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A Cyclic β-Strand Tripeptide with an α-Helix Like CD Spectrum

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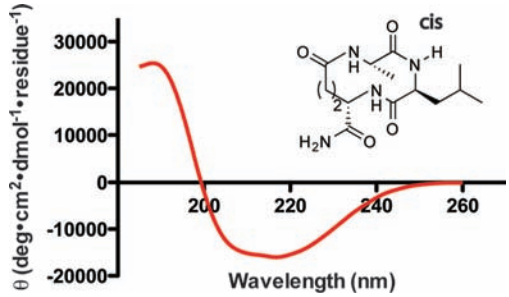
ABSTRACT



A protein α-helix is defined by 3.6 amino acids per turn. Cyclization of the tripeptide Alanine-Leucine-Glutamate through a side chain to the N-terminus lactam bond produces cyclo-(1,3)-[ALE]-NH_2 which displays a circular dichroism spectrum typical of an α-helix backbone. However, proton NMR spectra show a novel cyclic peptide featuring two non-hydrogen-bonded antiparallel β-strands connected by an Ala-Leu *cis*-amide bond. This example highlights that the common practice of characterizing α-helices by CD spectra alone can be misleading.

α-Helical and β-sheet domains of proteins mediate countless important biological functions, but corresponding short amino acid sequences in isolation are usually unstructured, with low biological activity, susceptibility to enzymatic degradation, and low membrane permeability.[1] Toward the miniaturization of proteins, small organic molecules are being sought to realize the potential of small protein surfaces[1] as biological probes, therapeutics, or diagnostics, either by stabilizing α-helical[2] or β-strand[3] peptide fragments or by acting as templates to spatially direct substituents analogous to amino acid side chain projections.[4,5] For example, the smallest known α-helices in water are cyclic pentapeptides,[2d] featuring a specific lactam bridge between side chains of the first and fifth amino acid residues. The resulting peptide backbone reproduces a single α-helical turn, with the characteristic repeating dihedral angles (ϕ −57°, ψ −47°) and the three classic overlapping 13-membered hydrogen-bonded rings formed by hydrogen bonds between i, i+1, and i+2 carbonyl oxygen atoms and i+4, i+5, and i+6 amide NH protons, respectively (Figure 1A).[2d] However, an idealized α-helical turn is defined by an even shorter sequence of just 3.6 amino acid residues, so we decided to examine helical propensity for a cyclic tripeptide cyclo-(1,3)-[ALE]-NH_2, in which Alanine-Leucine-Glutamate is cyclized through a side chain to the N-terminus lactam bridge. Interestingly, this compound displays a circular dichroism spectrum in water typical of an α-helix, but is it really α-helical?

Molecular modeling suggested to us that an idealized α-helix brings the N-terminus of an amino acid residue at

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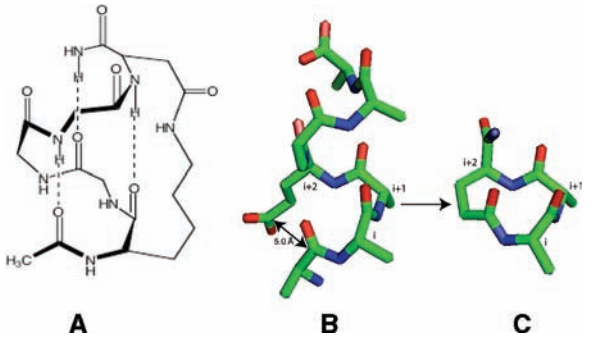

Figure 1. (A) Cyclic pentapeptide Ac-(1,5)-[KxxxD]-NH_2 showing three intramolecular backbone hydrogen bonds defining overlapping 13-membered H-bonded rings.[2d] (B) Idealised α-helix showing close approach of the carboxylate side chain of residue i+2 to the N-terminus of residue i, which can potentially be linked (C).

position i into close proximity with the side chain of a glutamate residue at position i+2 (Figure 1B). We thus decided to test whether linking the side chain of glutamate to the N-terminus of alanine in Ala-Leu-Glu would produce an α-helical peptide backbone in just a three-residue peptide. Connectivity between side chains of i and i+2 residues can stabilize a β-strand[3,5] or a γ-turn with a seven-membered hydrogen-bonded ring formed by a hydrogen bond between a carbonyl oxygen at residue i and an amide NH proton at residue i+2.[6] There are few examples of cyclic di- or tripeptides,[7] which can display reverse (γ- or β-) turns or hydrogen-bonded β-sheets.

Compounds Ac-Lys-Ala-Arg-Ala-Asp-NH_2, Ala-Leu-Asp-NH_2, Ala-Leu-D-Asp-NH_2, Ala-Leu-Glu-NH_2 (**1**), (cyclo-1,3)-[ALE]-NH_2 (**2**), and cyclo-(1,5)-Ac[KARAD]-NH_2 **3** were synthesized by Fmoc/HBTU solid-phase peptide synthesis methods, purified by semipreparative HPLC using ACN/H_2O gradients, and characterized by analytical rpHPLC, high-resolution MALDI-TOF, and ^{1}H NMR spectra. Compound **3** was prepared and characterized as previously reported.[2d] Peptides were cyclized with diphenylphosphoroazide/DIPEA at low micromolar concentrations in DMF at 4 °C. The cyclization yield of **2** was low (15%), while the shorter Ala-Leu-Asp-NH_2 and diastereomer Ala-Leu-(D-Glu)-NH_2 could not be cyclized through a (1,3) linkage.

In this field of research, chemists and biochemists typically characterize α-helicity in peptides of any length using circular dichroism spectroscopy, most often without any additional corroborating evidence. α-Helical peptides with >25 amino acid residues typically display two absorbance minima (222 and 208 nm, ratio 0.7−1.1) corresponding to $n-\pi^*$ and $\pi-\pi^*$ electronic transitions, while a positive absorbance maximum (~192 nm) derived from $\pi-\pi^*$ exciton splitting by residue side chains is also usually observed but not always with high intensity.[8] This does not necessarily mean that such CD spectra are only produced by α-helices nor that shorter peptides displaying such characteristics are necessarily α-helical.

The circular dichroism spectra of **2**, at 197 μM to 3.2 mM concentrations in 10 mM phosphate buffer (pH 7.4, 298 K), were recorded on a Jasco J-710 spectropolarimeter and recapitulate these "α-helix" CD spectral features with surprising fidelity (Figure 2). The absorbance from 206−225 nm (minimum 217 nm) and positive maximum (188 nm) would be accepted by most practioners to be diagnostic of an α-helix. Indeed, the spectrum is very similar (Figure 2) to the shortest reported α-helical peptide, Ac-(cyclo-1,5)-[KARAD]-NH_2 (**3**), which has been structurally validated.[2d] The ratio of molar ellipticities at the molar ellipticity minima is even closer to the ratio (1.1) for an idealized α-helix for **2** than **3**, although the positive absorbance is not as sharp or as intense for **2** as **3**. By contrast, linear analogue **1** has no significant absorptions (Figure 2) and is largely unstructured in water. Addition of 2,2,2-triflouroethanol to different concentrations of **2** in aqueous solution did not change the line shape or absorbance intensities, indicating that the compound has no unrealized helical propensity and that the structure was stable. Varying the concentration of **2** (197 μM to 3.2 mM) did not alter the absorbances or their intensities, suggesting no aggregation. CD spectra of helical peptides are usually temperature-dependent, but the line shape and molar elipticities for **2** were unchanged between 5 and 75 °C.

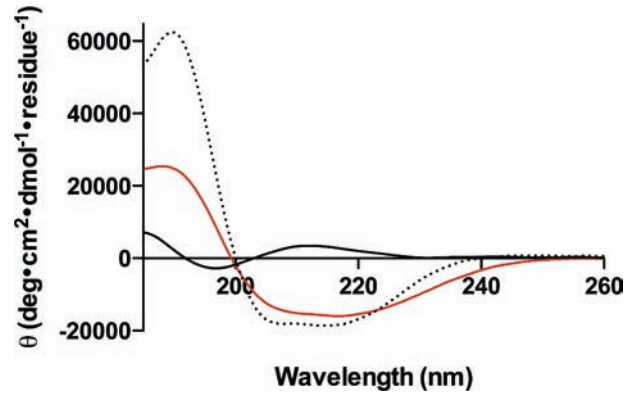

Figure 2. Circular dichroism spectra for **1** (black), **2** (red), and **3** (---) in aqueous 10 mM phosphate buffer (pH 7.4, 25 °C), showing similar CD spectra for **2** and **3** that would normally be taken to be indicative of α-helicity for **2**.

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The characteristic helix-like CD spectrum observed for **2** encouraged us to investigate its structure in more detail. The ^{1}H NMR spectrum (Figure 3) revealed uniformly large

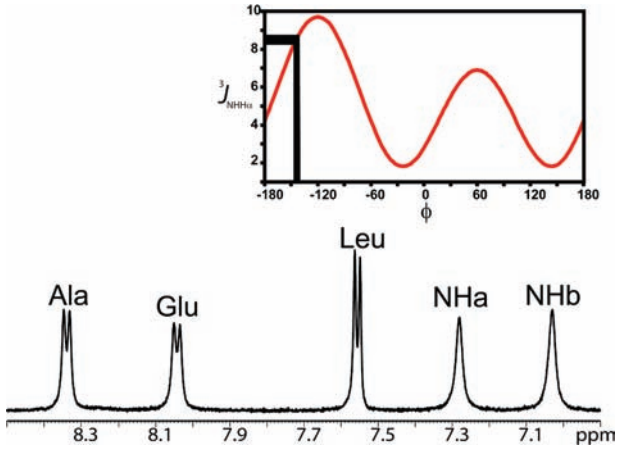

Figure 3. Amide NH region of 600 MHz ^{1}H NMR spectrum of **2** in 90% H_2O/10% D_2O (100 mM, 298 K). Bottom: assigned amide chemical shifts ($^3J_{HN\text{-}H\alpha}$ coupling constants) for Ala (8.7 Hz), Leu (8.7 Hz), and Glu (8.4 Hz). Top: Karplus plot[9] predicting −140° and −145° φ torsion angles from observed coupling constants for **2**.

$^3J_{NHCH\alpha}$ coupling constants (8.7, 8.7, 8.4 Hz at 298 K) for Ala, Leu, and Glu residues more typical of β-strand geometry, rather than <6 Hz expected for α-helical amides. Variable-temperature ^{1}H NMR spectra showed a single low temperature coefficient ($\Delta\delta/T$ −3.4 ppb/deg) for the C-terminal amide NHa proton suggesting its participation in a hydrogen-bonded ring. The closest (Leu) carbonyl oxygen is 3.3 Å away from this proton, while hydrogen bonding to other more distant oxygens would require an intervening water molecule. The NH did not undergo slow hydrogen/deuterium exchange expected for a hydrogen-bonded NH, whereas Leu2 and Glu3 amide protons exchanged more slowly.

ROESY and NOESY 2D ^{1}H NMR experiments were performed in both 90:10 (H_2O/D_2O) and 1:1 (H_2O/D_2O)/d_3-trifluoroethanol. The structure for **2** in H_2O/D_2O (9:1) was calculated from nine ROE distance restraints and three backbone ϕ-dihedral angle restraints (−120 ± 40°) inferred from $^3J_{NHCH_\alpha}$ coupling constants. There was a medium strength ROE between Cα protons of Ala and Leu residues revealing a *cis*-amide bond between them.[10] The other two amide bonds were in a trans conformation. The structures were calculated in XPLOR[11] using a dynamic simulated annealing protocol with a geometric force field and were energy minimized using a modified CHARMm[12] force field.

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No explicit hydrogen bond restraints were included in the structure calculations to prevent exclusion of any other conformations (e.g., γ-, β-, or α-turns). The 20 lowest energy structures for **2** (Figure 4) contained no distance (≥0.2 Å)

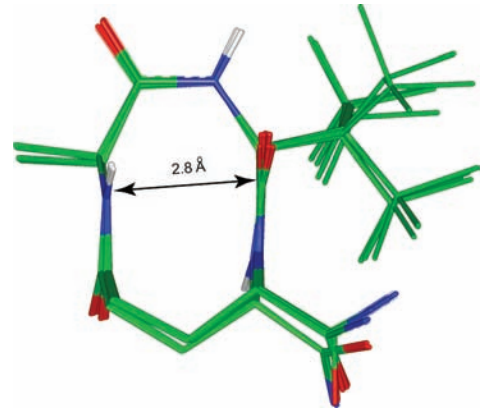

Figure 4. Superimposed backbones for the 20 lowest energy solution structures calculated for **2** in 90% H_2O:10% D_2O (average backbone heavy atoms pairwise rmsd 0.02 Å) showing antiparallel β-strands connected by *cis*-amide (top) and Glu side chain (bottom).

or dihedral angle (≥3°) violations and represent a highly convergent family of structures (average pairwise backbone rmsd 0.02 Å). The three-dimensional structure for **2** (Figure 4) is a conformationally constrained 11-membered macrocycle, in which the Leu-Glu and Glu-Ala amide bonds are in antiparallel extended β-strand conformations with the amide bond planes perpendicular to the plane of the cyclic peptide. These strands are 2.8 Å apart, about half the separation of β-strands in hydrogen bonded β-sheets, and are connected by the Ala-Leu *cis*-amide that acts as a turn motif between them. The alternative isomer with an Ala-

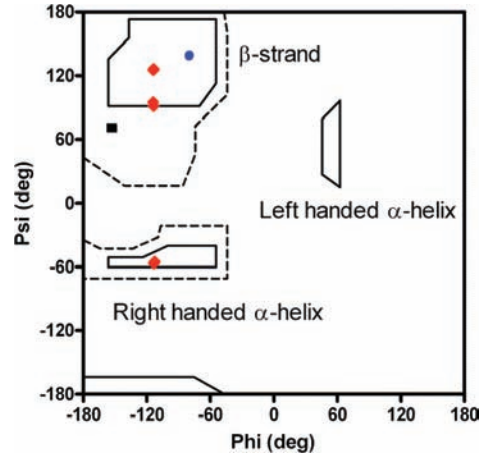

Figure 5. Ramachandran plot for the 10 lowest energy structures of **2**, showing Ala (black), Leu (blue), and Glu (red) φ/ψ angles vs regions allowed (solid line) or partially allowed (dashed line) for β-strands and α-helices.

Leu *trans*-amide was calculated to be ~60 kcal/mol higher in energy than the *cis*-isomer.

A Ramachandran plot (Figure 5) of φ and ψ angles for the 10 lowest energy calculated structures places Ala, Leu, and 2/3 of Glu residues in regions typical[13] of β-strand conformations, while 1/3 of the structures put the Glu residue in a space typical of α-helicity due to free rotation about the C-terminus. We conclude that the N-terminus to the Glu side chain (i→i+2) linkage is a little short to fix the macrocycle in an α-helical turn and instead enforces a pseudoplanar geometry with the aid of an Ala-Leu *cis*-amide bond. The resulting structure is the smallest β sheet structure ever reported, one that is highly novel in featuring two antiparallel but non-hydrogen bonded β-strands within a three-residue peptide.

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Note Added after ASAP Publication. The abstract and table of content graphic were incorrect in the version published ASAP on June 17, 2009; the corrected versions were published ASAP on June 24, 2009.

Supporting Information Available: Compound characterization data including HPLC, CD, mass, and NMR spectra and data (12 figures). This material is available free of charge via the Internet at http://pubs.acs.org.

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