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Design, machine synthesis, and NMR-solution structure of a β-heptapeptide forming a salt-bridge stabilised 3_{14}-helix in methanol and in water†

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Salt-bridge formation may be used to stabilise the 3_{14}-helical conformation of β-peptides in aqueous solution, as shown by a 2D-NMR spectroscopic investigation.

The design, synthesis, and characterisation of artificial molecules capable of forming specific and predictable secondary structures offer new possibilities in the field of molecular recognition, catalysis, and protein folding.[1] Thus, it is not surprising that unnatural oligomers like β-peptides have received considerable attention, since these molecules are able to form stable secondary structures with as few as six residues.[2] Although we are starting to understand some of the factors influencing the folding of these molecules, much work remains until we are able to correctly predict how a particular sequence of unconstrained building blocks will fold, especially in aqueous media. To investigate if salt-bridge formation can be used to stabilise β-peptidic secondary structures we set out to synthesise the β-heptapeptide **1** (Fig. 1).

The sequence of **1** was selected based on the following design principles: (*i*) we expect an *all*-β^3 peptide to form a 3_{14}-helix[2]; (*ii*) use of charged residues will ensure high water solubility; (*iii*) residues with positively and negatively charged side-chains should be positioned in an *i*/*i* + 3 disposition so that they are in juxtaposition on the ternary 3_{14}-helix, allowing for the two desired salt-bridges to be formed; (*iv*) β^3-HOrnithine rather than β^3-HLysine residues were chosen since this brings the charges closer together and less entropy is lost upon salt-bridge formation; (*v*) one side of the helix is covered by aliphatic side-chains to allow for intramolecular hydrophobic interactions, Fig. 1.

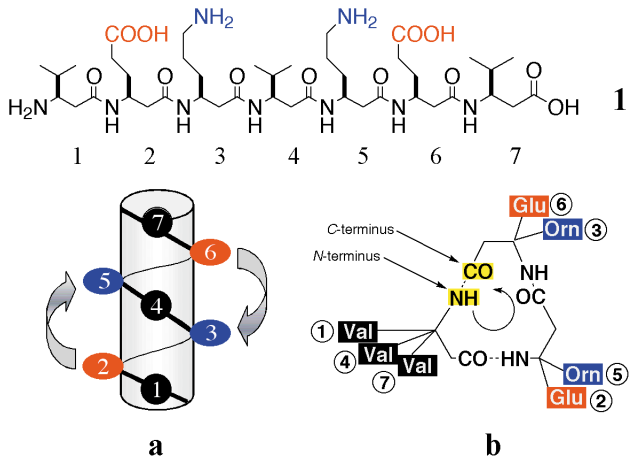

Fig. 1 Molecular formula of the β-heptapeptide **1** and schematic representations of a linear 3_{14}-helical structure from the side (**a**) and from top (**b**). Colour-code: Black = hydrophobic residues, Blue = positively charged residues, and Red = negatively charged residues.

† Electronic supplementary information (ESI) available: NMR spectroscopy and conc. dependent CD-spectra of **1**. See http://www.rsc.org/suppdata/cc/b1/b101085i/

The *all*-β^3-heptapeptide **1** was synthesised on *Wang* resin (0.25 mmol scale) using the standard *FastMoc* chemistry as implemented in an Applied Biosystem 433A automated peptide synthesiser. The protocol involved 45 min coupling times with four equiv. of the HBTU–HOBt activated β-amino acid (Fmoc-β^{3}HVal-OH, Fmoc-β^{3}HOrn(Boc)-OH, and Fmoc-β^{3}HGlu(OtBu)-OH prepared by Arndt-Eistert homologation),[3] 5 × 10 min deprotection, including NMP–DCM washings. The crude peptide was isolated in 78% purity, after TFA mediated removal of the protecting groups and cleavage from the resin. Purification by reverse phase HPLC (C18 column; MeCN–water gradient containing 0.1% TFA) afforded the pure peptide **1** in 65% yield, as characterised by its electrospray mass spectrum and complete assignment of the 500 MHz ^{1}H NMR spectrum. The main impurity (10%) was also isolated and characterised as *N*-Fmoc **1**, thus confirming previous observations that the standard Fmoc-cleavage, with 20% piperidine, is insufficient for complete Fmoc removal of β-peptides with chain-lengths exceeding six amino acids.[4] To the best of our knowledge, this is the first time a β-peptide was synthesised with standard coupling conditions on an automated synthesiser, suggesting that automated synthesis may be successfully applied for β-peptides of longer sequences and combinatorial libraries, especially if the deprotection protocol is modified according to our recently improved conditions.[4]

β-Peptide **1** exhibits excellent solubility in both water and MeOH. The far-UV circular dichroism (CD) spectra of **1** in MeOH and aqueous solution are shown in Fig. 2. As expected, the CD spectrum in MeOH displays a minimum at *ca*. 216 nm and a maximum at *ca*. 198 nm, characteristic of a 3_{14}-helix with (*M*)-chirality. Remarkably, when one compares the mean molar ellipticity of all β^3-peptides synthesised in our laboratory to date this peptide exhibits the strongest ellipticity of all in MeOH solution. The CD spectra recorded in aqueous solution also show the typical pattern of a 3_{14}-helical conformation.[4] Furthermore, the pH dependence study in Fig. 2 suggests that the two salt-bridges successfully increase the stability of the

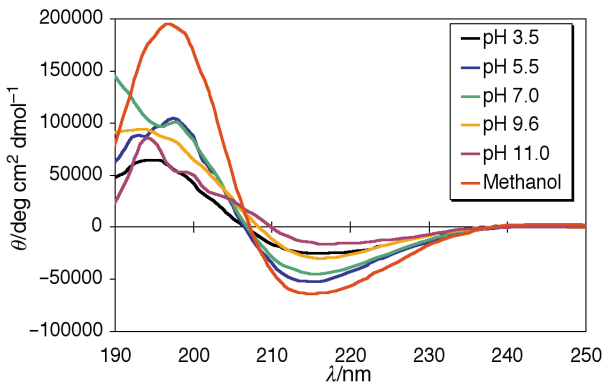

Fig. 2 CD-spectra of **1** in MeOH solution (red) and in aqueous solutions at different pH values (all measurements were done at 0.2 mM). The minimum near 215 nm, considered characteristic of a (*M*) 3_{14}-helical structure, shows maximal intensity at pH 5.5 (blue) consistent with a salt-bridge stabilisation.

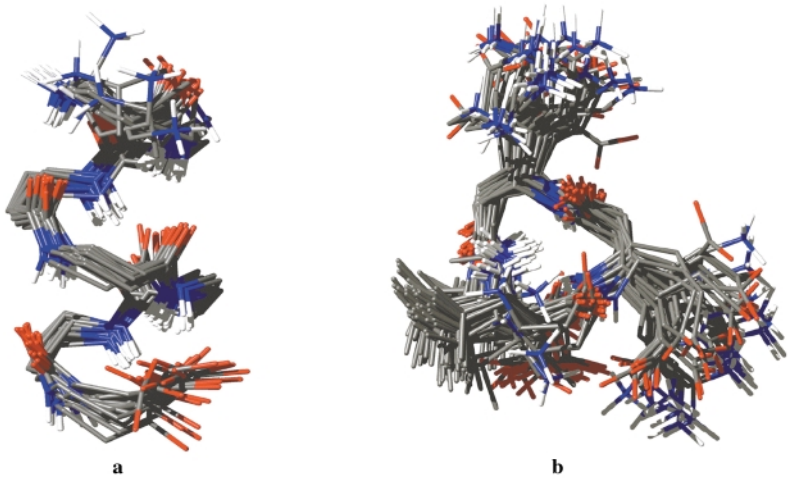

Fig. 3 Solution structure of the β-heptapeptide **1** in MeOH represented as a bundle of 25 lowest-energy structures obtained by simulated annealing, using NMR dihedral-angles and NOE-distance restraints, in the XPLOR programme. View along the (*M*) 3_{14}-helical axis with side-chains omitted (**a**), and top-view with side-chains (**b**).

helix since the strongest molar ellipticity is observed at pH 5.5, *i.e.* the pH value where the ornithine residues are expected to be protonated and the glutamic acid residues are still deprotonated. The CD spectra displayed only a slight change in the range 0.02 to 2 mM (see supporting information †), suggesting that no aggregation takes place in this concentration range. This observation is surprising if one considers the amphiphilic nature of the helix, and raises the question how to distribute the side-chains in order to achieve self-aggregation of 3_{14}-helical structures.

A detailed 2D-NMR spectroscopic study was undertaken to obtain high-resolution data on the actual conformation of **1** in water and in MeOH. The MeOH spectrum, recorded at 500 MHz, showed good separation of all signals and allowed complete assignment of all ^{1}H resonances in their respective spin system using DQF-COSY and TOCSY techniques; HSQC and HMBC experiments could then be used for sequence assignment. The large ^3J(NH; C(β)-H) coupling constants establish that the NH and C(β)-H protons are in an anti-periplanar arrangement. The diastereotopic CH_2(α) protons were assigned assuming that the axial protons exhibit a large, and the lateral protons a small, coupling with C(β), which is in agreement with the stronger NOEs observed from H-C(β) to the lateral H-C(α) protons than to the axial H-C(α) protons. ROESY spectra were acquired at three different mixing times (80, 150, and 300 ms), and a total of 72 NOEs were extracted from the spectra with mixing time 300 ms. Integration of the cross peak volumes followed by calibration allowed classification of the NOEs in three distance categories with the following upper bound distance limits: strong < 3.0 Å, medium < 3.5 Å, and weak < 4.5 Å. These distance restraints, together with 6 (NH, C(β)-H) dihedral angle restraints, derived from the coupling constants, were subjected to simulated annealing following the XPLOR protocol. The calculation produced a set of 25 structures with lowest restraint violations. These structures show a well-defined left-handed 3_{14}-helical conformation, Fig. 3. The side chains of β^3-HGlu and β^3-HOrn are on top of each other, thus allowing the designed salt bridges to form.

The NMR spectra recorded in aqueous solution at pH 5.5 showed more spectral overlap. Although it was possible to assign all resonances using a combination of the TOCSY, HMBC, and HSQC spectra, integration of the cross-peak volumes in the ROESY spectra was severely hampered by overlap. Nevertheless, the following NOEs could be unambiguously assigned: NH(2) and NH(4) to H-C(β) *i*+2 and *i*+3; NH(2) to NH(3); NH(3) to side chain protons of residue 6; H-C(β) of residue 4 to the lateral H-C(α) proton on residue 1. A qualitative treatment of these key NOEs, together with the large ^3J(NH; C(β)-H) coupling constants observed, imply the 3_{14}-helical conformation. Furthermore, no NOEs violating this conformation were observed. Based on the NMR spectroscopic data and the characteristic CD-spectra we conclude that the main conformation of β-peptide **1** is a 3_{14}-helix in MeOH as well as in water. Thus, since this is the first time a high resolution method supports the folding of a β-peptide without covalent constrains in water,[5] this study demonstrates that salt-bridge formation constitutes a valuable tool for the design of stabilised unnatural secondary structures in aqueous media.

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