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Alpha Helix Nucleation by a Simple Cyclic Tetrapeptide

Alpha Helix Nucleation

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The simple cyclic tetrapeptide cyclo-(1,4)-[Ala-Arg-Ala-homoGlu]-NH_2 (**3**) is shown to adopt an unusual α-turn structure, which is not α-helical but can nucleate α-helicity when attached to the N-terminus of either model peptides or two biologically relevant peptides. This new N-terminal helix-capping macrocycle provides very simple and rapid synthetic access to α-helical peptide structures.

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Introduction

The α-helical conformation dominates >30 % of a protein structure and is a key structural motif recognised in protein–protein interactions (PPIs).[1] Such α-helical 'hot spots' in proteins typically involve 1–5 helical turns[2] and are prime targets for developing smaller compounds that mimic them or inhibit PPIs.[3] However, short synthetic peptides of these lengths (<20 amino acid residues) do not form thermodynamically stable α-helices in water, which strongly competes for helix-defining hydrogen bonding backbone amides.[4] Many approaches have been investigated for stabilising an α-helical turn for potential use as a nucleator of α-helical structures when appended to a peptide sequence. Helix induction from the N-terminus requires helix nucleators that provide hydrogen bond acceptors (Fig. 1a),[5,6] whereas nucleators appended to the C-terminus of a peptide provide hydrogen bond donors (Fig. 1b).[7] Helix nucleators reduce the entropic penalty associated with the folding of the first α-helical turn, enabling helix propagation along the length of the peptide.[8] Here, we describe a new and simpler approach to an N-terminal helix nucleator.

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A peptide α-turn can be stabilised using five amino acid residues and is defined by a $C_\alpha(i)$–$C_\alpha(i+4)$ distance of <7 Å, specific ϕ and ψ dihedral angles at each $i+1$, $i+2$ and $i+3$ residue, and a 5-to-1 hydrogen bond that creates a 13-membered ring (Fig. 2a). This hydrogen-bonded ring is common to classical, canonical α-helix structures in peptides and proteins.[9] Pentapeptides, cyclised through connecting side chains at the first and the fifth amino acid residues (e.g. Ac-cyclo-(1,5)-[KARAD]-NH_2 (**1**), Fig. 2b), are able to reproduce this classical α-helical turn (Fig. 2c), which is easily recognised by a distinctive circular dichroism spectrum (Fig. 2d). However, even slight variations to the composition of the side chain-to-side chain linker, its positioning, or its length can profoundly reduce the extent of α-helicity and alter the structure.[10]

However, a perfect α-helical turn actually requires only 3.6 residues. Hence, it may be possible to stabilise an α-turn in a cyclic tetrapeptide instead of a cyclic pentapeptide. We have previously reported two cyclic tetrapeptides (**2** and **3**; Fig. 3) that were not α-helices, but were instead identified as mimics of two non-classical α-turns, type I-α_{RS} (**2**) and II-α_{LU} (**3**), with ϕ and ψ angles (see ahead) that deviated from a classical α-helical turn.[11] Here, we report the synthesis and solution structure of a cyclic tetrapeptide containing α-aminoadipic acid (also known as L-homoglutamic acid (homoE or hE)) namely cyclo-(1,4)-[ARAhE]-NH_2 (**4**) that approximates another type of non-classical α-turn but which, unlike **2** and **3**, exhibits α-helix-nucleating properties when appended to the N-terminus of peptides.

Results and Discussion

*Synthesis and Structure of **4***

Cyclic peptide **4** can be cyclised either in solution phase from commercially available Fmoc-hGlu(OtBu)-OH or in solid phase from Fmoc-hGlu(Alloc)-OH, which can easily be synthesised from hE as reported.[12] For this study, we synthesised cyclo-(1,4)-[ARAhE]-NH_2 (**4**) from its acyclic precursor **4a** on solid phase (Fig. 4a) so that it can be readily appended to the N-terminus of different peptide sequences. Additionally, we re-synthesised the known compounds Ac-cyclo-(1,5)-[KARAD]-NH_2 (**1**), Ac-cyclo-(1,4)-[DapARA] (**2**), and cyclo-(1,4)-[ARAE]-NH_2 (**3**) on solid phase for comparisons with **4**.

The conversion of **4a** to **4** resulted in a significant structural change as observed in the circular dichroism (CD) spectra in Fig. 4b. The CD spectrum for **4** showed a weak molar ellipticity maximum at λ ~190 nm, a strong minimum at ~200 nm, and a much weaker minimum at ~222 nm. This CD fingerprint has been associated with a 3_{10}-helix or β-turn type III and/or admixtures of α-helix and random coils. Two temperature-independent amide protons ($\Delta\delta/T \leq 4$ ppb/K) are consistent with two hydrogen bonds from the homoglutamate NH and one of the C-terminal NH protons (Fig. 4c). A 2D ROESY NMR spectrum for **4** in H_2O/D_2O (9 : 1) showed short and medium range rotating frame Overhauser effects (ROEs), such as $d_{NN}(i,i+1)$, $d_{\beta N}(i,i+1)$ and $d_{\alpha N}(i,i+2)$, indicating that **4** was not a classical α-helix like **1**. The solution structure for **4** was calculated from these ROEs, with the 20 lowest-energy NMR-derived solutions structures for **4** matching to some extent (Fig. 4d, rmsd 0.055 Å over 4 C_α atoms) to an α-turn type I-αRS in the crystal structure of human serum retinol binding protein (1RBP, residues 16–20). A Ramachandran plot of ϕ/ψ dihedral angles for **4** (Fig. 4e) is approximately consistent with an α-turn type

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rather than a classical α-helical α-turn. These ϕ/ψ angles differ from those in cyclic peptides **1–3** and from other turn types in proteins (Fig. 4f), perhaps being closest to but not identical with α-turn type I-αRS.

Table 1 compares amide coupling constants ($^3J_{NHH\alpha}$) and temperature dependence of amide NH chemical shifts for **1–4**. The latter data are consistent with two possible 5→1 hydrogen-bonding amide NH protons from the C-terminal amide and residue **4** in cyclic tetrapeptide **4** ($\Delta\delta/T \leq 4$ ppb/K), although there were no α-helix-like amide coupling constants ($^3J_{NHH\alpha} < 6$ Hz) (Fig. S3, Supplementary Material). Interestingly, a superposition of the 20 lowest-energy structures of **4** on an idealised α-helix turn (Fig. 5) showed significant deviations between the structures. However, the positions of the three carbonyl oxygen atoms almost overlap, suggesting the possibility of **4** acting as a helix nucleator.

In summary, NMR and CD spectral data and structure calculation support the configuration of **4** in featuring a non-classical α-turn, closer to type I-αRS than II-αLU or classical α-helical turn (Fig. 4f).

*Cyclic Tetrapeptide **4** as a Helix Nucleator*

To investigate the relative helix-nucleating capacities of **2**, **3**, and **4**, the latter were attached to a 9-residue model peptide sequence ARAARAARA to form the C-terminal-capped Ac-cyclo-(10,13)-ARAARAARA[DapARA] (**5**), N-terminal-capped cyclo-(1,4)-[ARAE]ARAARAARA-NH_2 (**6**), and cyclo-(1,4)-[ARAhE]ARAARAARA-NH_2 (**7**). For comparison purposes, the acyclic analogue Ac-ARAhEARAARAARA-NH_2 (**8**) was also synthesised as control peptide. The CD spectrum of **8** (Fig. 6, black curve) showed a strong negative molar ellipticity at $\lambda = 195$ nm and a very weak band at 222 nm, suggesting ≤2 % α-helicity. The CD spectra of **5** and **6** lacked α-helix ellipticity bands at 195 and 208 nm. However, they showed a negative ellipticity at 200 nm, as consistent with a random coil, whereas the strong band at 225 nm is likely due to cyclic tetrapeptides **2** and **3** (Fig. 3). Therefore, **2** and **3** did not induce α-helicity in this peptide sequence. However, **7** displayed an α-helical CD spectrum with two characteristic ellipticity minima at 207 and 222 nm and a maximum at 195 nm, with an α-helicity degree of 63 %.

The three-dimensional structure of **7** was determined by NMR spectroscopy. The $^3J_{NHH\alpha}$ coupling constants of **7** were all smaller than 6 Hz (except for residue Arg 9), and all α-protons shifted significantly upfield, as consistent with an α-helix along the length of the peptide (Fig. 7a). These two parameters changed even for the R-A-hE residues of the cyclic tetrapeptide, which clearly had become more α-helical than in the absence of the appended linear peptide. Several low amide temperature coefficients (<4 ppb/K) supported the presence of multiple intramolecular hydrogen bonds all along the peptide sequence of **7**. There were also many medium intensity $d_{NN}(i,i+1)$, $d_{\alpha N}(i,i+3)$, and $d_{\alpha N}(i,i+4)$ nuclear Overhauser effects (NOEs), characteristic of an α-helical structure. A Ramachandran plot of ϕ and ψ dihedral angles indicates that all peptide residues in **7** adopt an α-helical conformation (Fig. 7b). The calculated structure for **7** in water was an α-helix from N-terminus to C-terminus (Fig. 7c) and matched well an idealised α-helix model (RMSD 0.71 Å). Superimposition of the averaged structure of **4** alone on the N-terminal end of **7** (Fig. 7c) supports the $^3J_{NHH\alpha}$ and δCHα data above in indicating that the cyclic tetrapeptide had an altered structure in **7** versus **4**, becoming an α-helical α-turn in **7** due to the formation of three hydrogen bonds to attached peptide residues. The additional methylene carbon in the linker in **4** versus **3** is the reason for this

flexibility that makes only **4** an α-helix nucleator. Molecular dynamics simulations conducted in water (Fig. S1, Supplementary Material) support this greater flexibility in **4** when compared with that in **2** and **3**.[11] Therefore, **4** is sufficiently flexible to be able to adapt to a classical α-helical turn in the presence of hydrogen bonding amide NH partners from an appended sequence, which is required to facilitate α-helix nucleation.

The cyclic tetrapeptide residues were modified to investigate whether changes were tolerated without affecting α-helicity. The CD spectra of cyclo-(1,4)-[GAAhE]ARAARAARA-NH_2 (**9**) and cyclo-(1,4)-[AAAhE]ARAARAARA-NH_2 (**10**) (Fig. S2, Supplementary Material) indicated high α-helicity (69 % and 60 %, respectively). Introducing some flexibility to the cycle by replacing Ala with Gly slightly increased α-helicity from 63 % to 69 %, highlighting the capacity of the cyclic tetrapeptide to slightly alter its backbone structure and become more α-helical.

Having demonstrated the capacity of **4** to act as an N-terminal helix nucleator when attached to the model peptide **9**, we next examined similar cyclic tetrapeptides at the N-terminus of two biologically important sequences, HIF-1α and p53 proteins. The HIF-1α peptide sequence Ac-ELARALDQ-NH_2 (**11**) was compared with cyclo-(1,4)-[AELhE]RALDQ-NH_2 (**12**), and the p53 peptide sequence Ac-QEAFSDLWKLLS-NH_2 (**13**) was compared with cyclo-(1,4)-[AQEhE]FSDLWKLLS-NH_2 (**14**) and the cyclic pentapeptide analogue Ac-cyclo-(1,5)-[KAQED]FSDLWKLLS-NH_2 (**15**), which were all synthesised in solid phase using standard procedures (Supplementary Material). Circular dichroism spectra revealed that the uncapped linear peptides (**11** and **13**) had little α-helical character. N-terminal addition of cyclic tetrapeptide analogues of **4** increased α-helicity from 2 % in **11** to 35 % in **12**, and from 10 % in **13** to 30 % in **14**. Similar helicity between **14** and **15** (32 %) in phosphate buffer (pH 7.2, 298 K) indicated the same helix-capping ability for **1** and **4** when attached to the N-terminus of **13**. These results suggest that this type of cyclic tetrapeptide α-turn can effectively nucleate α-helicity in short non-helical peptide sequences to a similar extent as cyclic pentapeptides. Their ease of synthesis supports further studies on these types of cyclic tetrapeptide turn motifs as helix nucleators in short peptides with biological significance. We will report on further applications and activities of these helix-nucleating cyclic peptides in the future.

Conclusions

A simple cyclic tetrapeptide cyclo-(1,4)-[ARAhE]-NH_2 (**4**), easily synthesised on resin or in solution and readily appended to the N-terminus of peptide sequences, was structurally examined by CD and NMR spectroscopy; it was found to be folded into a non-classical[10] α-turn, closest to but not identical with type I-α_{RS} of known[10] α-turn structures in water (Fig. 4). The α-turn structure in **4** has three key oxygen atoms that project into similar three dimensional space as those in a classical α-helical turn found in canonical α-helical peptide and protein structures (Fig. 5). The homoglutamic acid side chain-to-main chain N-terminal amine linker in **4** can thus act as an N-terminal hydrogen bonding template or cap for nucleating α-helicity when attached to peptide sequences. Addition of macrocycle **4** to the N-terminus of a model peptide sequence (Figs 6 and 7c), or analogues of **4** to two biologically relevant peptides (Fig. 8), resulted in α-helix induction along the entire length of the peptide sequences examined. This new N-terminal helix nucleator appears to have advantages over other N-terminal helix nucleators[5,13–17] (Fig. 9) in being much easier to synthesise, involving simple condensation of a homoglutamic acid in solid

phase peptide syntheses and on-resin cyclisation, thus being a very accessible approach to making peptides α–α-helical for a variety of uses.

Supplementary Material

Synthetic methods, compound characterisation (mass spectrometry, analytical HPLC, NMR), CD and NMR methods for calculated structures, MD simulation, and other experimental data are available on the Journal's web-site.

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Fig. 1. (a) N- and (b) C-terminal helix nucleators provide hydrogen bond acceptors and donors, respectively, to make the first three hydrogen bonds that promote the formation of an α-helix. The box represents a template.

Fig. 2. (a) α-Turn showing ϕ and ψ dihedral angles, the 13-membered hydrogen bonded ring, and the $C_\alpha(i)$–$C_\alpha(i+4)$ distance. (b, c) Cyclic pentapeptide (**1**) mimics an α-helical turn. (d) Circular dichroism spectrum of **1** measured in 10 mM phosphate buffer (pH 7.2, 298 K) shows a classic α-helix fingerprint.[10]

Fig. 3. Side chain-to-main chain-cyclised tetrapeptides that mimic non-classical α-turns. Peptide **2** is α-turn type I-α_{RS}[10] and **3** is α-turn type II-α_{LU}.[10] The structure of peptide **4** is also an α-turn as characterised ahead. Comparative circular dichroism spectra are shown for these cyclic tetrapeptides measured in aqueous 10 mM phosphate buffer (pH 7.2, 298 K).

Fig. 4. Structure of **4**. (a) Tetrapeptide **4a** cyclised through glutamate side chain to N-terminus main chain lactam formation produced **4**; the latter feastures three oxygen atoms (red) as potential hydrogen bond acceptors for inducing α-helicity. (b) CD spectra of **4** and its acyclic precursor **4a** (both at 100 μM) measured in 10 mM phosphate buffer (pH 7.2, 298 K). (c) NMR data of **4** recorded in H_2O/D_2O (9 : 1) at 298 K. (d) Superimposition of 20 lowest-energy-calculated NMR solution structures of **4** (PDB ID 5TWI) (grey) versus a crystal structure of an I-α_{RS} turn (residues 16–20) from human serum retinol binding protein (PDB ID 1RBP) (pink); homoGlu side chain linker is shown in orange. (e) Ramachandran plot of ψ versus ϕ dihedral angles for average structure of **4**, showing Ala1 (diamond), Arg2 (triangle), Ala3 (circle), hGlu4 (square) compared with demarcated ϕ/ψ region for protein α-turn type I-α_{RS} (large squares).[10] (f) Comparative ϕ/ψ angles for **1–4** versus different helices and turns found in proteins. SPPS, solution-phase peptide synthesis; HATU, xxx; DIPEA, xxx.

Fig. 5. (a) 20 Lowest-energy NMR structures of **4** (PDB id 5TWI) (grey) measured in H_2O/D_2O (9 : 1) at 298 K superimposed on idealised α-helical turn (green), showing different helical pitch and deviation (double arrow lines) from idealised α-helix; homoGlu side chain linker is shown in orange. (b) Angle deviation from a canonical α-helix of three of the oxygen atoms in **4**.

Fig. 6. CD spectra of peptides **5–8** (50 μM) measured in 10 mM phosphate buffer (pH 7.2, 298 K): **5** (blue), **6** (green), **7** (red, 63 % α-helicity), and **8** (black).

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Fig. 7. Structure of cyclo-(1,4)-[ARAhE]ARAARAA-RA-NH_2 (**7**) in water at 298 K. (a) Summary of NMR data of **7**, as consistent with α-helix induction along the length of the peptide. (b) Ramachandran plot of ψ versus ϕ dihedral angles for average of 20 lowest-energy structures of **7**, coinciding with the region that defines a classical α-helix. (c) Superimposition of 20 lowest-energy NMR-derived structures for **7** (PDB ID 5TWW, grey; RMSD 0.524 Å) matching very well with an idealised α-helix (green ribbon, RMSD 0.714 Å). Side chain–main chain linker (orange), the first two hydrogen bonds (red), structural change from **4** (cyan) to **4** within **7** indicated by red arrow.

Fig. 8. CD spectra showing helix nucleation by cyclic tetrapeptide analogues of **4** appended to the N-terminus of p53 and HIF-1α peptide sequences in 10 mM phosphate buffer (pH 7.2, 298 K). (a) HIF-1α peptide Ac-ELGRALDQ-NH_2 (**11**, dotted line) versus cyclo-(1,4)-[AELhE]RALDQ-NH_2 (**12**, solid line; 35 % α-helicity). (b) p53 peptide Ac-QEAFSDLWKLLS-NH_2 (**13**, dotted line) versus cyclo-(1,4)-[AQEhE]FSDLWKLLS-NH_2 (**14**, solid line; 30 % α-helicity) versus Ac-cyclo-(1,5)-[KAQED]FSDLWKLLS-NH_2 (**15**, dashed line; 32 % α-helicity). Alpha helicity was calculated at λ = 222 nM (Supplementary Material).

Fig. 9. N-terminal helix-capping motifs highlighting hydrogen bond acceptors in the cap that support nucleation of the first two-to-three helical hydrogen bonds in **16**,[13] **17**,[14] **18**,[15] **19**,[16] **20**,[17] and **21**.[18]

Table 1. Comparative amide coupling constants ($^3J_{NHH\alpha}$) and temperature dependence of chemical shifts ($\Delta\delta/T$) for 1–4 in H_2O/D_2O (9 : 1) at 298 K

Cyclo-(1,5)-Ac[KARAD] (**1**)							
Residue	K1	A2	R3	A4	D5	NH	NH
$^3J_{NHH\alpha}$ [Hz]	3.6	3.6	5.4	5.6	6.9		
$\Delta\delta/T$ [ppb/K]	7	4	8	3	5	2	9
Cyclo-(1,4)-Ac[DapARA] (**2**)							
Residue	Dap1	A2	R3	A4			
$^3J_{NHH\alpha}$ [Hz]	6.9	7.1	7.0	8.6			
$\Delta\delta/T$ [ppb/K]	6	8	6	6			
Cyclo-(1,4)-[ARAE]-NH_2 (**3**)							
Residue	A1	R2	A3	E4		NH	NH
$^3J_{NHH\alpha}$ [Hz]	4.7	9.6	7.0	4.7			
$\Delta\delta/T$ [ppb/K]	7	7	8	6		6	7
Cyclo-(1,4)-[ARAhE]-NH_2 (**4**)							
Residue	A1	R2	A3	hE4		NH	NH
$^3J_{NHH\alpha}$ [Hz]	6.5	6.2	6.5	7.4			
$\Delta\delta/T$ [ppb/K]	8	6	7	3		2	7