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Unexpected conformational properties of a peptide constrained by an aliphatic link between the *i* and *i*+4 positions

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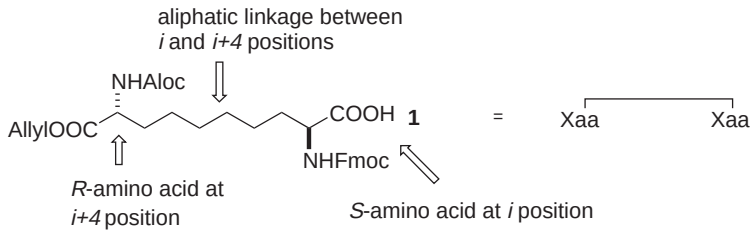
Abstract—A new method for the synthesis of cyclic peptides bearing unnatural aliphatic linkages between the *i* and *i*+4 positions results in a peptide with an unexpectedly strong β-turn propensity.

The importance of the α-helix as a motif in protein and peptide structure has inspired many approaches to the stabilisation of peptide α-helical conformation.[1] In particular, α-helical conformation has been induced or enhanced by synthesising peptides where the side-chains at the *i* and *i*+4 positions are covalently linked, thus directing these amino acids to align as required for an α-helix and reducing the conformational freedom of the peptide. Such covalent linkages have frequently been formed via amide bonds between amino acids such as Lys and Asp, Lys and Glu, Orn and Glu, and similar residues;[2,3] other, longer amide linkers have also been reported.[2] A wide range of possible linker structures and lengths between the *i* and *i*+4 positions appear to be equally successful in stabilising the helix structure. Introducing unnatural aliphatic linkages between the side-chains at the *i* and *i*+4 positions of a helix has considerable potential to enhance the biological properties and metabolic stability of the resulting peptides; however, the difficulties in synthesising such peptides have until recently prevented such studies. The only reliable and general method for introducing such linkages between the side-chains of peptides reported to date is the RCM methodology of Grubbs.[4]

We report herein a new method for the synthesis of peptides containing unnatural aliphatic linkages between the side-chains at the *i* and *i*+4 positions. Our methodology involves the synthesis of a bifunctional amino acid, with the appropriate aliphatic linker join-



test peptide **2** AcNH-Lys-Ala-Ala-Ala-Ala-Lys-Ala-Ala-Xaa-Ala-Lys-Ala-Xaa-Ala-Ala-Lys-$CONH_2$

control peptide **3** AcNH-Lys-Ala-Ala-Ala-Ala-Lys-Ala-Ala-Ala-Ala-Lys-Ala-Ala-Ala-Ala-Lys-$CONH_2$

Figure 1.

Keywords: peptides; circular dichroism; conformation; cyclisation.

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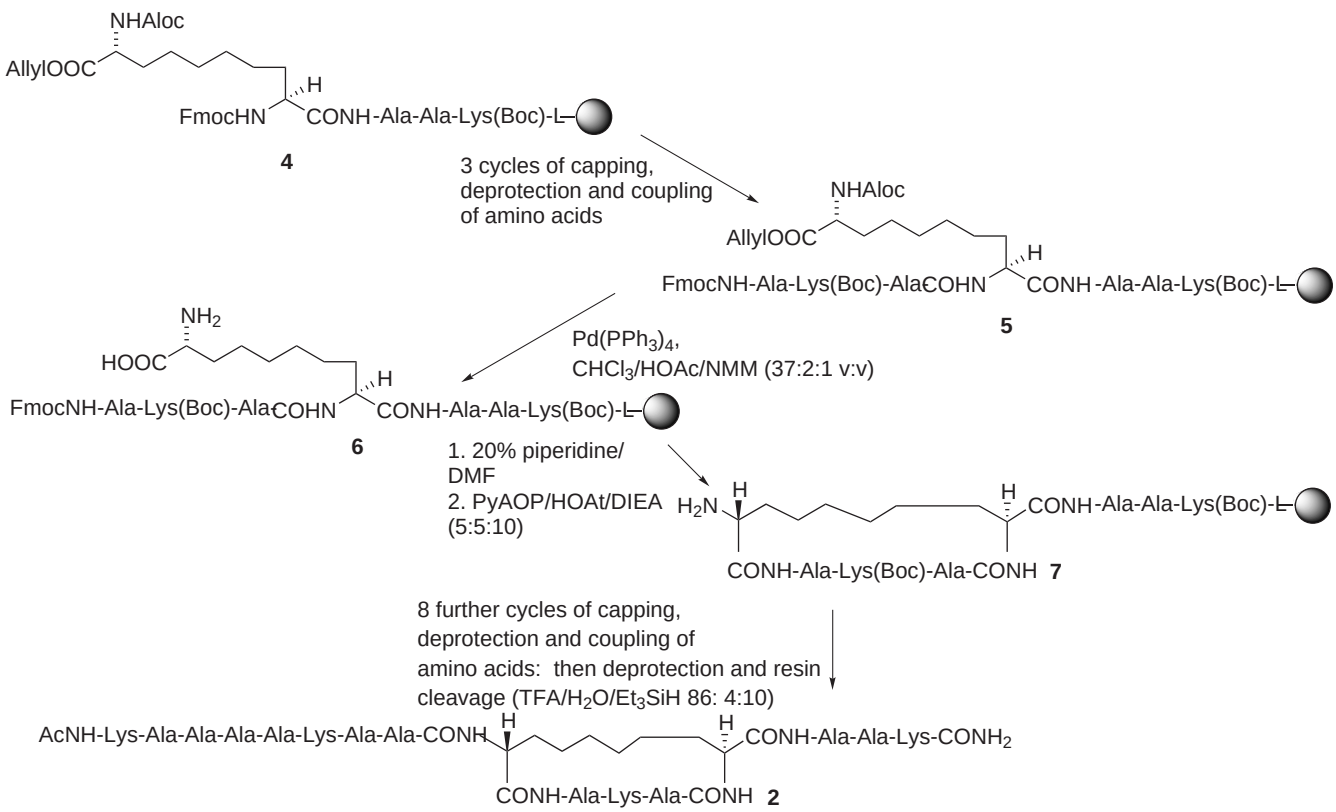
ing the chiral centres, orthogonally protected in such a way that first one, then the second amino acid moiety may be incorporated into the peptide. We also report CD studies carried out on the resulting peptide.

Preliminary modelling studies prompted us to introduce a hexyl linker between the *i* and *i*+4 positions, with the amino acid at the *i* position bearing the naturally occurring (*S*)-stereochemistry and that at the *i*+4 position the (*R*)-stereochemistry. We previously reported[5] the synthesis of bifunctional amino acid **1**, which effectively contains both the hexyl linker and both amino acid stereocentres, and is orthogonally protected in such a way that a cyclic peptide may be formed using solid-phase methods.

We aimed to study the effect of this hexyl linker on the helicity of an alanine-based model host peptide. Alanine-based peptides show significant helicity, even in water; this has been attributed to the high helix propensity of alanine itself. A range of such model host peptides have been designed and used for studying the helix stabilising/destabilising properties of other amino acids. As these peptides are relatively short, single amino acid substitutions have a large effect on the helical content, which can be measured by CD.[6] We chose to incorporate amino acid **1** into a variant of the AK peptide studied by Baldwin and co-workers,[7] bridging the 4- and 8-positions to give test peptide **2** (Fig. 1). For comparison purposes, we also synthesised control peptide **3** (which lacks the aliphatic bridge), using standard automated solid-phase peptide synthesis methods.

The synthesis of test peptide **2** was carried out using a Millipore 9050 continuous-flow peptide synthesiser, as follows. Fmoc-Lys(Boc)-OH was attached to Sieber amide resin[8] using DIC/HOAt; two standard cycles of capping, deprotection and coupling of Fmoc-Ala-OH followed. Amino acid **1** (3.3 equiv.) was then coupled, using HOAt/DIC (4 equiv.), to give linear peptide **4** (Scheme 1). Three further standard cycles of capping, deprotection and coupling followed, affording linear peptide **5**. The resin was then transferred to a Merrifield bubbler. Palladium catalysis[9] was used to selectively remove the allyl ester and allyloxycarbonyl (Aloc) protecting groups, affording **6**. The Fmoc group was then removed from the *N*-terminus of the peptide; this was followed by on-resin cyclisation using PyAOP/HOAt/DIEA (5:5:10)[10] to give cyclic peptide **7**. In contrast to other methods for the on-resin synthesis of cyclic peptides,[9,11] our approach provides a cyclic peptide, still attached to the resin, with a free *N*-terminus for further chain extension. Accordingly, the peptide synthesis was then completed, using standard cycles of capping, deprotection and coupling. Cleavage of the peptide from the resin, removal of the Lys side-chain protecting groups with TFA and purification by HPLC gave the desired peptide **2** in an overall yield of 15%. The peptide was characterised by ES-MS.

Structural studies of the test peptide **2** by CD revealed an unexpected result. Whereas the CD spectra of control peptide **3** at 4°C in H_2O, TFE, ethanediol–H_2O (2:1) and SDS (20 mM) respectively were dominated by the α-helix conformation, the test peptide **2** showed different CD spectra (Fig. 2) under the same conditions



Scheme 1.

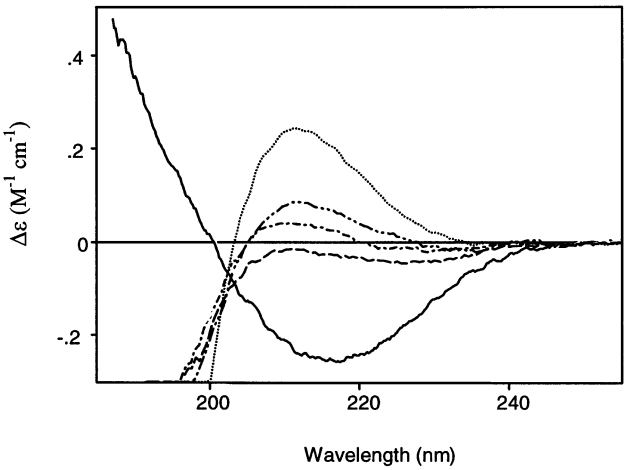


Figure 2. CD spectra of test peptide **2** at 4°C in H_2O (dash–dot), TFE (solid), ethanediol–H_2O (2:1) (dash at 4°C and dot at −92°C) and SDS 20 mM (dash–dot–dot).

of solvent and temperature. There is no evidence of a α-helix conformation at all in the constrained test peptide. Instead, the conformational behaviour of the test peptide **2** in H_2O, ethanediol–H_2O (2:1) and SDS 20 mM, respectively revealed a β-turn of type II[12] that increased in content on lowering the temperature. By contrast, in TFE the test peptide **2** showed a CD spectrum with a negative band at about 218 nm and a cross over at about 200 nm, indicating a β-turn of type I.[12]

In conclusion, the triply-orthogonal protecting group approach described in this paper constitutes a powerful and flexible method for introducing unnatural linkages between amino acid side-chains in peptides. The method is compatible with solid-phase peptide synthesis techniques, and can clearly be generalised to incorporate linkers of different length and structure. Unexpectedly, the hexyl linker chosen has been shown to induce a β-turn structure in a peptide sequence that has inherent α-helical propensity. This indicates that not all *i* to *i*+4 linkages are helix-stabilising.[13] It is notable that, in a recent study[14] in which aliphatic *i*, *i*+4 and *i*, *i*+7-linkages were formed in helical peptides using RCM, it proved impossible to effect ring closure with certain aliphatic *i*, *i*+4 combinations. Finally, our results also indicate a possible approach to the synthesis of β-turn mimetics and the stabilisation of β-sheets.

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