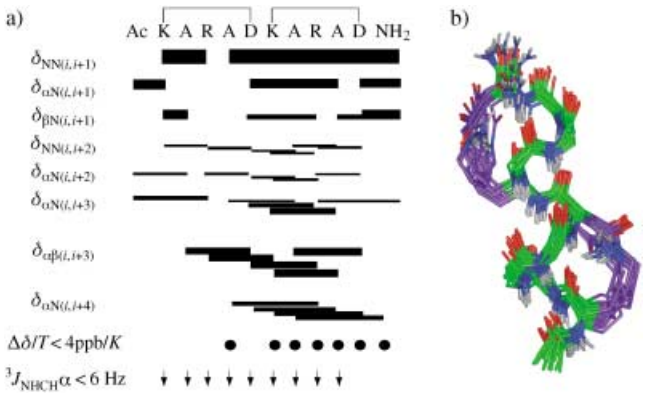

Figure 4. a) Sequential and medium range NOE interactions, temperature coefficients, and coupling constants for **2** in D_2O:H_2O (1:9). The intensities (strong <2.7 Å, medium <3.5 Å, weak <5.0 Å, very weak <6.0 Å) are proportional to the bar width. b) The 20 lowest energy structures for **2** superimposed (backbone, green; lactam bridges, purple; carbonyl O, red; amide N, blue; average backbone pairwise rmsd: 0.577 Å). The C-terminus is at the top and side chains are omitted for clarity.

included in the calculations. The final 20 lowest energy structures contained no dihedral angle ($>2°$) or distance (>0.17 Å) violations. The final structures (Figure 4b) indicate three well-defined α-helical turns for **2** in water, with lactam bridges in locations anticipated from Figure 1c.

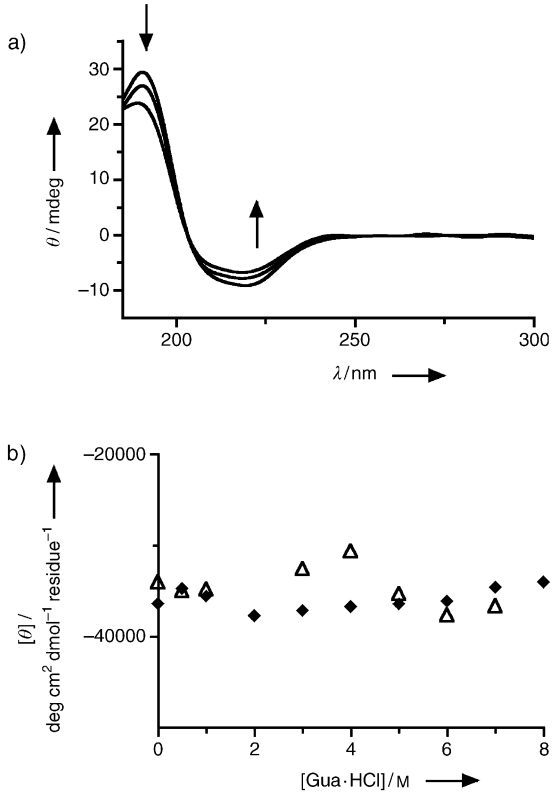

Figure 5. a) CD spectra for **2** in 10 mM phosphate buffer (pH 7.4) at variable temperatures (5, 35, 65 °C). b) Molar elipticity at 222 nm for **2** (◆) and **3** (△) with varying [guanidine·HCl] at 25 °C.

The helical macrocycles were conformationally very stable even under protein-denaturing conditions, as illustrated by the small dependence of their CD spectra on temperature between 5 and 65 °C (Figure 5a) and on the concentration of guanidine·HCl (Figure 5b). Compound **2** was also found to be highly resistant to proteolytic cleavage by trypsin (ca. 97 % intact after 2 h), whereas the linear peptide Ac-$(KARAD)_2$-NH_2 was degraded in 30 s.

In summary, we have described a promising new generic approach to mimicking α-helices by using sequences of consecutive macrocyclic pentapeptides (for example, cyclo(1→5)-Ac-[KARAD]-NH_2, **1**) to form 3-turn (for example, cyclo(1→5,6→10)-Ac-$[KARAD]_2$-NH_2, **2**) and 4-turn (for example, cyclo(1→5,6→10,11→15)-Ac-$[KARAD]_3$-NH_2, **3**) α-helices. These 10- and 15-residue peptides are easy to synthesize and form contiguous α-helical turns through two and three macrocycles that maintain high conformational stability in water, and are resistant to protein-denaturing conditions (65 °C, 8 M Gua·HCl, trypsin digestion). CD and 2D-NMR spectra provide compelling evidence of α-helicity, which was not increased by adding TFE. These multicyclic structures permit variation of up to 60 % of their component amino acids and thus appear suitable for general mimicry of short α-helical protein segments that bind receptors/ligands on one helical face, and thus confer the major advantages of conformational and proteolytic stability over linear peptides.

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