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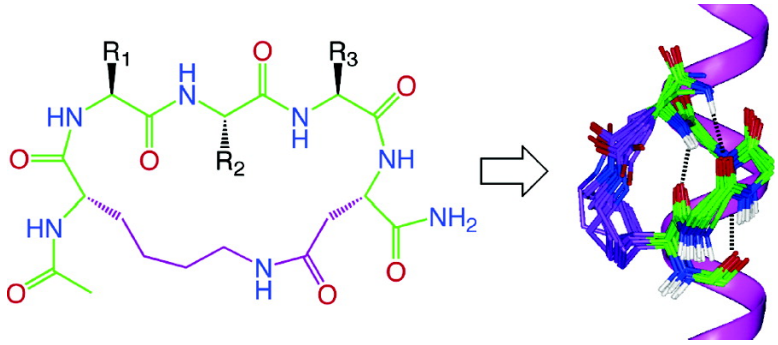

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Single Turn Peptide Alpha Helices with Exceptional Stability in Water

Nicholas E. Shepherd, Huy N. Hoang, Giovanni Abbenante, and David P. Fairlie*

Contribution from the Centre for Drug Design and Development, Institute for Molecular Bioscience, University of Queensland, Brisbane, Qld 4072, Australia

Received July 22, 2004; E-mail: d.fairlie@imb.uq.edu.au

Abstract: Cyclic pentapeptides are not known to exist in α-helical conformations. CD and NMR spectra show that specific 20-membered cyclic pentapeptides, Ac−(cyclo-1,5) [KxxxD]-NH_2 and Ac-(cyclo-2,6)-R[KxxxD]-NH_2, are highly α-helical structures in water and independent of concentration, TFE, denaturants, and proteases. These are the smallest α-helical peptides in water.

Introduction

Alpha-helical sequences of ≤15 amino acids are estimated to account for ∼30% of protein structure and frequently mediate biological processes through interactions with proteins, DNA, or RNA.[1] Short synthetic peptides corresponding to such α-helical recognition motifs tend not to display appreciable helical structure in water, away from the helix-stabilizing hydrophobic environments of proteins.[2] If short peptide α-helices could be stabilized or mimicked by small molecules, such compounds might be valuable chemical or biological probes and lead to development of novel pharmaceuticals, vaccines, diagnostics, biopolymers, and industrial agents.

The goal of structurally mimicking short α-helices with small molecules that have biological activity comparable to proteins has not yet been realized. Various strategies have been tried for biasing the peptide backbone toward the α-helical conformation, including helix-nucleating templates,[3] metal ion coordination,[4−6] unnatural amino acids,[7] noncovalent side chain interactions,[8] and covalent side chain linkers such as disulfide,[9] hydrazone,[10] aliphatic,[11] and lactam bridges.[12] Alternatively, more recent attempts have been made to mimic just the side chain components of an α-helix using nonpeptidic scaffolds such as oligoamide,[13] terphenyl,[14] terephthalamide,[15] and other[16] units which project two or three substituents into similar three-dimensional space as occupied by side chains of 2−3 turns of α-helical peptides.

A common approach to forcing peptides into bioactive conformations has been to constrain the peptide backbone via cyclization, either through side chain to side chain, main chain to side chain, or main chain to main chain linkages, often using amide, alkyl, alkenyl, or thioether connectors. When used in

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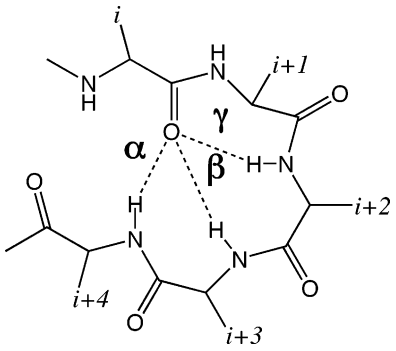

Figure 1. 7-, 10-, and 13-membered macrocycles formed via hydrogen bonds that respectively define γ-, β-, and α-turns.

conjunction with insertion of other molecular constraints, cyclization[17] has been a particularly successful strategy for mimicking γ or β turns that are respectively defined by 7- and 10-membered hydrogen-bonded pseudo-macrocyclic rings (Figure 1). Such rings are formed by a hydrogen bond between the carbonyl oxygen of residue i and the amide NH of residue $i + 2$ or $i + 3$. By contrast, an α-turn, the minimal repeating unit in an α-helix, is defined by a 13-membered ring formed by a hydrogen bond between a carbonyl oxygen of one residue (position i) and the amide NH of another residue (position $i + 4$) separated by three residues (Figure 1), suggesting that 5 residues is the theoretical minimum to define an α-turn. While peptide sequences longer than 10 residues have been constrained to varying degrees into α-helical conformations, shorter peptides have rarely been constrained into α-turns. Recently a pseudopeptide was shown to adopt an α-turn conformation in the crystal state; however it lacked a unique conformation in solution.[18] A hexapeptide inhibitor of calmodulin was found to adopt a distinct α-helical conformation in certain solvents,[19] and metallopentapeptides have been reported to exhibit solvent-dependent α-helicity.[6] However, restraining peptides to a substantial proportion of α-helicity in water remains a difficult challenge.

Perhaps the simplest and most common method of enhancing helical structures in long (≥15 residues) peptides has been the use of side chain to side chain amide linkages. This has generally involved forming a covalent bond between side chains of lysine/ornithine residues and aspartic/glutamic acid residues, separated by two ($i \rightarrow i + 3$) or three ($i \rightarrow i + 4$) intervening residues, or through longer linkers between i and $i + 7$ residues.[12] These constraints, although initially examined in model peptides, have been applied to a number of 15−30 residue peptides of biological significance[20] including PTH,[21] NPY,[22] CRF,[23] GCN4,[24] Galanin,[25] Dynorphin-A.[26] Despite the study of such bridged peptides for over two decades, there is still a lack of consensus as to the optimal residue combinations, ring sizes, and sequences that can effectively stabilize helical peptide conformations. Indeed, everything has been proposed from Asp-Lys,[12] Glu-Lys,[27] Lys-Glu,[28] and Lys-Asp,[25] though there is some consensus that i to $i + 4$ separation of linking residues is better than other separations. More recently, two overlapping lactam bridges have been used to stabilize a rigid hexapeptide α-helix, resistant to thermal degradation,[29] that has some limited templating capacity.[24] There are few studies[30] that report α-helicity for the theoretical minimum (pentapeptide) sequence needed to define an α-turn, the existence and properties of which are not well defined despite the likelihood that only one or a few turns of a protein helix need to be mimicked for agonist/antagonist biological activity.

We now report the surprising observation that simple cyclic pentapeptides, involving one specific type of side chain to side chain linkage that results in a 20-membered macrocycle, exhibit highly α-helical structures in water. Helicity is reasonably tolerant of amino acid substitution at three of the five component amino acids, and the cyclic pentapeptide has both conformational and chemical stability to protein denaturing/degrading conditions (8 M guanidine·HCl; trypsin; human plasma). These results suggest the use of one or more such cyclic pentapeptides or analogues as α-helical scaffolds that can project additional peptidic, cyclic, and nonpeptidic appendages into positions typical of side chains of α-helical peptides and protein segments. These minimalist single turn α-helical modules are now described.

Results

Macrocycle Synthesis. The solid phase synthesis of cyclic pentapeptides was achieved using standard Fmoc protocols to assemble linear peptides, with orthogonal allyl/alloc protecting groups on the respective lysine and aspartic acids (Scheme 1). Once assembly of the linear peptides was completed in sequences with N-terminal lysine/ornithine residues, the N-terminal Fmoc group was removed and acetylation was carried out before removal of the orthogonal protecting groups with $Pd(PPh_3)_4$ (Scheme 1). Macrolactamization was achieved with BOP/DIPEA in a mixture of polar solvents (DMSO/NMP) and in all cases went to completion as judged by mass spectrometry. Alternatively, for residues with N-terminal aspartic/glutamic acid residues, removal of the orthogonal protecting group and

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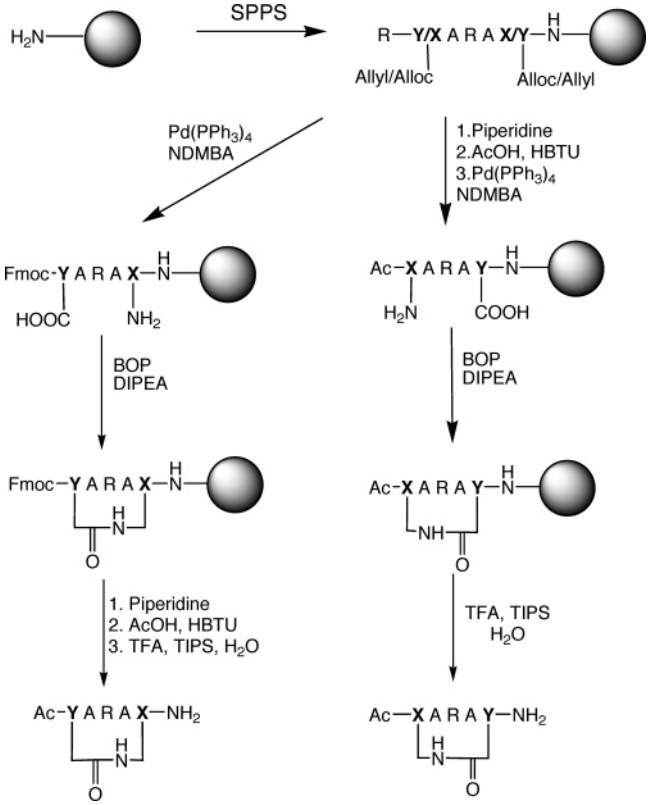
Scheme 1. Representative Syntheses for Cyclic Peptides (X = Lys/Orn, Y = Asp/Glu)



macrolactamization were best undertaken before the final Fmoc-deprotection and acetylation steps. Cleavage with TFA yielded the desired peptides (Scheme 1). In general this synthesis provided milligram quantities of the desired peptides, although yields were compromised by three competing side reactions.

When aspartic acid was at the N-terminus, side chain deprotection and cyclization was carried out before deprotection of the N-terminal Fmoc group and acetylation, to avoid piperidine-mediated aspartimide formation. The latter resulted in mixtures that could not be separated by rpHPLC, and also occurred during macrolactamization, when large excesses of coupling reagent and base were used. Instead, we used stoichiometric ratios of coupling reagent (1.2 equiv) and base (2 equiv).[31] Alternatively Sheppard's Hmb group[32] could be positioned on the preceding alanine residue, although lower yields were observed due to incomplete coupling of the last aspartic acid residue (even with prolonged coupling times), resulting in contamination of the desired product by the tetrapeptide deletion product.

A second minor problem occurred when glutamate was at the N-terminus. Side chain deprotection and cyclization, carried out before acetylation, resulted in higher yields as some piperidine-mediated pyroglutamate deletion peptide was observed in the alternative sequence of acetylation followed by cyclization. This was not a significant problem for Glu residues at the C-terminus, although yields of these products were lower.

A third problem occurred during cyclization, resulting in 30−70% of the product yield being dimers and higher oligomers attributed to the relatively high loading of trityl chloride (~1.0 mmol/g) and Rink-amide MBHA resin (~0.70 mmol/g) encouraging intermolecular coupling. In contrast to previous reports,[12,33] we found that formation of these byproducts was relatively independent of the coupling reagent, base, solvent, and time. Prolonged cyclization times (>24 h) generally yielded greater oligomerization. Although the side products could be removed by HPLC, switching to a low substitution resin (Tentagel S RAM 0.25 mmol/g)[34] significantly improved the yield and purity of the desired monocyclic products, with no more than 10% oligomers. In addition, we found that diluting high loading resins had little effect on oligomer formation, possibly because the distribution of anchored peptide may not be uniform.

Helix Characterization By Circular Dichroism Spectra. The helix stabilizing effects of $i \rightarrow i + 4$ lactam bridges in peptides are well documented;[12] however there are many inconsistencies in the literature as to the optimal bridge size and the order of bridging residues (see above). The lengths and sequences of the peptides examined by other workers have varied considerably, CD and/or NMR spectra have been used to measure α-helicity under quite different conditions, or α-helical content has simply been inferred from observations of enhanced biological activity. Consequently it has been very difficult to compare data and make accurate conclusions about key requirements for α-helicity in short peptides. We have chosen a systematic approach to examine factors that influence α-helicity in water by using circular dichroism spectroscopy to both qualitatively and quantitatively compare α-helicities in a range of pentapeptides, being the theoretical minimal α-turn unit. Previously, it has only been possible to study α-helical peptides in this size range by incorporating them into larger stabilizing systems and then subtracting the contribution of the stabilizing system from the CD spectrum.[5]

Twenty one compounds (Tables 1 and 2) were synthesized to determine whether a pentapeptide could be made α-helical through cyclization and to examine the effects on helicity of varying the length and orientation of (i, $i + 4$) side chain to side chain bridges, capping one or both ends of the pentapeptide through amide bond formation, altering the nature of the three residues between the bridging residues. We began with Ala and Arg (for solubility) residues at the center of the pentapeptide, as the α-helix inducing capacity of these residues is well documented.[35]

Helix Dependence On Ring Size. Lysine (K), ornithine (O), aspartic acid (D), and glutamic acid (E) were systematically substituted at i and $i + 4$ positions (positions 1 and 5) in the cyclic pentapeptide sequence Ac-(cyclo-1,5)-[XARAX]-NH_2 (Table 1, **1**−**8**). Figure 2 shows that a 20-membered macrocycle formed through a K(i) → D($i + 4$) linkage (i.e., **3**) is the bridge system that results in maximal helicity. To confirm that this was the direct result of the lactam constraint, a CD spectrum of the acyclic version (**9**) was acquired under identical conditions and gave a characteristic spectrum of an unstructured peptide.

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Table 1. Molar Elipticities ([θ] deg cm^2 dmol^{-1} residue^{-1}) at λ = 215, 207, and 190 nm, Ratios of Elipticites at 215/207 nm, and Percentage Helicity for Peptides **1**−**12** in 10 mM Phosphate Buffer (pH 7.4, 25 °C)

peptide	$[\theta]_{215}$	$[\theta]_{207}$	$[\theta]_{190}$	$\theta_{215}/\theta_{207}$	relative helicity[c]
Ac-(cyclo-1,5)-[**K**ARA**E**]-NH$_2$ (**1**)	−1068	−3393	−10611	0.31	0.08
Ac-(cyclo-1,5)-[**E**ARA**K**]-NH$_2$(**2**)	−7430	−12803	20735	0.58	0.58
Ac-(cyclo-1,5)-[**K**ARA**D**]-NH$_2$ (**3**)	−12757	−12211	38300	1.04	1.00
Ac-(cyclo-1,5)-[**D**ARA**K**]-NH$_2$ (**4**)	−7723	−10705	15600	0.72	0.60
Ac-(cyclo-1,5)-[**O**ARA**D**]-NH$_2$ (**5**)	92	−2077	−4613	−0.04	0
Ac-(cyclo-1,5)-[**D**ARA**O**]-NH$_2$ (**6**)	−4671	−9748	−6954	0.48	0.37
Ac-(cyclo-1,5)-[**O**ARA**E**]-NH$_2$ (**7**)	741	−3368	−16228	−0.22	0
Ac-(cyclo-1,5)-[**E**ARA**O**]-NH$_2$ (**8**)	2442	−1917	−11256	−1.27	0
Ac-KARAD-NH$_2$ (**9**)	−524	−5555	−7372	0.09	0.04
Ac-(cyclo-1,5)-[**K**ARA**D**]-OH (**10**)	207	−5643	−13953	−0.04	0
a	−625	−3195	−2659	0.20	0.06
H-(cyclo-1,5)-[**K**ARA**D**]-NH$_2$ (**11**)	−812	−2228	1355	0.36	0.06
b	−2590	−3327	8452	0.78	0.2
H-(cyclo-1,5)-[**K**ARA**D**]-OH (**12**)	−1033	−5737	−4966	0.18	0.08

[a] In 0.01 M HCl pH 2. [b] In 0.001 M NaOH pH 10. [c] $[\theta]_{215}$ (**3**)/$[\theta]_{215}$ (**x**) refer to "Quantitation of Helicity" section.

Table 2. Molar Elipticities ([θ] deg cm^2 dmol^{-1} residue^{-1}) at λ = 215, 207, and 190 nm, Ratios of Elipticites at 215/207 nm, and Percentage Helicity for Cyclic Peptides **13**−**21** in 10 mM Phosphate Buffer pH 7.4 at 25 °C

peptide	$[\theta]_{215}$	$[\theta]_{207}$	$[\theta]_{190}$	$\theta_{215}/\theta_{207}$	relative helicity[a]
Ac-(cyclo-2,6)-R[**K**AAA**D**]-NH$_2$ (**13**)	−13 537	−13 684	39 352	0.99	0.91
Ac-(cyclo-2,6)-R[**K**ALA**D**]-NH$_2$ (**14**)	−14 798	−15 165	46 621	0.98	1.00
Ac-(cyclo-2,6)-R[**K**AMA**D**]-NH$_2$ (**15**)	−11 853	−12 296	38 464	0.96	0.80
Ac-(cyclo-2,6)-R[**K**AQA**D**]-NH$_2$ (**16**)	−11 394	−12 279	36 865	0.93	0.84
Ac-(cyclo-2,6)-R[**K**AFA**D**]-NH$_2$ (**17**)	−8644	−9087	27 718	0.95	0.76
Ac-(cyclo-2,6)-R[**K**AGA**D**]-NH$_2$ (**18**)	−4874	−7678	10 036	0.63	0.32
Ac-(cyclo-2,6)-R[**K**GSA**D**]-NH$_2$ (**19**)	−4810	−6975	12 831	0.69	0.32
Ac-(cyclo-2,6)-R[**K**SSS**D**]-NH$_2$ (**20**)	−4432	−8017	6827	0.55	0.30
Ac-(cyclo-2,6)-R[**K**GGG**D**]-NH$_2$ (**21**)	−2131	−4868	−1593	0.44	0.14

[a] θ_{215} (**14**)/θ_{215} (**x**) refer to "Quantitation of Helicity" section.

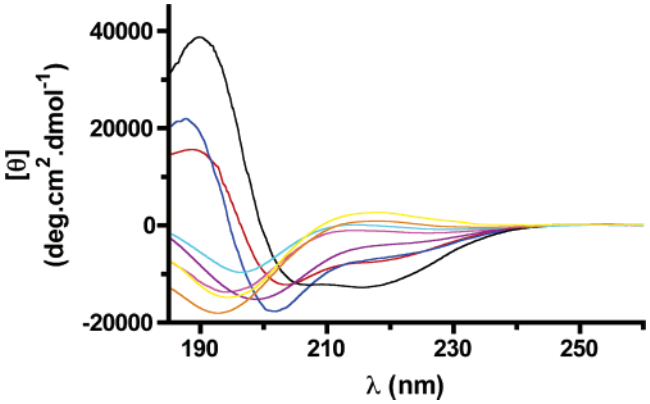

Figure 2. CD spectra of cyclic pentapeptides **1** (pilnk), **2** (blue), **3** (black), **4** (red), **5** (aqua), **6** (purple), **7** (orange), and **8** (yellow) in 10 mM phosphate buffer (pH 7.4, 25 °C).

Some helicity was observed for D(i) → K(i + 4) (e.g., **4**) and E(i) → K(i + 4) (e.g., **2**); however the negative shift and deeper minimum around 208 nm suggest that this may be 3_{10}-helicity. Thus it is clear that a 20-membered macrocycle is optimal for α-helicity, that an increase or decrease in ring size by one carbon significantly reduces helicity, and that in addition to ring size the position of the amide bond is also crucial. Reversing the position of the amide bridge in the 20-membered ring systems (**3** → **4**) or shifting the amide along by one carbon (**7**, **8**) has a detrimental effect on helicity.

Interestingly, the CD spectrum for **3** shows a slight shift in its minima to lower wavelengths compared with longer α-helical peptides (222 nm → 215 nm, 208 nm → 207 nm), as has been observed before in short fixed nucleus alanine helices.[5] Given that these are the first CD spectra of very short isolated α-helices, it is not surprising that their CD spectra differ from those of much longer helices. Theoretical studies[36] into the chiroptic properties of the α-helix have predicted that short α-helices should have different CD spectra from longer α-helices. The negative minimum at 215 nm is consistent with the long wavelength n → π^* transition commonly observed for α-helices and β-sheets in the 215−230 nm wavelength range.[37] The observed positive maximum at 190 nm and negative minimum at 207 nm characterize the structure as α-helix rather than β-sheet, as these bands can only arise from exciton splitting of the NV_1 transition by the interaction of electric dipole transition moments among amides in the well-defined geometry of the α-helix.[38] The relative intensities of these peaks for **3** mirror those observed for other α-helices; therefore we have quoted the intensities at 190 nm, 207 nm and 215 nm in Table 1.

Helix Dependence On Sequence. Certain residues are known to favor or disfavor α-helicity; therefore the residues in this system were altered in an attempt to gain insight into the helicity for these cycles. Initial solubility issues with hydrophobic pentapeptides prompted us to incorporate an additional arginine at the N-terminus. Nine K(i) → D(i + 4) side-chain cyclized hexapeptides (**13**−**21**) were synthesized with the three residues intervening the bridge systematically replaced by α-helix inducing (alanine, leucine, methionine, glutamine), α-helix indifferent (phenylalanine, serine), or α-helix breaking (glycine) residues (Table 2).

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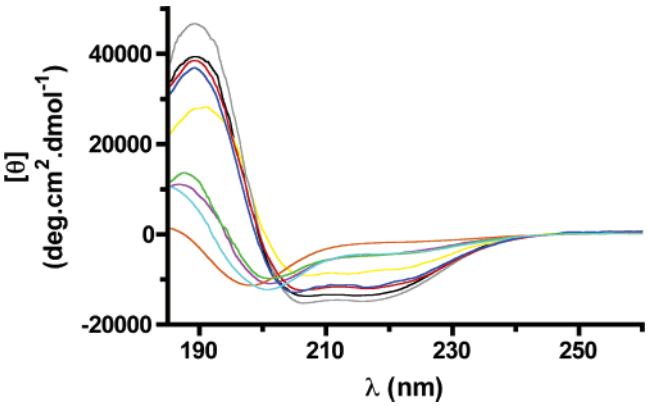

Figure 3. CD spectra of compounds **13** (black), **14** (grey), **15** (red), **16** (blue), **17** (yellow), **18** (purple), **19** (green), **20** (aqua), and **21** (orange) in 10 mM phosphate buffer (pH 7.4, 25 °C).

Figure 3 shows that helicity is indeed dependent on which residues intervene between the bridging residues. The helical structure is tolerant of substitution by α-helix inducing residues such as Ala (**13**), Leu (**14**), Met (**15**), Gln (**16**), and Phe (**17**), as demonstrated by the deep minima at 215/207 nm, high maximum at 190 nm, and high ratio $\theta_{215}/\theta_{207}$. There is some variation in the intensity at these wavelengths which closely mirrors the intrinsic α-helical propensity of specific amino acids determined in protein environments.[35] The system is quite delicate; however, replacing only the central residue by glycine (**18**) results in a substantial decrease in intensity at 215 nm, 207 nm, and 190 nm, along with the appearance of a deeper minimum at 201 nm that is commonly observed for 3_{10}-helicity/random coil structures. This reduction in α-helicity also results from placement of two (**19**) or three (**20**) helix disfavoring residues between the bridging residues, although based on the shape of their CD spectra there is some bias toward a helical conformation. For three α-helix breaking residues (**21**), total abolition of helicity was indicated by the single deep minimum at 200 nm characteristic of a random coil.

NMR Evidence For α-Helicity. The compelling CD spectra acquired for **3**, **13**−**16** prompted us to explore them further. Structural characterization was conducted for **3** and **13** using 1D and 2D ^{1}H NMR spectroscopy in 90% H_2O: 10% D_2O at 288 K (pH 4.0). 2D-TOCSY spectra at 600 MHz were used to identify resonances for each amino acid, and individual spin systems are shown in the Supporting Information (Figure S1). Due to the molecular weight of the macrocycle, ROESY instead of NOESY spectra had to be used to identify sequential connectivity and intraresidue NH−NH and NH−CH cross-peaks (Figures S2 and S3, Supporting Information). Spectral overlap in **3** prevented unambiguous identification of key long-range ROEs; however **13** gave well-defined resonances and was investigated further. There were a number of spectral features that are characteristic of a well-defined structure in the cyclic hexapeptide (**13**) and specifically characteristic of alpha helicity.

First, there were conspicuously low coupling constants ($^3J_{NHC\alpha H} < 6$ Hz) for all amide resonances except D6 (see Experimental Section), as normally observed in α-helical peptides.[39] Second, all of the residues displayed an upfield shift (0.18 to 0.32 ppm) for $\delta(H\alpha)$ relative to the corresponding residue in random coil structures (Figure S4, Supporting Information), this also being typical of helical peptides.[40] Third, there was a low temperature dependence for amide NH chemical shifts, with temperature coefficients ($\Delta\delta/T$) being ≤4 ppb/K for Ala3, Ala4, Ala5, Asp6, and one C-terminal amide NH (Figure S5, Supporting Information), consistent with their involvement in hydrogen bonds that characterize an α-helix.[41] Fourth, the observation in ROESY spectra of nonsequential medium range $d_{\alpha N}(i,i+4)$, $d_{\alpha N}(i,i+3)$, and $d_{\alpha\beta}(i,i+3)$ ROEs (Figure 4) suggest helical structure, and the particularly prominent $d_{\alpha N}(i,i+4)$ versus weak $d_{\alpha N}(i,i+2)$ ROEs support a high proportion of α-helicity rather than 3_{10}-helicity or turn conformations.

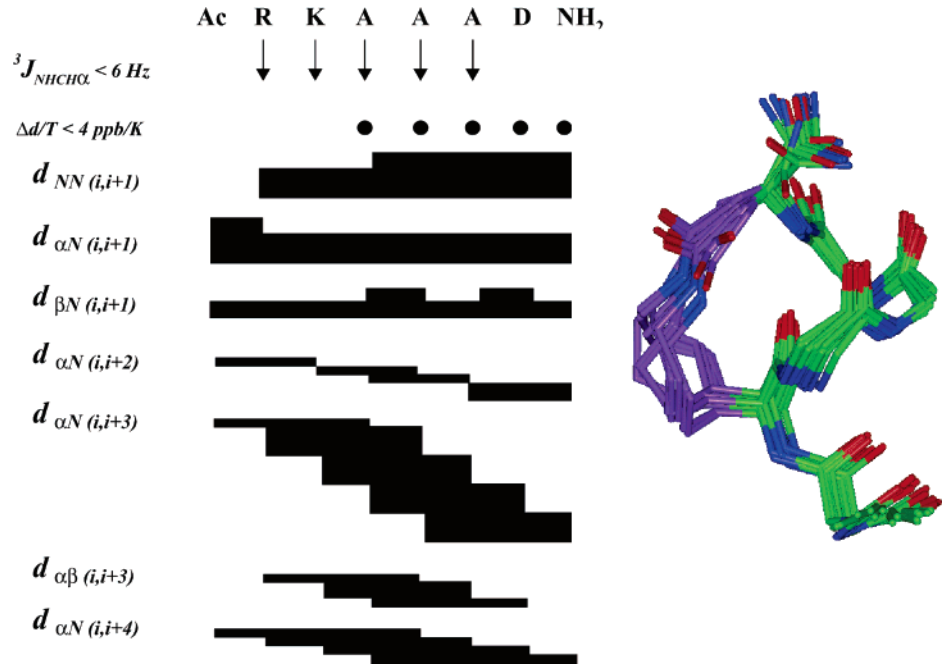

Figure 4. ROE summary diagram (left) and 20 lowest energy calculated structures (right, lactam bridge in purple) for Ac-(cyclo-2,6)-R[KAAAD]-NH_2 (**13**) in 90% H_2O:10% D_2O at 20 °C. Thickness of bars reflects intensity of ROEs.

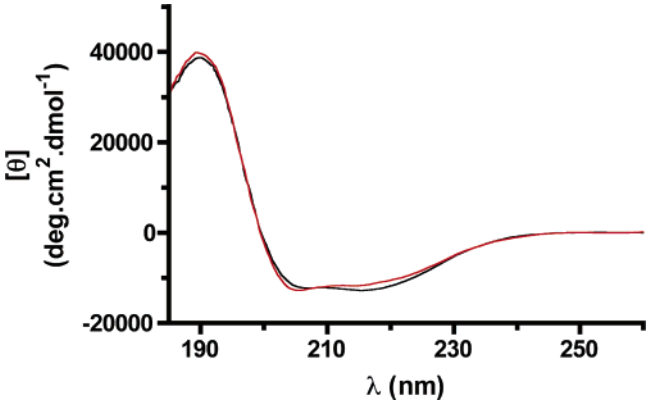

Figure 5. CD spectra of **3** in 10 mM phosphate buffer (black) (pH 7.4, 25 °C) and 50% TFE (red).

Solution Structure of 13. The three-dimensional structure for the hexapeptide **13** in 90% H_2O:10% D_2O at 20 °C was calculated using dynamic simulated annealing and energy minimization in Xplor (3.851)[20] from 81 ROE (26 sequential, 25 medium range, 30 intraresidue) distance restraints and four phi angle restraints ($^3J_{NHCH\alpha}$ 6 Hz, $\phi = -65 \pm 30°$). No explicit H-bond restraints were included in calculations. The final 16 lowest energy structures contained no dihedral angle (>2°) or distance (>0.1 Å) violations and are displayed in Figure 4. These lowest energy structures indicate a well defined α-turn, with four $i \rightarrow i + 4$ hydrogen bonds involving NH protons of the C-terminal amide, Asp6, Ala5, Ala4, and the CO oxygens of Ala3, Lys2, Arg1, and N-terminal amide. The backbone pairwise RMSD for this family of structures for Ac-(cyclo-2,6)-R[KAAAD]-NH_2 (**13**) in water is 0.35 Å, indicating a fairly tight structural convergence. When **13** was superimposed on an idealized textbook hexapeptide α-helix (i.e., backbone dihedral angles set $\psi = -47°$, $\phi = -57°$), the backbone pairwise RMSD = 0.81 Å. However, for just the cyclic pentapeptide component of **13**, the backbone pairwise RMSD = 0.22 Å over backbone carbon and nitrogen atoms. This structural evidence strongly supports the conclusion that the endocyclic residues are in a highly α-helical conformation, more so than the exocyclic Arg residue.

Effect of a Helix Stabilizing Solvent. To see whether the α-helicity exhibited by **3** in water could be increased, a CD spectrum was acquired for **3** in 50% aqueous TFE (a helix-inducing solvent which increases helicity for peptides[42]). Figure 5 shows that the presence of 50% TFE does not increase molar elipticity at 215 nm, suggesting that **3** is already maximally α-helical in water alone. This optimum α-helicity is also supported by the ratio $\theta_{215}/\theta_{207} > 1$ in water alone, as reported for an idealized (100%) α-helix.[43] Although there is some controversy regarding the accuracy of this ratio,[44] in our case it is at least still indicative of appreciable α-helicity.

Helix Dependence On Hydrogen Bonds. The NMR analysis indicated the presence of three hydrogen bonds within the pentapeptide cycle. To investigate the importance of these hydrogen bonds for the conformational stability of **3**, we synthesized a series of analogues with variable potential for forming intramolecular hydrogen bonds. Since 1,5-hydrogen bonds are characteristic of α-helicity, inclusion or exclusion of the N-terminal acetyl group or the C-terminal amide group allowed variation between one and three potential hydrogen bonds (Figure 6). Thus Ac-(cyclo-1,5)-[KARAD]-NH_2 (**3**) can form three potential intramolecular 1,5-hydrogen bonds (Figure 4), while Ac-(cyclo-1,5)-[KARAD]-OH (**10**) and H-(cyclo-1,5)-[KARAD]-NH_2 (**11**) can form only two hydrogen bonds, and H-(cyclo-1,5)-[KARAD]-OH (**12**) can only form one hydrogen bond (Table 1).

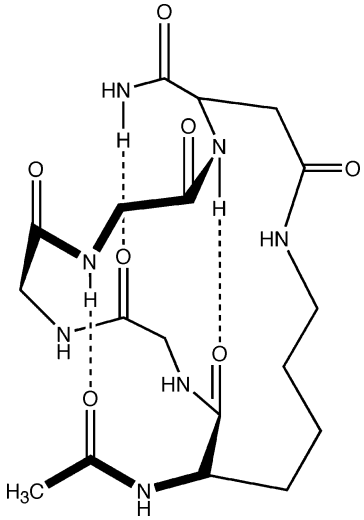

Figure 6. Three putative hydrogen bonds that define an α-helix.

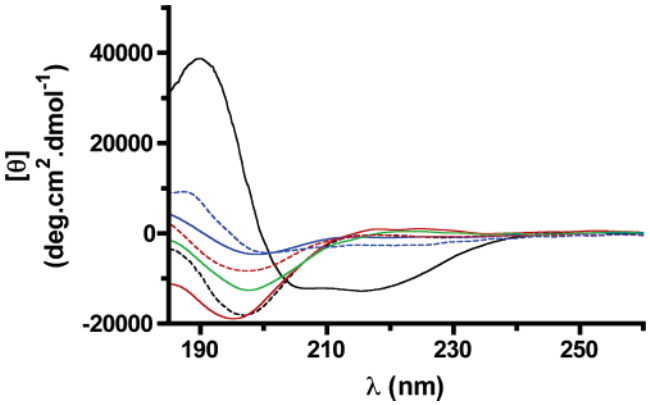

Figure 7. CD spectra of compounds **3** (black, solid), **9** (black, broken), **10** (red, solid), **11** (blue, solid), and **12** (green, solid) in 10 mM phosphate buffer (pH 7.4, 25 °C); **10** (red, broken) in 0.01 M HCl (pH 2) and **11** (blue, broken) in 0.001 M NaOH (pH 11).

Interestingly, only **3** showed α-helical structure in water at pH 7 (Figure 7), indicating that a minimum of three hydrogen bonds is required for α-helicity in a pentapeptide sequence. Since the effect of charges at the N- and C-termini might be expected to destabilize helicity, CD spectra were also recorded for **10** and **11** in 0.01 M HCl (pH 2) and 0.001 M NaOH (pH 11), ensuring that the uncapped termini were fully protonated and deprotonated, respectively. The pH had little effect on conformation; both **10** and **11** showed slight increases in helicity but remained largely unstructured. Compound **12** was not examined at high or low pH, as one of the termini will always be charged.

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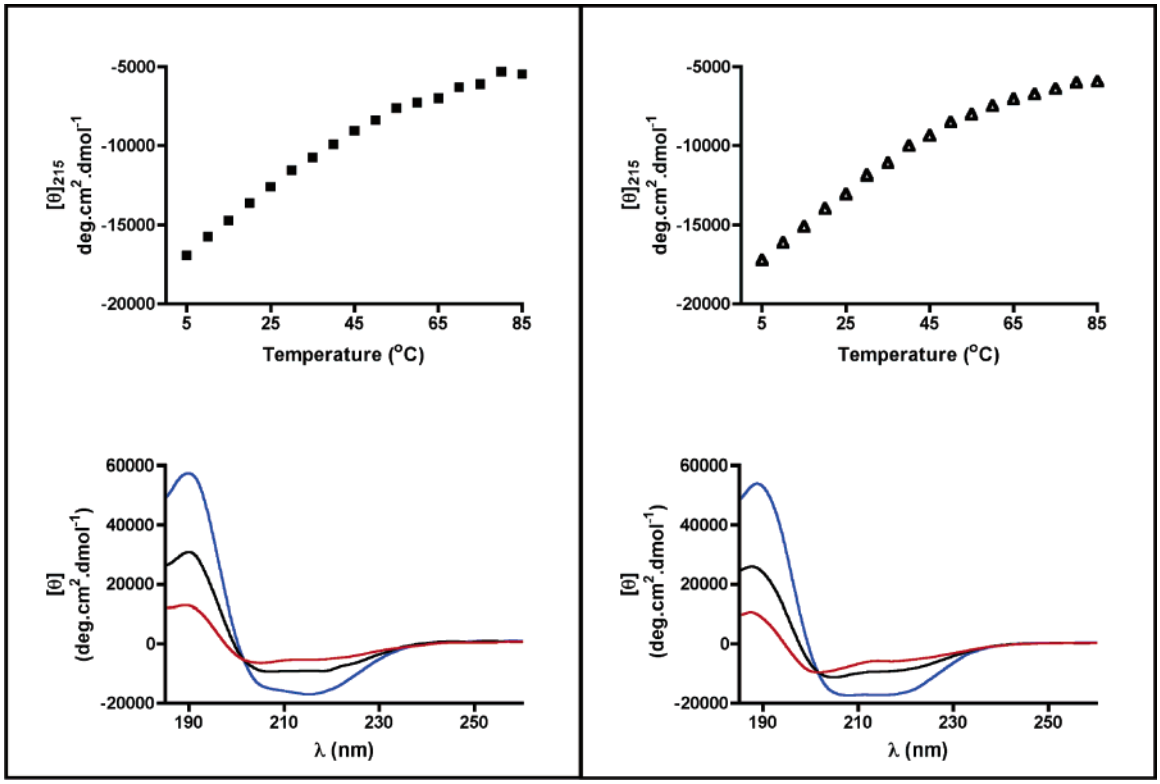

Figure 8. Dependence on temperature (5−85 °C) of mean residue ellipticity at 215 nm (top) and full CD spectrum (bottom) at 5 °C (blue), 45 °C (black), and 85 °C (red) for **3**, Ac-(cyclo-1,5)-[KARAD]-NH_2 (left panel), and **13**, Ac-(cyclo-2,6)-R[KAAAD]-NH_2 (right panel).

Table 3. Amide $^3J_{NH-CH\alpha}$ Coupling Constants for **3** and **13** at Low (5 °C) and High (65 °C) Temperatures

	K	A	R	A	D
5 °C	3.2 Hz	3.4 Hz	5.9 Hz	4.5 Hz	7.0 Hz
65 °C	4.5 Hz	4.8 Hz	6.9 Hz	5.1 Hz	7.6 Hz

	R	K	A	A	A	D
5 °C	4.4 Hz	4.5 Hz	3.8 Hz[a]	4.4 Hz	4.7 Hz	7.3 Hz[a]
65 °C	6.9 Hz	5.5 Hz	5.4 Hz	5.5 Hz	6.2 Hz	7.8 Hz

[a] $^3J_{NH-CH\alpha}$ coupling constant at 10 °C since these overlapped at 5 °C.

Helix Dependence On Temperature. The stabilities of **3** and **13** were investigated by thermal denaturation experiments. Figure 8 (upper) shows the temperature dependence of molar elipticity at 215 nm for **3** and **13**, and is consistent with some unwinding of the helix. Figure 8 (lower) shows CD spectra for these compounds at 5, 45, and 85 °C. There is a gradual increase in signal intensity at 215 nm with temperature, and a corresponding shift in the minimum from 208 to 203 nm above 45 °C, as expected for some helix unwinding.

NMR data were also consistent with some helix unwinding for **3** and **13**. We were unable to detect medium/long-range NOE/ROEs at 65 °C for **3** and **13**, which may be due to some helix unwinding. Second, all $^3J_{NH-CH\alpha}$ coupling constants for **3** and **13** increase by 20−30% between 5 °C and 65 °C (Table 3). However, three of four amide NH coupling constants ($^3J_{NH-CH\alpha}$) for **3** and three of five coupling constants for **13** that were <6 Hz at 5 °C were also <6 Hz at 65 °C, indicating retention of some α-helicity at 65 °C.

Stability of 3 to Denaturants. Using the molar elipticity at 215 nm as an indicator of α-helicity, Figure 9 clearly shows that macrocycle **3** is conformationally stable, and the pentapeptide maintains full α-helicity even in the presence of 8 M guanidinium chloride. These conditions readily denature peptides and proteins, but do not affect α-helicity in **3**.

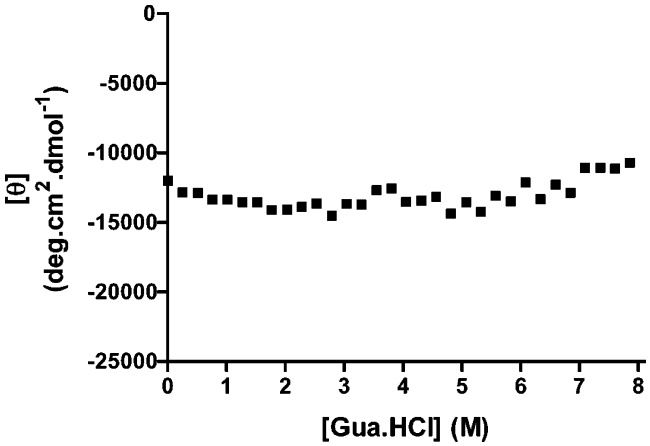

Figure 9. Variation in molar elipticity of **3** at 215 nm with increasing [guanidine· HCl] at 25 °C.

Proteolytic Stability of the Helix. Compound **3** and its acyclic version **9** were incubated with trypsin (pH 8.2), and aliquots were removed at intervals between 1 and 155 min and analyzed by LCMS. The cyclic compound remained intact over this period, whereas the acyclic analogue completely hydrolyzed within a few minutes to fragments Ac-KAR-OH, H-ARAD-NH_2, H-AR-OH, and H-AD-NH_2. Similarly the cyclic compound **3** was not degraded by human serum (1 h, 37 °C). These results prove that the peptide sequence in **3**, being held in an α-helical conformation, is not susceptible to recognition by the proteolytic enzyme. This is consistent with other observations that only an extended or linear peptide conformation is recognized, not only by trypsin but also by all proteolytic

enzymes.[45] Presumably the cycle is too tightly locked into an α-helical turn to permit significant unwinding to the extended format needed for recognition by trypsin.

Quantitation of Helicity. Assuming that mean α-helix content (f_H) is linearly related to ellipticity at 222 nm, or in our case 215 nm ($[\theta]_{obs215}$), then the equation for calculating helix content is

$$f_H = ([\theta]_{obs215} - [\theta]_C)/([\theta]_{\infty 215} - [\theta]_C) \quad (1)$$

Luo and Baldwin[42] determined that the random coil ($[\theta]_C$) and infinite α-helix ($[\theta]_{\infty 222}$) molar ellipticities are temperature-dependent based on the equations:

$$[\theta]_C = 2220 - 53T \quad (2a)$$

$$[\theta]_{\infty 215} = (-44\,000 + 250T)(1 - k/N_p) \quad (2b)$$

where T is temperature in degrees Celsius, N_p is the number of peptide units, and k is a finite length correction.

Despite the widespread use of eq 1, a key problem is the implementation of a suitable k factor for which a range of values between 2.4 and 4.5 have been used.[46] Baldwin has suggested using $k = 3.0$ for carboxyamidated peptides and 4.0 for unblocked peptides.[47] For **3**, variation of the k factor from 2.4 to 4.5 resulted in 56−135% helicity, so it was clear that the k factor can impact significantly on reported values of α-helical content. There is no empirical method to determine a suitable k factor, and the problem of choosing one becomes particularly acute for short peptides. Given our compelling high-resolution NMR data and CD studies in the presence of benign, helix stabilizing, and helix destabilizing environments, we believe it is highly likely that the equilibrium between random coil, partial helix, and pure α-helix is shifted significantly toward pure α-helix such that f_H is ∼1 and the contribution of $[\theta]_C$ is ∼0. Based on the assumption that **3** has 100% α-helicity, we can derive $k \approx 4$, which is within the suggested range. If we apply this value to eq 2b, then the percentage helicities for the α-helical compounds in Tables 1 and 2 are 100% (**3**), 83% (**13**), 91% (**14**), 73% (**15**), 70% (**16**), and 53% (**17**).

There are, however, problems associated with this derivation. First, despite the significance of the Luo−Baldwin study, it does not reflect the properties of very short peptides. Second it is based on extrapolating TFE titration data to CD data acquired in water alone.[44] Third, their study is calibrated to ellipticity at 222 nm, whereas the absolute minimum in these peptides occurs at 215 nm. Given these difficulties, we have expressed relative, rather than absolute, helicities for our peptides in Tables 1 and 2.

Discussion

This paper has reported the first 5-residue peptides that display essentially complete α-helicity in water, making them the shortest and most stable peptide α-helices known. This result, confirmed by NMR-derived structure determination in water, was unique for pentapeptides cyclized through amide formation specifically between lysine and aspartate at positions i and $i + 4$, respectively. Their α-helical nature has been convincingly established by circular dichroism and ^{1}H NMR spectra, neither of which were concentration dependent, thus ruling out α-helicity due to aggregation. Failure to enhance α-helicity (especially of **3**) using 50% TFE, failure to diminish it with 8 M guanidine·HCl, and lack of success in degrading it with proteolytic enzymes support our conclusion of an exceptionally high proportion of α-helical conformers in the structural ensemble, especially for compound **3**.

Conventional head to tail cyclic pentapeptides are traditionally associated with various types of β- and γ-turn conformations in solution,[17,48] although in water they tend to display no well defined structure at all. In the literature of peptide hormones there is evidence that lactam bridges, particularly (i, $i + 4$) linkages in pentapeptide components, can increase helicity in longer peptides and enhance bioactivity. However there is considerable disparity in the α-helix stabilizing effects of various lactam bridges, with little agreement about which sequences, ring sizes, and ring compositions impart the highest α-helicity[12] and suggestions that effectiveness is case dependent.[27] In this paper, we have systematically examined the effects of different lactam bridges on α-helix stability in simple pentapeptides, cyclized through side chain to side chain coupling. We have shown that cyclic pentapeptides of defined size (20-membered rings) and specific composition have the capacity to adopt a single α-turn conformation that is remarkably stable in water. Spectral data from circular dichroism and 2D ^{1}H NMR strongly support α-helicity for such cycles in water, even under severe peptide-denaturing conditions such as high concentrations of guanidine hydrochloride (≤8 M). These results also demonstrate that a single lactam bridge can effectively stabilize α-helicity in short peptides, which contrasts with the best results to date[49] where two overlapping lactam bridges were required to necessitate α-helix stabilization in short peptides.

A feature of this work is the finding that appreciable α-helicity is dependent not only on the cyclic constraint that produces a 20-membered ring but also on accompanying formation of three intramolecular 13-membered hydrogen bonded rings. Thus three $i \rightarrow i + 4$ hydrogen bonds together with the cyclic constraint would appear to be the minimum requirements to stabilize an α-turn, since either removal of just one of these hydrogen bonds or minor modification to the cyclic restraint was sufficient to collapse the α-helical structure. It is worth pointing out that, for a conventional uncapped peptide, three $1 \rightarrow 5$ hydrogen bonds would require a minimum sequence of seven amino acids. In the absence of the thermodynamic stability afforded by the cyclic constraint, it is therefore unsurprising that acyclic peptides can only be highly α-helical if they are much longer.

The significant difference observed in the RMSD values between the α-helical hexapeptide structure **13** and its cyclic

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pentapeptide structural component is consistent with the need for the cyclization restraint, rather than just three (or four) hydrogen bonds, for α-helicity. Although NMR data at low temperature did indicate that the exocyclic Arg residue in **13** was in an α-helical environment created by four hydrogen bonds and the cyclization constraint, the deviation from an idealized α-helix was larger for the hexapeptide over the pentapeptide, reflecting less α-helicity for this residue which is outside the cycle. This does not necessarily mean that the cyclic pentapeptide cannot transmit α-helicity to multiple attached exocyclic residues, only that it is not very effective in inducing helicity in a single attached residue. This slight fall off in helical integrity outside the cycle is not too surprising, since the termini of protein/peptide α-helices are normally quite disordered in solution structures, and certainly more disordered than found for the ends of **13**.

The helix stability is dependent upon sequence, those residues known to favor α-helicity in proteins also favor α-helicity in these cyclic pentapeptides. However, unlike proteins, these simple systems are not complicated by effects of side chain packing, folding, and intra- or inter- molecular interactions other than with solvent and thus would appear to offer excellent opportunities to investigate effects of individual natural or unnatural amino acid components on the α-helix. There is however the possibility of steric interactions between the lactam bridge and adjacent residues. Since the cycles remained intact under peptide-denaturing conditions, they may be useful as templates in longer peptides for studying unfolding/refolding and for "seeding" structure in proteins and polypeptides. The stability of the cycles under peptide-degrading conditions (trypsin, human serum) also suggests that they may have useful α-helix-mimicking properties in biologically relevant environments.

In another context the observations in this paper complete the picture for cyclic peptides as mimetics of the key elements of protein structure. While $i \rightarrow i + 4$ side chain to side chain cyclization herein has produced a stable α-turn with a 13-membered hydrogen bonded ring in pentapeptides, $i \rightarrow i + 3$ side chain to side chain cyclization is known to produce the β-turn with a 10-membered hydrogen bonded ring (multiples of which constitute the 3_{10}-helix) in tetrapeptides, and $i \rightarrow i + 2$ side chain to side chain cyclization has produced the γ-turn involving a 7-membered hydrogen bonded ring as well as the beta strand, depending upon the constraints in the cycle, in tripeptides. Clearly cyclization, together with appropriate use of molecular constraints in peptide sequences,[1b,17] can be systematically and effectively used to mimic any of the fundamental structural elements of proteins.

In summary, the high conformational and proteolytic stability of these α-helical cyclic pentapeptides suggests their use as single turn α-helical modules, with capacity for decoration by peptidic, cyclic, or nonpeptidic appendages, to mimic bioactive peptide or protein α-helical segments.

Experimental Section

General. Fmoc-Asp(OAllyl)-OH, Fmoc-Lys(Alloc)-OH, Tentagel-S-RAM resin, and tetrakis(triphenylphosphino)palladium were obtained from Sigma-Aldrich (Sydney, Australia). Boc-Lys(Fmoc)-OH, Rink Amide MBHA resin and other L-amino acids were obtained from Novabiochem (Melbourne, Australia). Benzotriazol-1-yl-1,1,3,3-tetramethyluronium (HBTU) and benzotriazol-1-yloxy-tris(dimethylamino)-phosphonium (BOP) were obtained from Richelieu Biotechnologies (Quebec Canada). All other reagents were of peptide synthesis grade and obtained from Auspep (Melbourne, Australia).

Synthesis Of Compounds 1−21. General Procedure: Peptides were prepared on 0.25 mmol scale by manual stepwise solid-phase peptide synthesis using HCTU/DIPEA activation for Fmoc chemistry[50] on Rink Amide MBHA resin (substitution 0.78 mmol g^{-1}), Tentagel S RAM resin (substitution 0.25 mmol g^{-1}), or Trityl chloride resin (substitution 1.0 mmol g^{-1}). Amino acid (4 equiv) and diisopropylethylamine (DIPEA, 8 equiv) were employed in each coupling step (45 min), except for Fmoc-Asp(OAllyl)-OH and Fmoc-Lys(Alloc)-OH where only 2 equiv were used. Fmoc deprotections were achieved with 3×5 min treatments with excess 1:1 piperidine/DMF. Coupling yields were monitored by quantitative ninhydrin assay,[51] and double couplings were employed for yields below 99.6%. After the assembly was complete, the allyl ester of aspartic acid and allyl carbamate of lysine were removed by treating the peptide resin with $Pd(PPh_3)_4$ (0.1 equiv) and *N*,*N*-dimethylbarbituric acid (4 equiv), in DCM, under argon and in the dark for 2 h; this procedure was repeated once. After the peptide was washed with DCM, DMF, and 0.5% diethyldithiocarbamate in DMF, 2 mg of resin were subjected to cleavage and the progress of the reaction was monitored by MS. This process was repeated if necessary.

Cyclization was effected on-resin using 1.5 equiv of BOP and 2 equiv of DIPEA in DMSO/NMP (1:4). The reaction was monitored by cleavage of ~2 mg resin and subjecting the residue to MS; total reaction time was <24 h. The peptides were simultaneously deprotected and cleaved from the resin by a 2 h treatment of the washed and dried resin in 95% TFA, 2.5% TIPS, 2.5% H_2O, or 1% TFA in DCM (15 μL per 10 mg resin). The solution was then filtered, the filtrate was concentrated in vacuo, and the peptide was precipitated with cold diethyl ether. The peptide precipitate was filtered, washed with copious amounts of diethyl ether, redissolved in 1:1 acetonitrile/water, and lyophilised. The crude peptides were purified by rp-HPLC (R_{t1}:Vydac C18 column, 300 Å. 22×250 mm^2, 214 nm, solvent A = 0.1% TFA in H_2O, solvent B = 0.1% TFA, 10% H_2O in acetonitrile. Gradient: 0% B to 100% B over 30 min. R_{t2}:Phenomenex C18 column, 300 Å. 22×250 mm^2, 214 nm, solvent A = 0.1% TFA in H_2O, solvent B = 20.1% TFA, 10% H_2O in acetonitrile. Gradient: 0% B to 100% B over 30 min). ^{1}H NMR was carried out in H_2O/D_2O (9:1) at 298 K.

CD Spectroscopy. CD experiments were performed on a Jasco Model J-710 spectropolarimeter which was routinely calibrated with (1*S*)-(+)-10-camphorsulfonic acid. Spectra were recorded in a 0.1 cm Jasco cell between 260 and 185 nm at 50 nm/min with a bandwidth of 1.0 nm, response time of 2 s, resolution step width of 0.1 nm, and sensitivity of 20, 50, or 100 mdeg. Each spectrum represents the average of five scans with smoothing to reduce noise. Peptide samples for CD spectroscopy were dissolved in 18 MΩ distilled water (~3−4 mg/mL). Each stock solution (500 μL) was then diluted 1:1 with phosphate buffer pH 7.4. Solutions were then prepared for each sample with final concentrations ranging from 50 to 800 μM in 10 mM sodium phosphate buffer (pH 7.4), with or without additives (2,2,2-trifluoroethanol (TFE), guanidine·HCl, 0.01 M HCl, 0.001 M NaOH). Over this concentration range the CD intensity was found to be independent of concentration ruling out aggregation as a stabilizing effect. The remaining 500 μL from the initial stock solution was used for accurate concentration determination.

Guanidine·HCl denaturation experiments were performed according to Creighton;[52] briefly a saturated stock solution of guanidine hydrochloride was prepared in distilled water, and the concentration was

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accurately determined by refractive index measurement. Solutions (32) were then prepared increasing the concentration of guanidine·HCl from 0 to 8.11 M in approximately 0.25 M increments, with a standard peptide concentration of 200 μM. The solutions were allowed to incubate overnight at room temperature and run the following day.

Temperature experiments were conducted between 5 and 85 °C in 5 °C increments. Temperature control was achieved using a Neslab RTE-111 circulating water bath and an PTC-423 thermostated cell holder. Once the desired temperature was reached, a 10 min equilibration time was allowed before CD spectra were recorded as described above.

Accurate concentrations of stock solutions were determined by 1D ^{1}H NMR spectroscopy based on integrations of nonexchangeable proton signals using the method of Larive et al.[53] Briefly 475 μL of the initial peptide stock solution was mixed with 50 μL of D_2O and spiked with an internal standard, 25 μL of 10.077 mM DSS (the concentration of which had carefully been back-calibrated by NMR from a standard solution of L-tryptophan, the concentration of which was determined by UV $\epsilon_{278} = 5579$[54]). 1D ^{1}H NMR spectra were then recorded with presaturation and a relaxation delay (d1) of 30 s to allow for full relaxation of peptide and DSS ^{1}H signals to facilitate accurate integration of proton signals with $S/N > 250{:}1$. This method provides reproducible concentrations within ±2%.

Circular Dichroism Data Analysis. CD data in ellipticity was converted to mean *peptide* ellipticity using the equation:

$$[\theta] = \theta/(10 \times C \times N_p \times l)$$

where θ is the ellipticity in millidegrees, C is the peptide molar concentration (M), l is the cell path length (cm), and N_p is the number of peptide units (i.e., pentapeptides, $N_p = 6$; hexapeptides, $N_p = 7$; **10** and **11**, $N_p = 5$; **12**, $N_p = 4$).

Relative helicities were employed to allow scaling of compounds **1**−**22** by dividing the $[\theta]_{obs215}$(**3**)/$[\theta]_{obs215}$(**x**) for pentapeptides and $[\theta]_{obs215}$(**14**)/$[\theta]_{obs215}$(**x**) for hexapeptides.

NMR Spectroscopy. Samples for NMR analysis of hexapeptide **13** were prepared by dissolving the peptide (2 mg) in 450 μL of H_2O and 50 μL of D_2O (5 mmol) and adjusting the pH of the solution to 4.5 by adding HCl or NaOH and stirring for 30 min. 1D and 2D ^{1}H NMR spectra were recorded on Bruker Avance DRX-600 spectrometers at 288 K. All spectra were recorded in the phase sensitive mode using time proportional phasing incrementation.[55] 2D experiments included TOCSY using MLEV-17 spin lock sequence with a mixing time of 80−100 ms and included ROESY with a mixing time of 250 ms. Water suppression was achieved using watergate W5 pulse sequences with gradients using double echo.[56] 2D TOCSY and ROESY experiments were recorded over 6620.5 Hz with 4096 complex data points in F2 and 512−1024 increments in F1 with 16 and 48 scans per increment, respectively. Spectra were processed using XWINNMR (Bruker, Germany). The *t*1 dimensions of all 2D spectra were zero filled with 2048 real data points, and 90° phase-shifted sine bell window functions applied in both dimensions followed by Fourier transformation and fifth order polynomial baseline correction. Chemical shifts were referenced to TSP an internal standard at 0.00 ppm. Processed spectra were analyzed using the program SparkyNMR[57] and assigned using the sequential assignment technique[58]

Structure Calculations. Cross-peaks in ROESY spectra were integrated and calibrated in SparkyNMR, and distance constraints, from ROE intensities and placed into four groups: strong (2.7 Å upper limit), medium (3.5 Å upper limit), weak (5 Å upper limit), and very weak (6 Å upper limit). Corrections for pseudo atoms were added to distance constraints where needed. Backbone dihedral angle restraints were inferred from $^3J_{NHCH\alpha}$ coupling constants in 1D spectra at 288 K, and ϕ was restrained to $-65 \pm 30°$ for $^3J_{NHCH\alpha} \leq 6$Hz. Peptide bond ω angles were all set to trans, and structures were calculated without explicit hydrogen bond restraints. Initial structures were generated using XPLOR 3.851. Starting structures with randomized ϕ and ψ angles and extended side chains were generated using an ab initio simulated annealing protocol.[59] The calculations were performed using the standard force field parameter set (PARALLHDG·PRO) and topology file (TOPALLHDG·PRO) in XPLOR with in house modifications to generated lactam bridges between lysine and aspartic acid residues. Refinement of structures was achieved using the conjugate gradient Powell alogrithm with 1000 cycles of energy minimization and a refined force field based on the program CHARMm.[60] Structures were visualized with MOLMOL[61] and InsightII.[62]

Trypsin Digestion. A standard solution of Ac-KARAD-NH_2 and Ac-(cyclo-1,5)-KARAD-NH_2 (1 mg/mL) was prepared in 100 mM ammonium carbonate buffer at pH ≈ 8.2. To 100 μL of each solution was added 1 mg/mL trypsin (1 μL). The digestion was conducted at room temperature with 5 μL aliquots taken at 1, 4, 8, 28, 48, 55, 110, and 155 min. Aliquots were diluted with 5 μL of 3% TFA to stop the reaction and analyzed by LC-MS using a 2.1 × 150 mm^2 Phenomenex 300A C18 5 μm column, with a 3% per minute linear gradient of 0−60% acetonitrile over 20 min. The amount of starting material left in each sample was quantified by determination of total ion counts for the molecular ion.

Serum Stability. Standard solutions of Ac-KARAD-NH_2 and Ac-(cyclo-1,5)-KARAD-NH_2 (1 mg/mL) were prepared in water. Each peptide (200 μL) was added to human serum (800 μL) and incubated at 37 °C. Acetonitrile/water 3:1 (300 μL)was added to aliquots (100 μL) of serum at 5, 15, 30, 45, and 60 min to precipitate serum proteins, which were removed by centrifugation. The decanted supernatant was analyzed by LC-MS MS with a 2.1 × 150 mm^2 Phenomenex 300A C18 5 μm column, using a 3% per minute linear gradient from 0%−60% acetonitrile over 20 min. The amount of starting material left in each sample was quantified by determination of total ion counts for the molecular ion.

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Supporting Information Available: ^{1}H NMR, HPLC, and HRMS characterization data for all compounds (**1**−**21**). 2D spectral data, variable temperature, and distance restraints used for the calculation of the NMR structure of **13**. This material is available free of charge via the Internet at http://pubs.acs.org.

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