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Stabilisation of Peptide Foldamers in an Aqueous Medium by Incorporation of Azapeptide Building Blocks

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Foldamers stabilised by long-range H-bonding interactions constitute a thoroughly studied class of protein-mimicking materials and are currently attracting considerable interest.[1] There are two major approaches to the modification of natural α-amino acid building blocks: 1) homologation of the amino acid residues (this results in aliphatic β- and γ-peptides[2] and aromatic oligoamide foldamers)[3] and 2) replacement of the amide moiety with functional groups capable of forming a strong H-bonding network (this leads to intriguing foldameric oligoureas[4] and azapeptides).[5] The secondary structure models of both helical oligoureas[4b,6] and strand-like homooligo-azapeptides[5d,7] display bifurcated H-bonds in the backbone; this arrangement is theoretically predicted for azapeptides.[8] Although our preliminary DFT calculations indicate that the stabilisation energy calculated for azapeptide H-bonds is at least 10% higher, even in a non-bifurcated arrangement, than that of the parent peptide–peptide H-bond, helical geometry for azapeptides has not been found experimentally thus far. This is possibly due to the preferred short-range bifurcations that stabilise the strand-like geometries.

Because of their promising biomedical applications,[9] the stabilisation of foldamers in an aqueous medium is a great current challenge, and various methods have been proposed to stabilise them: macrodipole stabilisation,[10] sidechain ion pairs[11] and disulfide bridges.[12] All of these approaches utilise either sidechains or chain termination to attain an energetically favourable construction; this increases their stability in H-bond-destroying water. Although polar solvents can successfully compete for the H-bond pillars, ureas and azapeptides potentially offer stronger H-bonds in the backbone, and thus are reasonable candidates as foldamer-stabilising building blocks.

In this work, a novel backbone stabilisation approach is proposed as a means of increasing the stability of the foldameric structures in an aqueous medium. We demonstrate that incorporation of 1-aminoproline aza-ACPC (ACPC = 2-aminocyclopentanecarboxylic acid) building blocks into β-peptide chains leads to well-defined secondary structures that are unusually stable in water.

Oligomers of the ACPC diastereomers can form four major types of secondary structures:[13,14] a homochiral H12 helix, an alternating heterochiral H10/12 helix, a homochiral non-polar strand and an alternating heterochiral polar strand. While the H10/12 helix constructed from ACPC residues partially retains its structure in an aqueous medium, the H12 helix does not fold in water. In an aqueous medium the folding of the H12 helix can be facilitated by substituting nitrogen in the sidechain moiety of the five-membered ring.[15] The non-polar and polar strands of ACPC oligomers exhibit fibrillar aggregates in a polar solvent, due to infinite sheet formation. Because of their versatility, the ACPC oligomers provide an ideal starting model through which to study the effects of azapeptide incorporation on secondary structure preference and stability. In this work, the following sequences were studied (Scheme 1): Ac-[(1*S*,2*R*)-ACPC-2*S*-aza-ACPC]$_3$-NH_2 (**1**), Ac-[(1*R*,2*S*)-ACPC-2*S*-aza-ACPC]$_3$-NH_2 (**2**), Ac-[(1*S*,2*S*)-ACPC-2*S*-aza-ACPC]$_3$-NH_2 (**3**) and Ac-[(1*R*,2*R*)-ACPC-2*S*-aza-ACPC]$_3$-NH_2 (**4**).

The four Boc-ACPC diastereomers and Boc-2*S*-aza-ACPC were prepared by using literature methods.[16] Chain assembly was carried out on a solid support, with Boc chemistry and DCC/HOBt coupling, by following standard methodology. The products were isolated by RP-HPLC (RP = reverse phase) and characterised by means of ESI-

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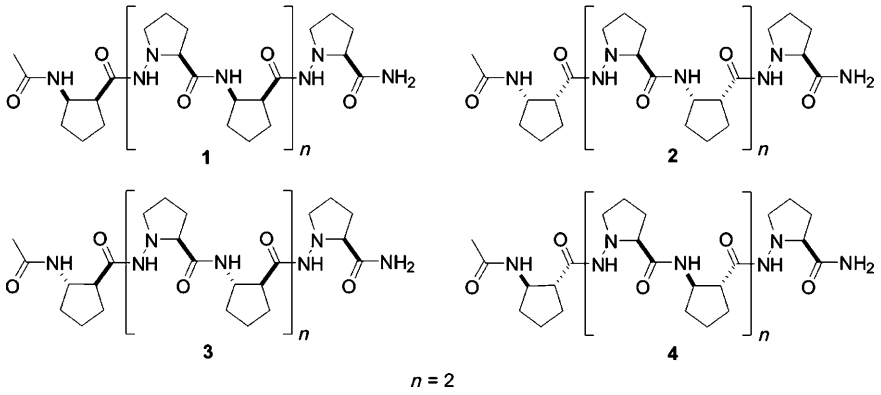

Scheme 1. Structures of the Ac-[*cis*- and *trans*-ACPC-2*S*-aza-ACPC]$_3$-NH_2 oligomers.

MS. A complete ^{1}H NMR signal assignment was achieved for **1**–**4** through the combined use of COSY, TOCSY and ROESY. The concentrations were 4 mM, and various solvents were utilised, including aqueous buffer (pH 7.4) at both 277 K and 298 K, and CD_3OH and [D_6]DMSO at 298 K. The solubilities of **1**–**4** were excellent under the studied conditions. In an aqueous medium, line broadening was observed for the azapeptide-NH signals at 298 K (Figure S1); this could be eliminated at the lower temperature. Self association was tested in aqueous solution by means of DOSY and TEM measurements, but aggregation phenomena were not detected (Figure S2-S3). The line broadening is attributed to the inherently higher rate of aza-NH–solvent exchange, which is due to the considerably higher H-bond acidity of the azapeptide-NH moiety.[17] This is supported by the relatively intense azapeptide NH–solute chemical exchange ROESY crosspeaks in water. It is important that ^{1}H resonances of quaternary nitrogen origin (protonated aza nitrogen) were not detected in the NMR spectra recorded in the organic solvents or in aqueous buffer at pH 7.4.

For assessment of the conformational stabilities of the peptides, the NH chemical shift temperature gradients ($\Delta\delta/\Delta T$)[18] were measured in [D_6]DMSO in the range 298–318 K (Figure 1). Unfortunately, $\Delta\delta/\Delta T$ reference data for the azapeptide moiety could not be found in the literature. In our models, the $\Delta\delta/\Delta T$ values were systematically lower than those for the amide hydrogens, but this can be explained by the increased H-bond acidity. The values obtained for the non-terminal amides ($\Delta\delta/\Delta T < 3$ ppb/K) in **1**, **3** and **4** clearly indicate strong H-bonding interactions attributable to the well-defined secondary structure. For **2**, only NH_3 displayed strong shielding; this suggests a partial aperiodic fold. $^3J_{NHCH}$ coupling constants were also extracted (see Supporting Information). These values were not sensitive to temperature variations; this reinforced the view that the relatively quick proton exchange for the azapeptide NH is not a consequence of conformational inhomogeneity.

To determine the high-resolution structures, ROESY experiments were run in CD_3OH or [D_6]DMSO at 298 K and in aqueous buffer at both 298 K and 277 K. In all the solvents applied, signal-rich ROESY spectra were obtained, and a number of long-range (i–i+2) NOE interactions could readily be observed for **1** and **4** (Figure 2); this offered useful input for the further structure refinement. Importantly, long-range NOEs were also detected in the aqueous medium at 298 K for the slowly-exchanging amide nuclei and for all nuclei at 277 K; this is exceptional for such short oligomers. For **2**, a single i–i+4 NOE was found, in good accord with a shielded NH_3. No long-range interaction was detected for **3**.

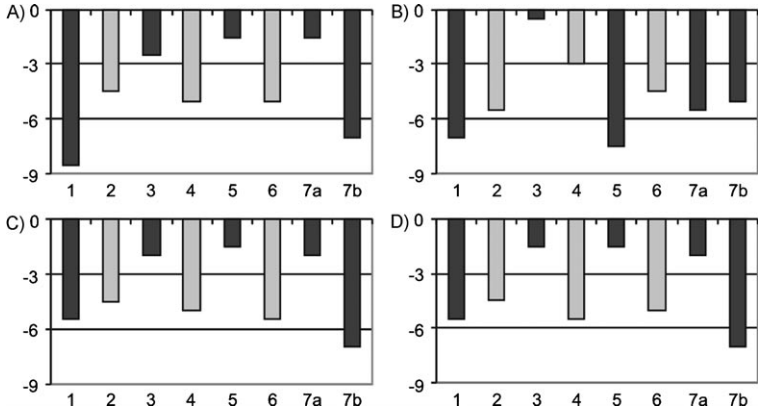

Figure 1. Temperature gradients of the chemical shifts for the protons of the β-peptide (black) and azapeptide (grey) moieties measured in [D_6]DMSO. Panel A) **1**, B) **2**, C) **3** and D) **4**.

Since the backbone contains basic nitrogen atoms, the pH can affect the folding process and consequently the NMR features. The NMR experiments were repeated in water buffered to pH 2.0 for **1** and **4** in which the long-range NOEs at neutral pH indicated helical folding. The acidic medium resulted in three new signals in the ^{1}H NMR spectra that could be assigned to the protonated aza nitrogen atoms. Interestingly, neither chemical shift changes nor broadening were observed for the peptide NH signals upon decrease of the pH. Moreover, the long-range NOE signals remained intact. These findings strongly suggest that protonation takes place on the aza-ACPC residues in water at sufficiently acidic pH, but backbone protonation cannot induce unfolding of the helical structures (see ECD results).

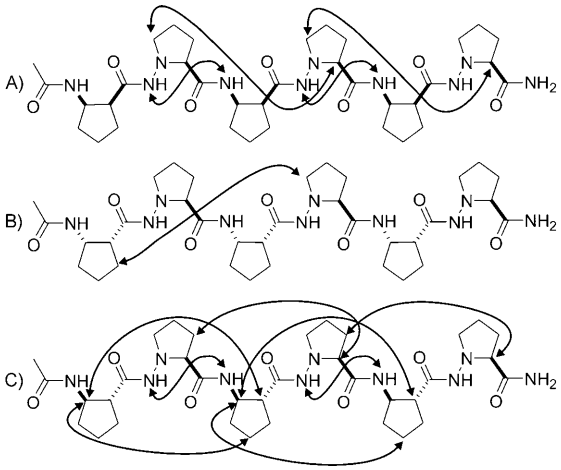

Figure 2. Characteristic long-range NOE interactions observed. A) **1**, B) **2** and C) **4**.

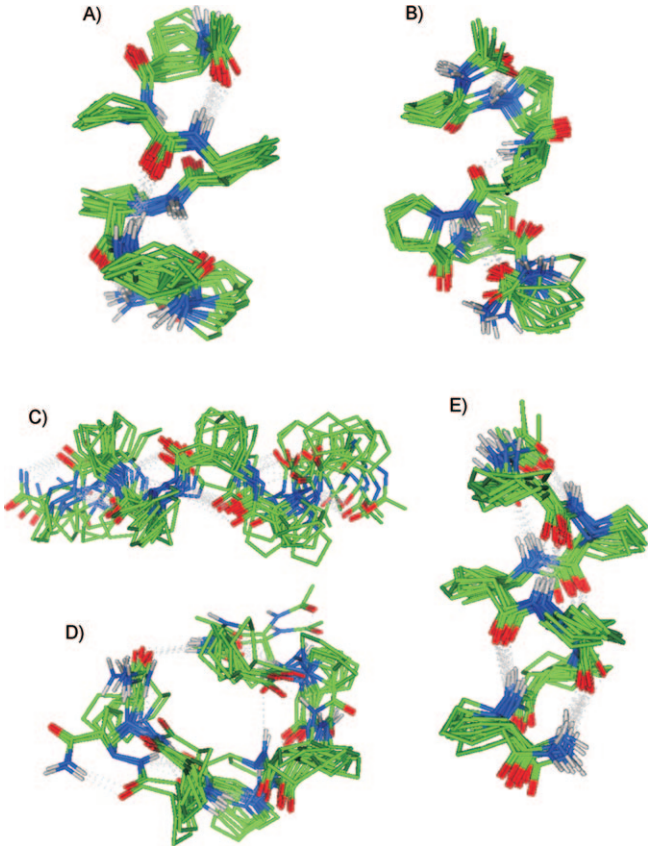

Figure 3. The ten lowest-energy structures obtained from the NMR structure refinement for A) **1**, B) **2**, C) **3** (cluster 1), D) **3** (cluster 2) and E) **4**.

For the 3D structural refinement, an NMR restrained hybrid Monte Carlo molecular dynamics (MCMD) conformational search was carried out by using the MMFF94x force field, and cluster analyses were performed for the resulting conformational ensembles. Since the NMR results pointed to unprotonated backbones in organic solvents and in water at pH 7.4, the initial structures were constructed without protonation. For **1**, the long-range restraints defined a single cluster corresponding to the alternating H10/12 helix (RMSV = 0.007 Å). For **4**, the structure refinement resulted in a single cluster corresponding to the H12 helix (RMSV = 0.003 Å). No consistent violations of distance restraints were observed, and the NOEs predicted by the ten lowest-energy conformations were detected in the spectra (Figure 3). Inspection of the refined helical structures revealed that the lone pairs of the aza nitrogen atoms are not involved directly in the backbone H-bonds and are accessible from the solvent. The molecular mechanics calculations carried out with the implicit solvation model (water) showed that both helices remain stable even with a fully protonated backbone, and this is in accord with NMR findings (Figure S4).

For **2**, the calculations resulted in a single cluster, which is a meandering structure in which NH_3 is involved in a bifurcated H-bond. On the other hand, the NOE interactions predicted in the C-terminal part of the sequence by the low-energy structures could not be identified in the ROESY spectrum; this suggests that **2** exhibits a fairly stable turn-like motif in the N terminal, together with a flexible C terminal. Two clusters with similar energies were found for **3**, both of which exhibit 8-membered H-bonded pseudo-rings: 1) a bent 8-strand cluster and 2) a circular fold cluster. Since strong shielding was indicated by the temperature gradient data, it can be concluded that the stable 8-membered rings are constant features of the structure, but the overall shape of the molecule undergoes fluctuation.

The lowest-energy conformations for **1** and **4** were selected and further optimised. For the ab initio quantum chemical geometry optimisations,[19] the HF/3-21G level of theory in a vacuum was utilised first, as this has been reported to provide a good approximation to the geometry of the β-peptides.[20] The structures converged to the corresponding local minimum of the potential energy surface. To take into account the effects of more diffuse basis sets and the electron correlation, the optimisations were performed at the B3LYP/6-311G** level and the vibration circular dichroism (VCD) curves were simulated at this level of theory. The VCD spectra recorded for **1** and **4** in [D_6]DMSO exhibit a well-defined negative couplet between 1650 and 1700 cm^{-1}, which can be assigned to the coupled amide-I vibrational modes with higher contributions from the middle and N-terminal amino acid residues. They show fairly good agreement with the ab initio calculated theoretical VCD curves, especially in reproducing the negative couplet-like feature in the amide-I region; this strongly suggests that the left-handed helical folds have been captured correctly with NMR structure refinements and ab initio modelling. Moreover, the correspondence between the curves is indicative of the conformational stability of the secondary structures (Figure 4). The higher intensity of the amide-I VCD couplet seen in the case of **4** is compatible with a more regular structure of the H12 helix as compared to the H10/12 helix proposed for **1**. There are, however, some discrepancies between the calcu-

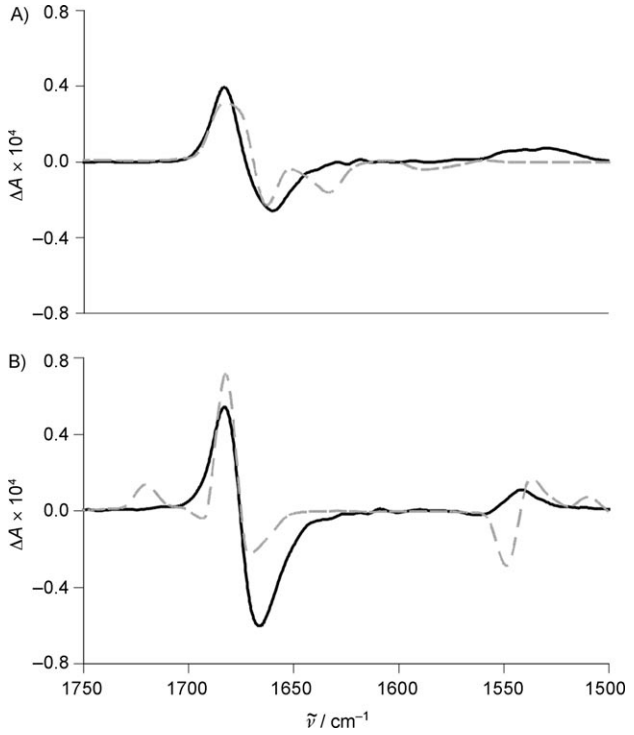

Figure 4. VCD curves for A) **1** and B) **4**, measured in [D_6]DMSO (thick black, continuous line), and the calculated results (dashed grey line, given in scaled VCD units).

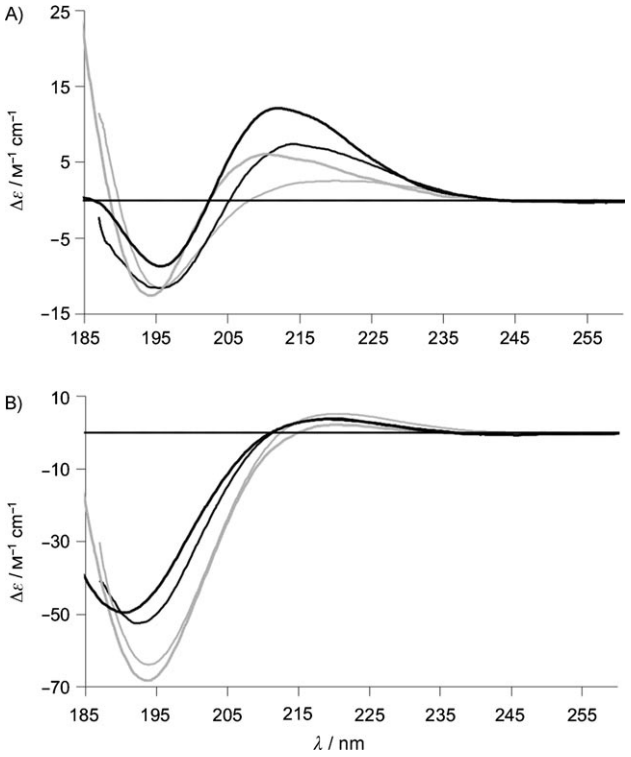

Figure 5. ECD curves measured in aqueous buffer with pH 7.4 (thick curves) and in MeOH (thin curves). A) **1** (grey) and **4** (black); B) **2** (black) and **3** (grey).

lated and experimental VCD curves, such as some additional high- or low-wavenumber amide-I features in the calculated spectra and differences in the less characteristic and rather weak amide-II region between 1500 and 1570 cm^{-1}. (This is mostly due to the coupled in-plane bending vibrations of the amide NH bonds). These differences may result from small deviations from the calculated geometries, especially at the more flexible C-terminal part of the helices (note that VCD is very sensitive to small deviations in the dihedral angles), as well as from specific interactions with the solvent molecules, as recently shown in the case of Ac-Ala-NHMe by comparative VCD studies in organic solvents and solid noble gas matrices.[21]

Electronic circular dichroism (ECD) measurements were carried out to gain supporting evidence for the presence of the structures in aqueous buffers and MeOH solutions (Figure 5). Symmetric positive Cotton effects were found for both **1** and **4**. The positive bands at around 210 nm and the negative bands at around 195 nm indicate their helix geometry. For both **2** and **3**, the largely asymmetric features with the negative bands at around 190 nm indicate the presence of elongated strand-like structures.[14] In line with the NMR findings, the ECD curves in an aqueous medium are similar to those in MeOH; this suggests the presence of highly stable secondary structures that can withstand the destructuring effect of the water. Moreover, the ECD curves of the helical foldamers (**1** and **4**) recorded at physiological and acidic pH were virtually identical (Figure 6). This is in agreement with NMR findings and indicates that the studied

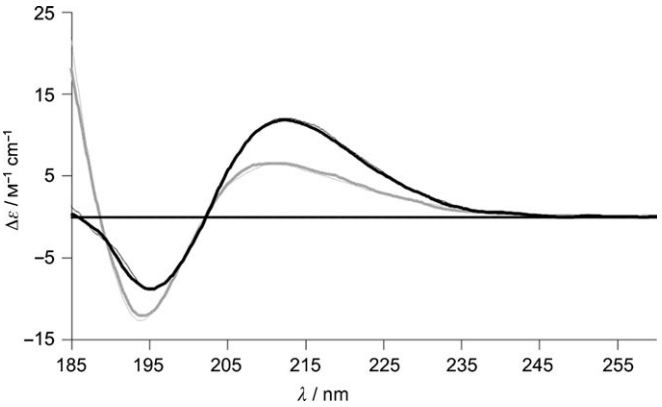

Figure 6. ECD curves of **1** (grey) and **4** (black) measured in buffers with pH 7.4 (thick curves) or pH 3 (thin curves).

helices do not undergo protonation-induced unfolding even at pH 2.0.

The effects of the incorporation of aza-ACPC building blocks into the β-peptide chain were tested. The NMR structural refinements based on the ROESY data revealed that the secondary structure type enforced by the stereochemical pattern along the backbone is well accommodated by the azapeptide moieties. The alternating stereochemical pattern with *cis*-ACPC residues in the sequence induced the H10/12 helix (**1**), while the homochiral sequence with *trans*-ACPC monomers gave rise to the H12 helix (**4**). The existence of the helical folds is strongly supported by the ECD

and the VCD findings. Interestingly, the incorporation of the aza-ACPC building blocks in an alternating manner prevented the i–$i+1$ sequential H-bonding interactions observed in the X-ray structures of the homo-oligo-azapeptides,[7] and thereby facilitated formation of the helix for **1** and **4**. The long-range NOE interactions for these geometries were also found in water, and the ECD intensities were not decreased in an aqueous medium; this indicates that they are highly stable, even in a destructuring solvent. Although elevated proton concentration resulted in protonated backbones for both **1** and **4**, no signs of unfolding were observed. It is likely that the free nitrogen lone pairs are accessible, and the positive charges are shielded by the solvent and the counter ions. While the homochiral *cis*-ACPC and the heterochiral alternating *trans*-ACPC oligomers exhibited fibril-forming strands,[22] **2** and **3** displayed a propensity for the formation of intramolecular H-bonds. The NMR behaviour of the azapeptide-NH resonances strongly suggested that their H-bond acidity is higher than that of the amide protons. This thereby explains the stronger H-bonds, and in turn the secondary structure-stabilising effect of the azapeptide moiety.

Besides their stabilisation effect, the aza-ACPC residues increased the water solubilities of the foldameric structures over a wide pH range; this underlines the future applicability of these building blocks in foldamer design, especially in the field of bioactive foldamers.

Experimental Section

Peptide synthesis: The synthesised hybrid oligomers were numbered as follows: H-[(1*S*,2*R*)-ACPC-2*S*-aza-ACPC]$_3$-NH_2 (**1**), H-[(1*R*,2*S*)-ACPC-2*S*-aza-ACPC]$_3$-NH_2 (**2**) H-[(1*S*,2S)-ACPC-2*S*-aza-ACPC]$_3$-NH_2 (**3**) and H-[(1*R*,2*R*)-ACPC-2*S*-aza-ACPC]$_3$-NH_2 (**4**). The sequences were synthesised by using a solid-phase technique that utilised *t*Boc chemistry.[23] The 2*S*-aza-ACPC (L-*N*-aminoproline) was synthesised in the following manner: 1-nitrosoproline was prepared from L-proline by using a standard literature procedure (3 M sulfuric acid and sodium nitrite).[16d] The resulting 1-nitrosoproline was dissolved in acetic acid (50 *v*/*v* %) and was reduced with zinc dust. The resulting compound was Boc protected in sodium hydrocarbonate solution by reaction with di-*tert*-butyl dicarbonate. The peptide chains were elongated on an MBHA resin (1.03 mmol g^{-1}) and the syntheses were carried out manually. Couplings of the ACPC and aza-ACPC isomers were performed with DCC/HOBt, without difficulties. The incorporation of hydrazino building blocks with an unprotected alpha nitrogen usually leads to numerous side reactions,[24] but in our case the imino nitrogen avoided these side reactions. The N-terminal acetylation was achieved with Ac_2O (20 % in DCM). The completed peptide resins were treated with liquid HF/dimethyl sulfide/*p*-cresol/*p*-thiocresol (86:6:4:2, *v*/*v*) at 0 °C for 1 h. The HF was then removed and the resulting free peptides were solubilised in aqueous acetic acid (10 %), filtered and lyophilised. The crude peptides were investigated by RP-HPLC on a Phenomenex Jupiter C18 column (4.6×250 mm). The solvent system used was as follows: TFA (0.1 %) in water, acetonitrile (80 %) in water, gradient: 25 %→45 % in 15 min, flow rate 1.2 mL min^{-1}, detection at 220 nm. The model peptides were characterised by mass spectrometry with a Finnigan TSQ 7000 tandem quadrupole mass spectrometer equipped with an electrospray ion source. The above peptides were purified on an HPLC system on a 21.2 mm semipreparative column.

NMR experiments: NMR measurements were performed on Bruker AV600 spectrometers with a multinuclear probe with a z-gradient coil in 4 mM CD_3OD or [D_6]DMSO at 298 K, and in aqueous phosphate buffer solutions at 277 K and 298 K. The ROESY measurements were carried out with a WATERGATE solvent suppression scheme. For the ROESY spinlock, mixing times of 225 and 400 ms were used; the number of scans was 64. The TOCSY measurements were made with homonuclear Hartman–Hahn transfer with the MLEV17 sequence, with a mixing time of 80 ms; the number of scans was 32. For all the 2D spectra, 2024 time domain points and 512 increments were applied. The processing was carried out by using a cosine-bell window function, with single zero filling and automatic baseline correction. The PFGSE NMR measurements were performed by using the stimulated echo and longitudinal eddy current delay (LED) sequence with water suppression. A time of 2 ms was used for the dephasing/refocusing gradient pulse length (δ), and 250 ms for the diffusion delay (Δ). The gradient strength was changed quadratically (from 5 % to 95 % of the maximum value B-AFPA 10 A gradient amplifier), and the number of steps was 32. Each measurement was run with 256 scans and 2 K time domain points. For the processing, an exponential window function and single zero filling were applied. During the diffusion measurements, the fluctuation of the temperature was less than 0.1 K. Prior to the NMR scans, all the samples were equilibrated for 30 min. The aggregation numbers were calculated from the Stokes-Einstein equation and lactose was utilised as an external volume standard.

VCD experiments: VCD spectra at a resolution of 4 cm^{-1} were recorded in DCM and [D_6]DMSO solutions with a Bruker PMA 37 VCD/PM-IRRAS module connected to an Equinox 55 FTIR spectrometer. The ZnSe photoelastic modulator of the instrument was set to 1600 cm^{-1}, and an optical filter with the transmission range 1960–1250 cm^{-1} was used in order to increase the sensitivity in the carbonyl region. The instrument was calibrated for VCD intensity with a CdS multiplewave plate. A CaF_2 cell with a path length of 0.207 mm and a sample concentration of 10 mg mL^{-1} were used. VCD spectra were obtained as averages of 25000 scans, corresponding to a measurement time of 7 h. Baseline correction was achieved by subtracting the spectrum of the solvent obtained under the same conditions.

CD measurements: CD spectra were measured on a Jasco J810 instrument at 25 °C in a 0.02 cm cell. Eight spectra were accumulated for each sample. The baseline spectrum recorded with the solvent only was subtracted from the raw data. The concentration of the sample solutions was 4 mM in CD_3OH and 1 mM in the aqueous buffers.

Molecular mechanics calculations: Molecular mechanical simulations were carried out in the Molecular Operating Environment (MOE) of the Chemical Computing Group. For the energy calculations, the MMFF94x force field was used, without a cut-off for van der Waals and Coulomb interactions, and the distance-dependent dielectric constant (ε_r) was set to $\varepsilon = 1.8$ (corresponding to CH_3OH). For the protonated backbones, the implicit water model of GB/VI (Generalized Born) was applied. The conformational sampling was carried out by using the hybrid MCMD simulation (as implemented in MOE) at 300 K with a random MC sampling step after every ten MD steps. The MC-MD was run with a step size of 2 fs for 20 ns, and the conformations were saved after every 1000 MD steps, and this resulted in 10^4 structures. For the NMR-restrained simulation, the upper distance limits were calculated by using the isolated spin pair approximation and classified by following the standard method (strong: 2.5 Å, medium: 3.5 Å, and weak: 5.0 Å). The lower limit was set to 1.8 Å. Restraints were applied as a flat-bottomed quadratic penalty term with a force constant of 5 kcal Å^{-2}. The final conformations were minimised to a gradient of 0.05 kcal mol^{-1} and the minimisation was applied in a cascade manner, using the steepest-descent, conjugate gradient and truncated Newton algorithm.

Ab initio calculations: The optimisations were carried out in two steps with the Gaussian 03 program: first by using the HF/3–21G basis set, and then by using density-functional theory at the B3LYP/6–311G** level with a default setup. For the protonated models, the level of B3LYP/6–31G* with PCM water model was utilised. The theoretical VCD spectra of **1** and **4** were calculated with the Gaussian 03 program at the B3LYP/6–31G* level of theory, for geometry optimised in vacuum at the same

level. The calculated vibrational frequencies were scaled by a factor of 0.963. VCD curves were simulated from the calculated wavenumber and rotatory strength data by using Lorentzian band shape and a half-width at a half-height value of 6 cm^{-1}.

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Keywords: amino acids · chirality · foldamers · peptides · peptidomimetics

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