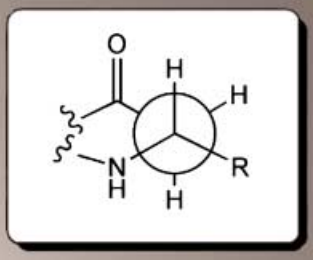
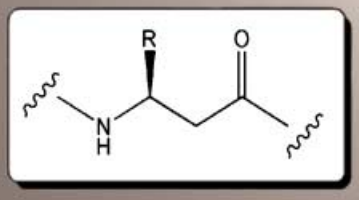


β-peptidic helices made in old and new ETH laboratories

Salt bridges stabilize helix in protic solvents (MeOH, H_2O)



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Design, Synthesis and Structural Investigations of a β-Peptide Forming a 3_{14}-Helix Stabilized by Electrostatic Interactions

Magnus Rueping, Yogesh R. Mahajan, Bernhard Jaun, and Dieter Seebach*[a]

Abstract: Two different strategies have been employed for the synthesis of Fmoc-protected β^3-homoarginine; the Arndt–Eistert homologation of α-arginine and the guanidinylation of β^3-homoornithine. Solid-phase β-peptide synthesis was used for the preparation of β-heptapeptide **1**, which was designed to form a helix stabilized by electrostatic interactions through positively (β^3hArg) and negatively charged (β^3hGlu) amino acid residues. CD measurements and corresponding NMR investigations in MeOH and aqueous solutions do indeed show that the β-peptidic 3_{14}-helix can be stabilized by salt-bridge formation.

Keywords: β-peptides · electrostatic interactions · helical structures · NMR spectroscopy · salt bridges

Introduction

Electrostatic interactions are important in many biological processes, such as enzyme catalysis, protein–protein and protein–nucleic acid binding, protein folding, flexibility and stability. Close range electrostatic interactions, that is, salt-bridges and their networks have been shown to contribute to peptide and protein stability.[1] Salt bridges are formed by amino acid residues with opposite charges (Asp or Glu with Arg, Orn, Lys or His), which are often near each other in the amino acid sequence. The strength of a salt bridge is determined by the geometry and distance of interaction, degree of exposure to solvent, and effects of neighboring charged residues. Many charged residues that form salt-bridges are found in α-helical conformations where the oppositely charged side chains occur one turn apart on the same face of the helix in *i* and *i*+3,4 positions.

Various synthetic oligomers with conformations similar to those in natural peptides and proteins have recently been studied to increase our understanding of protein folding and stability.[2] Especially, β- and γ-peptides (peptides consisting of chiral β- and γ-amino acid residues) have received considerable attention.[2,3] These peptides have several attractive characteristics: they are structurally related to the ubiquitous α-peptides; they can be designed to fold into secondary structures, such as helices,[4,5] turns[6] and sheets;[4,6a] and they are resistant to proteolytic degradation by common proteases and peptidases,[7] suggesting that they might be useful peptidomimetics.

Short β-peptides consisting solely of β^3-amino acids, that are derived from natural L-amino acids via Arndt–Eistert homologation, have been shown to fold into left-handed 3_{14}-helical structures with the side chains of residues *i* and *i*+3 in juxtapositions at a distance of approximately 5 Å.[3] The role of specific side-chain interactions of residues *i* and *i*+3 in stabilizing the helical structure of β-peptide 3_{14}-helices has already been addressed. Previously, we showed, that introduction of a conformational constraint by covalently linking β^3hCys side chains in *i* and *i*+3 positions stabilizes the 3_{14}-helix.[8] In addition, we and others were able to demonstrate that polar side chains, as well as properly chosen charged side chains of β-amino acids can lead to helical conformations in aqueous solution.[9] However, these results relied mainly on CD-spectroscopic measurements,[9c–e] which are not always incisive.[10]

Now, we describe a combined and detailed CD- and NMR-spectroscopic structural analysis of a β-heptapeptide **1** designed to be stabilized by electrostatic interactions (Figure 1).

We selected β-peptide **1**, an all-β^3-peptide, which is expected to form a 3_{14}-helix, and introduced negatively and positively charged residues in *i* and *i*+3 positions to allow electrostatic interactions. The potential salt bridges are established by β^3hGlu (residues 2 and 6) and β^3hArg (residues 3 and 5). β^3hArg was specifically chosen, rather than β^3hOrn or β^3hLys, as the guanidinium group is known to play an important role in many organic and biological proc-

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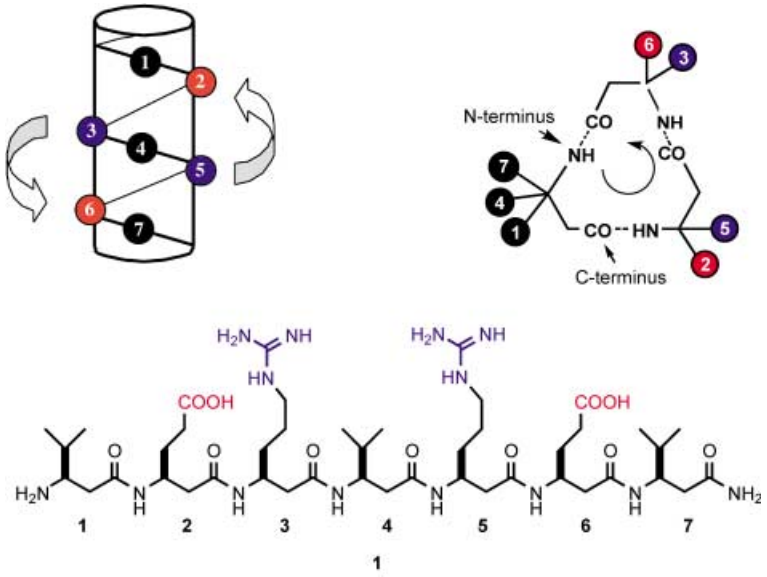


Figure 1. Schematic representation of a 3_{14}-helical structure of β^3-heptapeptide **1** from the side and top. Color code: black = hydrophobic residues (β^3hVal), red = negatively charged residues (β^3hVal) and blue = positively charged residues (β^3hArg).

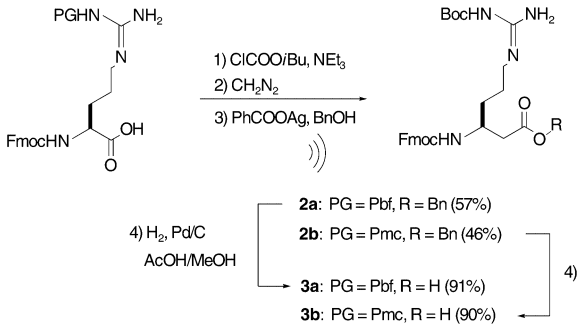
esses involving the binding of negatively charged substrates for example, synthetic molecular receptors,[11] protein–nucleic acid interactions,[12] DNA/RNA recognition and cellular uptake.[13,14] Furthermore, the guanidinium group is basic (pK_a ~12, that is, the positive charge is maintained over a wide pH range), planar, and demonstrates directionality in hydrogen-bonding interactions. The remaining three β^3-amino acid residues of heptapeptide **1** were provided by βhVal residues to facilitate helix formation by hydrophobic interactions. In addition the C-terminus was amidated to prevent interactions between the otherwise free carboxylic acid and the βhArg residues.

Results and Discussion

Preparation of β^3-homoarginine derivatives: The trifunctional guanidine group displays a strong nucleophilic character, and thus if it is improperly protected, side reactions occur such as intramolecular cyclisation to δ-lactam derivatives, acylation followed by decomposition to ornithine, and intramolecular cyclisation to 4-carboxy-2-imino-1,3-diazacycloheptane derivatives.[15,16] Many different protecting groups based on nitro, urethane, arylsulphonyl or aryl derivatives have been proposed, but so far none of them satisfies the basic requirements for an ideal protecting group, that is, robust protection to prevent undesired side reactions, and clean and smooth removal under mild conditions.[16] Nevertheless, acid-labile protections, such as $N^{\omega},N^{\omega'}$-bis(Boc), N^{ω}-Pmc[17] or N^{ω}-Pbf[18] are reported to be amenable to solid-phase peptide synthesis using the Fmoc strategy.

For the synthesis of β^3-homoarginine, two different approaches were chosen. The first approach involves the use of the Arndt–Eistert homologation method which has been successfully applied to the synthesis of various β^3-amino acids starting from the corresponding α-amino acids[19,20] including N^{α}-Boc- and N^{α}-Z-protected arginine derivatives.[21] The second approach relies on the guanidinylation[22] of a suitably protected ornithine derivative, a strategy that has long been used in α-peptide chemistry to synthesize arginine-containing peptides from the appropriate ornithine-containing precursors.[23]

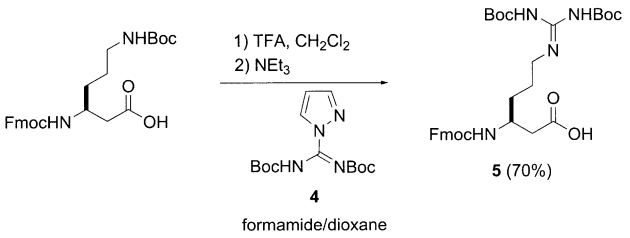
In accordance with the Arndt–Eistert homologation procedure of N^{α}-Fmoc-protected α-amino acids developed in our group,[20] the mixed anhydrides of commercially available N^{α}-Fmoc-protected α-arginine, with either a Pmc or Pbf protecting group on the N^{ω}-position, were converted to the corresponding Fmoc-protected diazoketones which were subsequently homologated using a modification of the base-free, silver(I)-catalysed, ultrasound-promoted Wolff rearrangement protocol of Sewald and coworkers.[24] In a typical homologation procedure used, the diazoketones were ultrasonicated in the presence of catalytic amounts of PhCOOAg and BnOH to provide the benzyl esters **2a** and **2b** in moderate yields (57 and 46%, respectively over two steps). Finally, debenzylation (H_2, Pd/C) of the fully protected β-amino acids **2** furnished the desired compounds, Fmoc-β^3hArg$^{\omega}$(Pbf)-OH **3a** and Fmoc-β^3hArg$^{\omega}$(Pmc)-OH[25] **3b** in 91 and 90%, respectively (Scheme 1).



Scheme 1. Synthesis of β^3-homoarginine derivatives via Arndt–Eistert homologation of the corresponding α-amino acids.

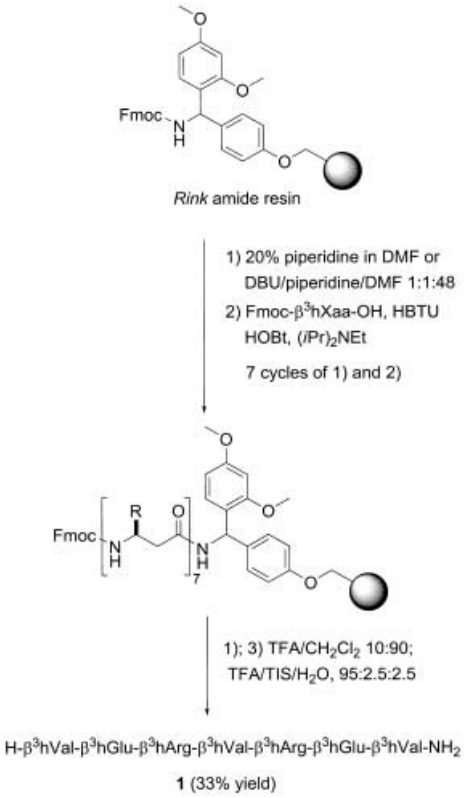
Fmoc-β^3hOrn(Boc)-OH, which was required for the ornithine-to-arginine transformation, was obtained by homologation of Fmoc-(*S*)-Orn(Boc)-OH using the Arndt–Eistert reaction[20] in combination with the ultrasound-promoted Wolff rearrangement. Fmoc-β^3hOrn(Boc)-OH was Boc-deprotected (TFA) and subsequently treated with *N*,*N*′-bis(Boc)-1-amidinopyrazole[26] (**4**) under basic conditions

(Et_3N) in formamide/dioxane to afford Fmoc-β^3hArg$^{\omega,\omega'}$-$(Boc)_2$-OH **5** in 70% yield over two steps (Scheme 2).



Scheme 2. Guanidinylation of Fmoc-β^3hOrn(TFA)-OH using *N*,*N*′-bis(Boc)-1-amidinopyrazole **4**.

Synthesis of β^3-heptapeptide 1: β-Peptide **1** was envisaged to be synthesized by coupling Fmoc-protected-amino acids on a Rink amide resin.[27] The Fmoc-protected amino groups on the Rink amide resin were liberated upon treatment with 20% piperidine in DMF. Anchoring of the first amino acid was achieved by reacting the resin with Fmoc-β^3-amino acids (3–5 equiv with respect to the resin loading) activated by HBTU/HOBt/(iPr)$_2$NEt at RT in DMF for 2–4 h. Completion of the coupling was confirmed by a TNBS test.[28] For elongating the peptide chain, the Fmoc-protecting group of the anchored amino acid was removed using a combination of 20% piperidine in DMF and DBU/piperidine/DMF 1:1:48. The amino acids were coupled under conditions similar to those used for anchoring, namely HBTU/HOBt/(iPr)$_2$NEt. The deprotection and coupling cycles were repeated six times in total to furnish the Fmoc-protected β-heptapeptides on the resin. The Fmoc-protecting group was comfortably removed from the resin-bound β-peptide **1** using the standard DBU/piperidine protocol. Subsequently, the resin-bound peptide was cleaved off the resin with 10% TFA in CH_2Cl_2, and the side-chain-protected peptide thus obtained was further subjected to acidolysis in the presence of a scavenger (TFA/(iPr)$_3$SiH/H_2O 95:2.5:2.5) to afford the crude β-peptide **1**. After preparative HPLC, the β-peptide **1** (33% yield) was isolated in >98% purity (Scheme 3).



Scheme 3. Solid-phase synthesis of β-heptapeptide **1** on a Rink amide resin.

Structural Analysis

CD-Spectroscopic measurements: Circular dichroism spectroscopy is a frequently used method for analyzing α-peptidic structures. Although, for β-peptides, the correlation between CD pattern and secondary structure is not yet fully established, for certain β-peptides, CD spectra have been correlated with secondary structures when used in combination with other spectroscopic techniques. Thus, CD measurements and corresponding NMR investigations have established that β^3-peptides forming a 3_{14}-helical structure exhibit a characteristic CD pattern (a negative Cotton effect near 215 nm and zero crossing at ca. 207 nm).[4, 8b, 9a, b, 29] As anticipated, the CD spectra of β^3-heptapeptide **1** in MeOH and aqueous solution display a pattern characteristic of a 3_{14}-helix (Figure 2a, b). However, the Cotton effect at 215 nm gradually decreases with increasing amount of water, indicating a loss of secondary structure (Figure 2a).

Remarkably, when one compares the mean molar ellipticity of all β^3-heptapeptides synthesized in our laboratory, heptapeptide **1** exhibits the strongest ellipticity in MeOH. Additionally, the pH dependence of the CD spectrum in aqueous solution supports our view that electrostatic inter-

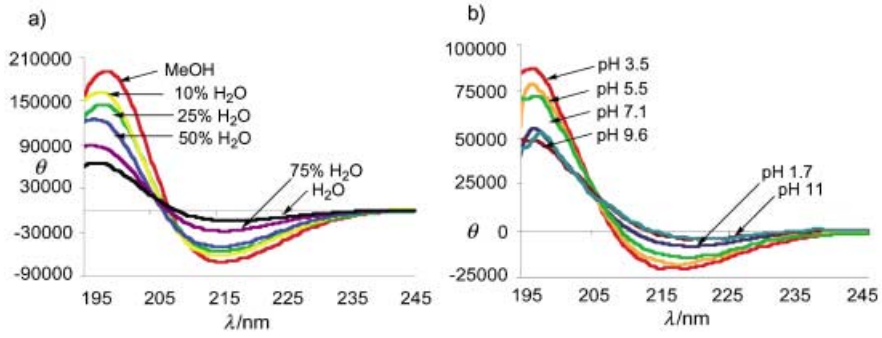


Figure 2. CD Spectra of **1** a) in MeOH and H_2O; b) in H_2O at different pH values. The spectra were recorded at a concentration of 0.2 mM at 20°C and are not normalized to the number of residues.

actions strongly stabilize the helical conformation of peptide **1** (Figure 2b). The ellipticity decreases at pH values above the pK_a values expected for the basic β^{3}hArg side chains and the terminal amino group, and below the pK_a values of the acidic β^{3}hGlu side chains. The strongest molar ellipticity is observed at pH 3.5, the value where the arginine residues are anticipated to be protonated and the glutamic acid residues to be deprotonated (Figure 2b). These observations clearly indicate that electrostatic interactions can be used to stabilize the β-peptidic 3_{14}-helix, while non salt-bridge-forming β-peptides without any conformational constraints do not show the characteristic CD pattern in aqueous solutions.[29]

NMR Spectroscopy: In order to obtain more detailed information on the secondary structure and its stability towards unfolding, a detailed NMR-spectroscopic investigation of β^3-heptapeptide **1** was carried out. First, we examined peptide **1** in MeOH.[30] The presence of a regular secondary structure was indicated by a large dispersion of the chemical shifts, as well as by the observation of large and small values for the vicinal coupling constants ^3J(H-C(β),H_{ax}-C(α)) and ^3J(H-C(β),H_{la}-C(α)), respectively. The amino acid spin systems were assigned using DQF-COSY and TOCSY techniques, whereas the sequence assignment was derived from $d_{\alpha N}(i,i+1)$ and $d_{N,N}(i,i+1)$ sequential NOEs and HSQC/HMBC correlations. Large ^3J(NH,H−C(β)) coupling constants (~9 Hz) established that the NH and H-C(β) protons are in an antiperiplanar arrangement. The diastereotopic H-C(α) protons were assigned assuming that the axial protons exhibit a large, and the lateral protons a small coupling with H-C(β). This is in agreement with stronger NOEs being observed from H-C(β) to the lateral H_{la}-C(α) protons, compared with the axial H_{ax}-C(α) protons, and with stronger NOEs from NH_{i+1} to the axial H_{ax}-C(α)$_i$ protons.

To determine the three-dimensional structure, ROESY spectra at different mixing times (150, 300 ms) were recorded, the NOE values were extracted, calibrated and classified according to their volume into strong, medium and weak distance categories. Examination of the NOEs and comparison with former β^3-peptides showed that all the characteristic cross-peaks[8a] for a 3_{14}-helical conformation were present for peptide **1** in MeOH. In fact, they were either comparable or even stronger than the corresponding NOEs earlier observed for other β^3-peptides[5e] suggesting that β^3-heptapeptide **1** forms the most stable 3_{14}-helix we have observed to date. The NOE derived distances as well as the dihedral angles derived from coupling constants and NOEs were used in a restrained molecular dynamics simulated annealing protocol.[30] The calculation yielded 20 structures that could be clustered into a well-defined left-handed 3_{14}-helix with side chains of β^{3}hGlu and β^{3}hArg on top of each other, in keeping with the presence of salt-bridges between these charged groups (Figure 3).

Subsequently, β^3-heptapeptide **1** was examined in an aqueous solution. Unfortunately, poor dispersion of the chemical shifts and overlapping resonance signals hampered complete assignment by NMR and at the same time indicated that peptide **1** may adopt a more extended or partially unwound conformation in aqueous solution as also indicated by the CD measurements.[29] In order to obtain information about the transition from a stable and well-defined 3_{14}-helix in MeOH to either a more extended or multiconformational state in aqueous solution, we carried out an NMR titration experiment starting with pure MeOH and adding increasing amounts of water (Figure 4). To our surprise, up to a concentration of ~25% H_2O (*v*/*v*) little change of the amide chemical shifts was observed and the dispersion of the amide and side chain NH chemical shifts actually increased, causing us to wonder whether the 3_{14}-helical structure may be maintained or even further stabilized by the presence of up to 25% (*v*/*v*) of water?

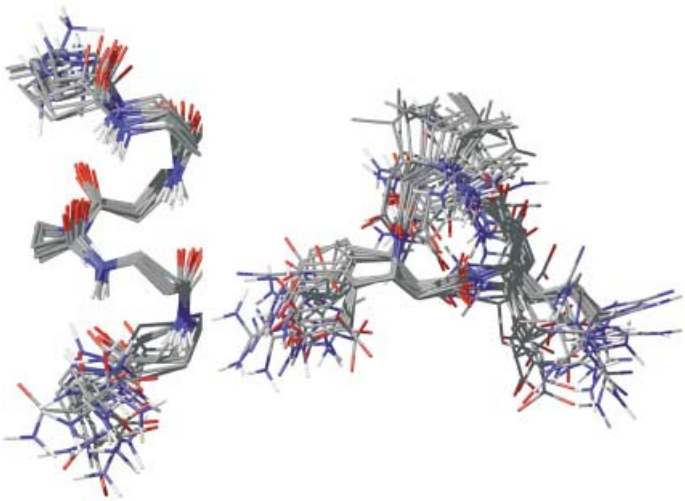


Figure 3. NMR Structure of β-heptapeptide **1** in MeOH. The peptide forms a well-defined 3_{14}-helical structure. Side view without side chains (left) and top view with side chains (right).

Therefore, a detailed NMR structural investigation of peptide **1** in a MeOH/water (3:1) mixture was carried out. The obtained spectra were analyzed along the same lines as described for the MeOH solution.[30] Again, NOEs typical of the 3_{14}-helix are found in the ROESY spectra, but some of these NOEs are considerable weaker than those observed in MeOH. A simulated annealing calculation was carried out to test whether the NMR derived restraints were still consistent with a single conformation. The resulting structures with the lowest restraint violation formed a bundle of structures (Figure 5) that has the shape of a 3_{14}-helical conformation. However, due to the lower number of restraints (NOEs) and lower intensity of the cross-peaks, the 3_{14}-helix is now less well-defined than in MeOH. This indicates that, although the 3_{14}-helix is still present, other conformations become increasingly populated when water is added.

Conclusion

In summary, we have synthesized β^3-homoarginine via two different strategies—method A: Arndt–Eistert homologation of α-arginine and method B: guanidinylation of an appropriately protected β^3-homoornithine derivative—suitable for solid-phase peptide synthesis. Solid-phase β-peptide synthesis was used to prepare β^3-heptapeptide **1**. The Fmoc

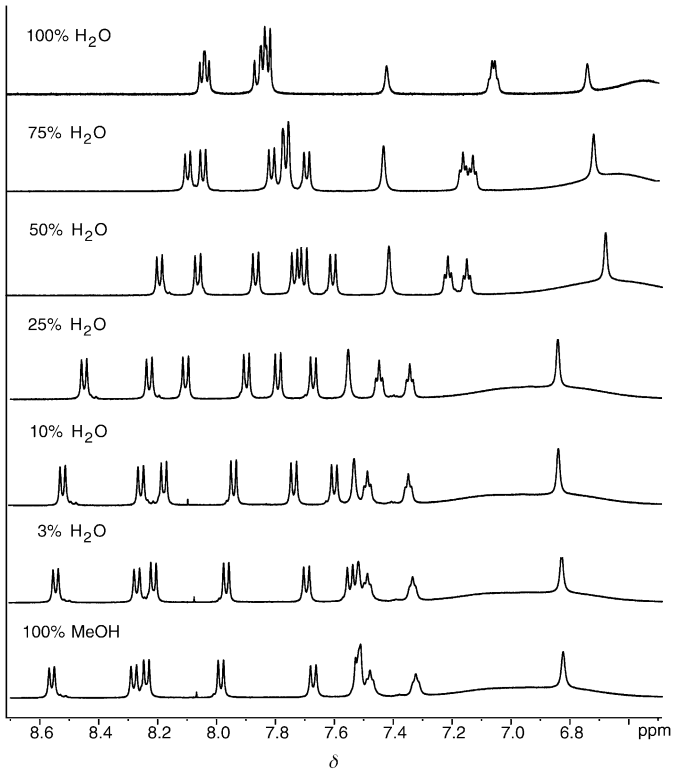


Figure 4. ^{1}H NMR Spectroscopic titration study of a MeOH solution of **1** with water. Up to a water concentration of ~25% (*v*/*v*) no considerable change of the amide chemical shifts can be observed; but the dispersion of the amide and the side-chain NH protons even increases. From 50 to 75% water, the amide protons move together and at 100% water their assignment becomes difficult.

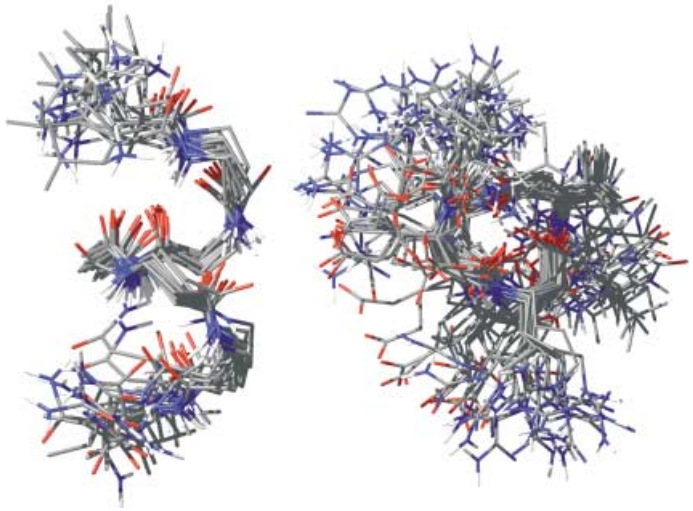


Figure 5. NMR Structure of β-heptapeptide **1** in 3:1 MeOH/H_2O. Side view without side chains (left) and top view with side chains (right).

strategy and the acid labile Rink amide resin allowed a mild cleavage of the final β^3-heptapeptide amide **1**. Based on the strong ellipticity in the CD spectrum and the corresponding NMR-structural investigations we conclude that β^3-heptapeptide adopts a stable 3_{14}-helical conformation in MeOH. The CD- and NMR-titration studies indicate a gradual loss of secondary structure as the water content increases in the medium. This observation suggests that the transition from the folded to the unfolded structures proceeds progressively, which would be in agreement with the proposed non-cooperative folding of β-peptides.[31] Furthermore, it confirms our previous findings that hydrogen-bonding is more important in stabilizing β-peptidic helices than the β-amino acid residues backbone.[32]

The surprisingly increased dispersion of the NH chemical shifts at a water concentration of 25% (*v*/*v*) led us to the first NMR structural analysis of a β-peptide 3_{14}-helical structure in a mixture of methanol and water. This result, in conjunction with the pH-dependent CD measurements, clearly demonstrates that electrostatic interactions can be utilized to stabilize secondary structures, while similar to α-peptides, non salt-bridge-forming peptides without any conformational constraints remain unstructured under these conditions.[29b]

Thus, we believe that these findings are of great importance with respect to our goals of designing β-peptidic tertiary structures, and of investigating the influences of β-peptidic secondary structures on the folding, stability, activity of mixed α/β-peptides and proteins.

Experimental Section

General methods: Starting materials and reagents: THF was distilled from K under an Ar atmosphere prior to use. Solvents for chromatography and workup procedures were distilled over anhydrous $CaSO_4$, P_2O_5 or KOH/$FeSO_4$ (Et_2O). Et_3N and $(i\text{Pr})_2\text{NEt}$ were distilled from CaH_2. Amino acid derivatives were purchased from Novabiochem. All other reagents were used as received from Fluka or Aldrich.

Caution: The generation and handling of CH_2N_2 requires special precautions.[33]

Reactions carried out with the exclusion of light were performed in flasks completely wrapped in aluminium foil. Acronyms: Pbf = 2,2,4,6,7-pentamethyl-dihydrobenzofurane-5-sulfonyl, Pmc = 2,2,5,7,8-pentamethyl-chromane-6-sulfonyl, HBTU = 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, HOBt = 1-hydroxybenzotriazole, NMM = *N*-methyl morpholine, TNBS = 2,4,6-trinitro-benzenesulfonic acid, DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, TFA = trifluoroacetic acid, TIS = triisopropylsilane.

Equipment: Thin-layer chromatography (TLC): silica gel 60 F_{254} plates (Merck); detection with UV and dipping into a solution of "Mo-stain" (25 g phosphomolybdic acid, 10 g $Ce(SO_4)_2{\cdot}H_2O$, 60 mL conc. H_2SO_4 and 940 mL H_2O) followed by heating. Flash column chromatography (FC): silica gel 60 (40–63 μm, Fluka) at 0.2–0.3 bar. Analytical HPLC: Knauer HPLC system (pump type WellChrom K-1000 Maxi-Star, degasser, UV detector (variable-wavelength monitor)), column: Nucleosil 100–5 C_8 (250 × 4 mm, Macherey–Nagel). Preparative HPLC: Merck/Hitachi HPLC system (pump type L-6250, UV detector L-4000) column: Nucleosil 100-7 C_8 (250 × 21 mm, Macherey–Nagel). Optical rotations: Perkin–Elmer 241 polarimeter (10 cm, 1 mL cell) at RT. CD Spectra: Jasco J-710 spectropolarimeter recording from 190 to 250 nm at 20 °C; 1 mm cell; average of 5 scans; peptide concentration 0.2 mM; smoothing was done by Jasco software. Solvents: MeOH (HPLC grade), aq. buffers: pH 1.7, 3.5: 0.1 M AcOK/AcOH, pH 5.7, pH 7.0 and 7.9: 0.1 M KH_2PO_4/K_2HPO_4;[34] pH 9.6 and 11.0: 0.05 M $NaHCO_3$/NaOH.[35] IR Spectra: Perkin–Elmer-782 spectrophotometer. $CHCl_3$ (Fluka) was filtered over Alumina N, Akt. I (ICN Biomedicals GmbH, Germany) before use. NMR Spectra: Bruker AMX 500 (^{1}H: 500 MHz, ^{13}C: 125 MHz) or Varian Gemini 300 or Varian Mercury 300 (^{1}H: 300 MHz, ^{13}C: 75 MHz); chemical shifts δ in ppm downfield from internal Me_4Si ($\delta = 0$). MS: IonSpec Ultima (MALDI FT-MS, high resolution MS (HRMS), in a 2,5-dihydroxy-

benzoic acid (DHB) matrix). Elemental analyses were performed by the Microanalytical Laboratory, Laboratorium für Organische Chemie, ETH-Zürich.

Reversed-phase (RP) HPLC analysis and purification: RP-HPLC Analysis was performed on a Nucleosil 100-5 C_8 column (250×4 mm, Macherey–Nagel) with a linear gradient of A (0.1% TFA in H_2O) and B (MeCN) at a flow rate of 1 mL min^{-1} (Knauer HPLC system); UV detection at 220 nm; t_R in min. RP-HPLC purification was performed on a Nucleosil 100-7 C_8 column (250×21 mm, Macherey–Nagel) with a linear gradient of A and B at a flow rate of 20 mL min^{-1} (Merck/Hitachi system), UV detection at 215 nm.

Heptapeptide 1: The Rink amide resin[27] (410 mg, 0.25 mmol; loading 0.61 mmol g^{-1}) was swelled in DMF (6 mL) for 30 min and Fmoc deprotected using 20% piperidine in DMF (6 mL, 3×20 min) under Ar bubbling. A solution of Fmoc-β^{3}hVal-OH (4.0 equiv), HBTU (3.8 equiv) and HOBt (4.0 equiv) in DMF (4 mL) and (iPr)$_2$NEt (7.8 equiv) were added successively to the resin and the suspension was mixed for 1–2 h by Ar bubbling. The coupling was monitored with TNBS test.[28] The resin was then filtered and washed with DMF (6 mL, 6×1 min) prior to the following Fmoc deprotection step. By measuring the absorbance of the benzofulvene/piperidine adduct the loading was determined to be 82%. The Fmoc group was removed using 20% piperidine in DMF (6 mL, 2× 10 min), DBU/piperidine/DMF 1:1:48 (6 mL, 3×10 min), 20% piperidine in DMF (6 mL, 10 min) under Ar bubbling. The resin was filtered and washed with DMF (50 mL mmol^{-1}, 6×3 min). For each coupling step, a solution of the Fmoc-β^3-amino acid (4/5 equiv), HBTU (3.8/4.8 equiv) and HOBt (4/5 equiv) in DMF (4 mL) and (iPr)$_2$EtN (7.8/9.6 equiv) were added successively to the resin and the suspension was mixed by Ar bubbling for 1–2 h. In case of a positive TNBS test (indicating incomplete coupling), the suspension was allowed to react further for 1–2 h. The resin was then filtered and washed with DMF (6 mL, 6×1 min). After coupling the last amino acid, the Fmoc group was cleaved and the resin washed with DMF (6 mL, 6×1 min), DCM (6 mL, 6×1 min) and MeOH (6 mL, 3×1 min). Drying overnight under *hv* afforded the Fmoc-deprotected peptide-resin (568 mg).

The dry Fmoc-deprotected peptide-resin (160 mg) was treated with a mixture of CH_2Cl_2/TFA/TIS 90:9:1 (5×3 mL), allowing the solvent to pass through the resin bed slowly. Excess TFA/CH_2Cl_2 was evaporated and the side-chain protecting groups removed by stirring the oily residue in TFA/TIS/H_2O 95:2.5:2.5 for 3 h. The solvent was evaporated, co-evaporated with CH_2Cl_2, to yield an oily residue. The precipitate formed upon addition of cold Et_2O to the oily residue was separated by decanting the solvent. The precipitate was dissolved in H_2O/dioxane solution and lyophilized to yield 94 mg of the crude peptide. Purification of the crude peptide by RP-HPLC (10–20% B in 40 min) afforded the TFA salt of **1** (19 mg, 33%) as a white fluffy solid. Analytical RP-HPLC (17–27% B in 40 min) t_R 38.7 min, purity >98%. ^{1}H NMR (500 MHz, D_2O/H_2O 1:9): δ=0.87 (d, J(H,H)=6.7 Hz, 6H; Me), 0.88 (d, J(H,H)=6.5, 6H; Me), 0.99 (d, J(H,H)=6.8, 3H; Me), 1.00 (d, J(H,H)=6.9 Hz, 3H; Me), 1.44–1.66 (m, 9H), 1.68–1.79 (m, 4H), 1.83–1.88 (m, 2H), 1.91–1.99 (m, 1H), 2.26–2.58 (m, 17H; 8 CH_2CO, CHHCO), 2.69 (dd, J(H,H)=16.2, 4.5 Hz, 1H; CHHCO), 3.17–3.18 (m, 4H; CH_2N), 3.44–3.48 (m, 1H), 3.53–3.54 (m, 1H; CHN), 4.03–4.09 (m, 2H; CHN), 4.16–4.22 (m, 4H; CHN), 6.83 (s, 1H; NH), 6.64 (brs, 7H), 7.15 (brs, 2H, 2NH), 7.52 (s, 1H; NH), 7.19–7.96 (m, 3H; 4NH), 7.96 (d, J(H,H)=10 Hz, 1H; NH), 8.12 (d, J(H,H)=9.7 Hz, 1H; NH), 8.14 (d, J(H,H)=9.6 Hz, 1H; NH); ^{13}C NMR (125 MHz, D_2O/H_2O 1:9): δ=19.9, 20.0, 20.9, 21.1, 27.3, 27.9, 28.1, 31.9, 32.7, 33.6, 33.7, 34.6, 37.3, 40.4, 41.1, 49.3, 49.4, 49.5, 55.1, 55.2, 57.1, 61.2, 72.7, 159.5, 159.6, 165.5, 165.8, 174.6, 174.9, 175.1, 175.8, 179.7, 180.8; IR (KBr): $\tilde{\nu}$ =3307 (m), 2969 (w), 1654 (s), 1560 (w), 1438 (w), 1207 (s), 1136 (s), 982 (w), 841 (w), 801 (m), 723 (m), 602 (w), 518 (w), 418 cm^{-1} (w); MS (MALDI): m/z (%): 1006.6 (10), 1005.6 (19) [M+Na]$^+$, 985.6 (16), 984.6 (54), 983.6 (100) [M+H]$^+$, 967.6 (10), 966.6 (20), 941.6 (10); HRMS: calcd for [$C_{44}H_{83}N_{14}O_{11}$]$^+$: 983.6360; found 983.6349.

Compound 2a: Fmoc-(*S*)-Arg(Pbf)-OH (9.73 g, 15.00 mmol) was dissolved in THF (43 mL) under Ar and cooled to −20 °C. After addition of $ClCO_2i$Bu (2.04 mL, 15.75 mmol) and NMM (1.74 mL, 15.75 mmol), the mixture was stirred at −20 °C for 30 min. The resulting white suspension was allowed to warm up to −5 °C and a solution of CH_2N_2 in Et_2O was added until the rich yellow colour persisted. Stirring was continued for 4 h as the mixture was allowed to warm to RT. Excess CH_2N_2 was destroyed by vigorous stirring. The mixture was then diluted with Et_2O and washed with sat. aq. $NaHCO_3$, 1 N HCl, and brine. The organic phase was dried ($MgSO_4$) and concentrated under reduced pressure. FC (EtOAc/hexane 8:2→9:1) afforded Fmoc-(*S*)-Arg(Pbf)-CHN$_2$ (6.73 g, 63%) as a yellow foam. R_f=0.3 (EtOAc/hexane 9:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ=1.42 (s, 6H; Me), 1.47–1.62 (m, 3H; CH_2), 1.80–1.85 (m, 1H; CH_2), 2.06 (s, 3H; Me), 2.49 (s, 3H; Me), 2.57 (s, 3H; Me), 2.90 (s, 2H; CH_2), 3.22 (brm, 2H; CH_2N), 4.11–4.16 (m, 2H; CHCH$_2$O, CHN), 4.30–4.42 (m, 2H; CHCH_2O), 5.53 (s, 1H; CHN_2), 5.99 (d, J(H,H)=8.1 Hz, 1H; NHFmoc), 6.23 (brs, 3H; NH), 7.25–7.33 (m, 2H; arom.), 7.36 (t, J(H,H)=7.5 Hz, 2H; arom.), 7.55 (d, J(H,H)=7.2 Hz, 2H; arom.), 7.72 (d, J(H,H)=7.5 Hz, 2H; arom.); ^{13}C NMR (75 MHz, $CDCl_3$): δ=12.6, 18.1, 19.4, 25.3, 28.7, 29.9, 40.8, 43.3, 47.2, 66.9, 86.4, 117.5, 119.9, 124.6, 124.9, 126.9, 127.6, 132.2, 132.5, 138.2, 141.2, 143.5, 156.1, 156.3, 158.7; IR ($CHCl_3$): $\tilde{\nu}$ = 3432 (w), 3347 (w), 3007 (w), 2977 (w), 2111 (s), 1719 (s), 1624 (s), 1556 (s), 1508 (m), 1451 (m), 1370 (s), 1150 (m), 1105 (s), 1035 (w), 852 (w), 658 cm^{-1} (w); MS (MALDI): m/z (%): 822 (11), 821 (25), 695 (9) [M+Na]$^+$, 686 (15), 685 (35), 683 (15), 670 (12), 669 (33), 668 (35), 667 (90), 663 (11), 646 (16), 645 (38), 548 (13), 547 (40), 529 (22), 467 (10), 457 (11), 421 (18), 411 (33), 395 (22), 394 (14), 393 (59), 389 (16), 374 (22), 373 (100), 277 (17), 273 (16), 199 (15); HRMS: calcd for [$C_{35}H_{40}N_6O_6SNa$]$^+$: 695.2622; found: 695.2621.

$PhCO_2Ag$ (0.12 g, 0.5 mmol) was added to a solution of Fmoc-(*S*)-Arg(Pbf)-CHN$_2$ (3.36 g, 5.0 mmol) in THF/BnOH (8.5:1.5, 8.0 mL). The resulting mixture was ultrasonicated for 4 h in the dark at RT. After removing the bulk of THF under reduced pressure, the residue was dissolved in EtOAc and washed with sat. aq. $Na_2S_2O_3$ (2×), sat. aq. $NaHCO_3$ (2×), sat. aq. NH_4Cl solutions and brine. The organic phase was dried ($MgSO_4$) and concentrated under reduced pressure. FC (EtOAc/pentane 1:1 → 7:3) afforded **2a** (3.44 g, 91%) as white foam. R_f=0.22 (EtOAc/pentane 3:1); $[\alpha]_D^{RT}$=−12.4 (c=1.00 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ=1.42 (s, 6H; 2Me), 1.42–1.63 (m, 3H), 1.66 (brm, 1H), 2.06 (s, 3H; Me), 2.49 (s, 3H; Me), 2.49–2.57 (m, 2H; CH_2COOBn), 2.57 (s, 3H; Me), 2.89 (s, 3H; Me), 3.19 (m, 2H; CH_2N), 3.96 (brs, 1H; CHNHFmoc), 4.13 (t, J(H,H)=7.2 Hz, 1H; CHCH$_2$O), 4.34 (d, J(H,H)=6.2 Hz, 2H; CHCH_2O), 5.09 (s, 2H; CH_2Ph), 5.48 (d, J(H,H)=9.0 Hz, 1H; NHFmoc), 6.05 (brs, 3H; 3NH), 7.23–7.39 (m, 9H), 7.55 (d, J(H,H)=7.5 Hz, 2H), 7.73 (d, J(H,H)=7.5 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=12.6, 18.0, 19.4, 25.6, 28.7, 32.1, 39.6, 40.9, 43.3, 47.3, 47.5, 66.6, 66.8, 86.3, 117.4, 119.9, 124.5, 126.9, 127.6, 128.2, 128.3, 128.5, 132.2, 132.9, 135.4, 138.2, 141.2, 143.6, 143.7, 155.9, 156.3, 158.6, 171.0; IR ($CHCl_3$): $\tilde{\nu}$=3621 (w), 3430 (w), 2976 (m), 1723 (m), 1621 (m), 1558 (m), 1514 (m), 1451 (m), 1390 (w), 1248 (m), 1106 (m), 1046 (m), 877 (w), 658 cm^{-1} (w); MS (MALDI): m/z (%): 776 (15), 775 (29) [M+Na]$^+$, 502 (33), 501 (100) [M−Pbf+2H]$^+$, 305 (40); elemental analysis calcd (%) for $C_{42}H_{48}N_4O_7S$ (752.9): C 67.00, H 6.43, N 7.44; found: 66.75, H 6.49, N 7.33.

Compound 2b: Fmoc-(*S*)-Arg(Pmc)-CHN$_2$ was synthesized from Fmoc-(*S*)-Arg(Pmc)-OH (9.94 g, 15.0 mmol) in a procedure analogous to that of Fmoc-(*S*)-Arg(Pbf)-CHN$_2$. FC (EtOAc/hexane 8:2→9:1) afforded Fmoc-(*S*)-Arg(Pmc)-CHN$_2$ (5.70 g, 55%) as a yellow foam. R_f=0.3 (AcOEt/hexane 90:10); ^{1}H NMR (300 MHz, $CDCl_3$): δ=1.26 (s, 6H; Me), 1.58 (brm, 3H; CH_2), 1.72–1.80 (m, 1H; CH_2), 1.74 (t, J(H,H)= 6.7 Hz, 2H; CH_2), 2.08 (s, 3H; Me), 2.54 (s, 3H; Me), 2.57 (s, 3H; Me), 3.22 (brm, 2H; CH_2N), 4.09–4.16 (m, 2H; CHCH$_2$O, CHN), 4.30–4.37 (m, 2H; CHCH_2O), 5.51 (s, 1H; CHN_2), 5.99 (d, J(H,H)=7.8 Hz, NH), 6.13 (brs, NH), 6.22 (brs, 2H; NH), 7.24–7.27 (m, 2H; arom.), 7.35 (t, J(H,H)=7.3 Hz, 2H; arom.), 7.54 (d, J(H,H)=7.5 Hz, 2H; arom.), 7.72 (d, J(H,H)=7.5 Hz, 2H; arom.); ^{13}C NMR (75 MHz, $CDCl_3$): δ=12.3, 17.6, 18.7, 21.5, 25.3, 26.8, 29.9, 32.8, 40.8, 47.2, 66.9, 73.7, 117.9, 119.9, 124.1, 125.0, 126.9, 127.6, 132.9, 134.8, 135.4, 141.2, 143.6, 153.6, 156.1, 156.3; IR ($CHCl_3$): $\tilde{\nu}$ = 3345 (w), 2879 (w), 2944 (w), 2111 (m), 1720 (m), 1624 (s), 1552 (s), 1509 (m), 1450 (m), 1370 (m), 1299 (m), 1166 (w), 1110 (s), 1046 (w), 834 (w), 657 cm^{-1} (w); MS (ESI, pos.): m/z: 725.0 (20) [M+K]$^+$, 710.0 (24), 709.0 (52) [M+Na]$^+$, 687.0 (100); HRMS: calcd for [$C_{32}H_{46}N_4O_6SNa$]$^+$: 681.2723; found: 681.2726.

Compound **2b** was synthesized from Fmoc-(*S*)-Arg(Pmc)-CHN$_2$ (3.36 g, 5.0 mmol) in a procedure analogous to that of **2a**. FC (EtOAc/pentane 3:2→5:1) afforded **2b** (6.33 g, 83%) as white foam. R_f=0.32 (EtOAc/pentane 3:1); $[\alpha]_D^{RT}$=−12.7 (c=0.82 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ=1.27 (s, 6H; Me), 1.31–1.61 (m, 4H; 2CH_2), 1.75 (t, J(H,H)=

6.7, 2H; CH_2), 2.08 (s, 3H; Me), 2.50–2.60 (m, 2H; CH_2COOBn), 2.55 (s, 3H; Me), 2.57 (s, 3H; Me), 3.96 (brm, 1H; C*H*NHFmoc), 4.14 (t, J(H,H) = 6.7, 1H; C*H*CH_2O), 4.34–4.37 (m, 2H; CHCH_2O), 5.09 (s, 2H; CH_2Ph), 5.43 (d, J(H,H) = 9.3 Hz, 1H; NHFmoc), 5.96 (brs, 3H; NH), 7.24–7.39 (m, 9H), 7.54 (d, J(H,H) = 7.5 Hz, 2H), 7.74 (d, J(H,H) = 7.5 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 12.2, 17.6, 18.6, 25.4, 26.8, 32.1, 32.8, 39.5, 40.9, 47.2, 66.6, 66.7, 73.6, 117.8, 119.9, 123.9, 124.9, 126.9, 127.2, 128.2, 128.3, 128.5, 133.2, 134.8, 135.3, 135.4, 141.1, 143.5, 143.6, 153.4, 155.7, 156.3, 171.0; IR ($CHCl_3$): $\tilde{\nu}$ = 3430 (w), 3352 (w), 3008 (w), 2946 (w), 1722 (m), 1621 (m), 1553 (s), 1513 (m), 1451 (m), 1385 (w), 1353 (w), 1299 (m), 1262 (m), 1167 (m), 1111 (s), 1013 (w), 657 cm^{-1} (w); MS (MALDI): *m*/*z* (%): 805 (8) $[M+K]^+$, 791 (16), 790 (49), 789 (100) $[M+Na]^+$, 502 (33), 501 (98) $[M-Pmc+2H]^+$, 481 (11), 305 (59); elemental analysis calcd (%) for $C_{43}H_{50}N_4O_7S$ (766.9): C 67.34, H 6.57, N 7.31 found C 67.37, H 6.73, N 7.28.

Compound 3a: A few drops of AcOH and 10% Pd/C (140 mg) under Ar were added to a solution of **2a** (1.51 g, 2.0 mmol) in methanol (20 mL). The apparatus was evacuated, flushed three times with H_2 and the mixture was stirred under an atmosphere of H_2 for 2 h and 30 min. The mixture was diluted with MeOH, filtered through Celite and concentrated under reduced pressure. FC (CH_2Cl_2/MeOH/AcOH 95:5:1→90:10:1) afforded **3a** (1.21 g, 91%) as a white foam. R_f = 0.14 (CH_2Cl_2/MeOH/AcOH 95:5:1); $[\alpha]_D^{RT} = -6.86$ (c = 1.02, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$, signals of rotamers are given in italics): δ = 1.39 (s, 6H; Me), 1.56 (br, 4H; CH_2), 2.04 (s, 3H; Me), 2.25–2.31 (m, 2H; CH_2CO), 2.47 (s, 3H; Me), 2.54 (s, 3H; Me), 2.86 (s, 3H; Me), 3.17 (brs, 2H; CH_2CMe$_2$), *3.59*, 3.94 (brs, 1H; CHN), 4.10–4.16 (t, J(H,H) = 6.9 Hz, 1H; C*H*CH_2O), *4.30*, 4.47 (br, 2H; CHCH_2O), 5.81 (d, J(H,H) = 8.4 Hz, 1H; NH), 6.36 (br, 3H; NH), 7.20–7.34 (m, 4H; arom.), 7.51 (d, J(H,H) = 7.2 Hz, 2H; arom.), 7.68 (d, J(H,H) = 7.5 Hz, 2H; arom.); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 12.6, 18.0, 19.4, 25.5, 28.6, 31.4, 39.6, 40.8, 43.2, 47.8, 66.8, 86.4, 117.6, 119.8, 124.7, 124.9, 126.9, 127.5, 132.3, 138.3, 141.0, 143.5, 143.6, 156.2, 156.3, 158.8, 174.9; IR ($CHCl_3$): $\tilde{\nu}$ = 3436 (w), 3348 (w), 3008 (w), 2976 (w), 1713 (s), 1622 (m), 1555 (s), 1451 (m), 1408 (m), 1371 (w), 1107 (s), 909 (w), 658 (w), 621 (w) cm^{-1}; MS (MALDI): *m*/*z* (%): 686 (32), 685 (79) $[M+Na]^+$, 412 (22), 411 (100), 394 (18), 393 (79), 215 (18).

Compound 3b: Compound **3b** was synthesized starting from **2b** (5.50 g, 7.3 mmol) in a procedure analogous to that of **3a**. FC (CH_2Cl_2/MeOH/AcOH 95:5:1) afforded **3b** (4.45 g, 90%) as a white foam. R_f = (CH_2Cl_2/MeOH/AcOH 95:5:1); $[\alpha]_D^{RT} = -5.2$ (c = 1.12 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$, signals of rotamers are given in italics): δ = 1.26 (s, 6H; Me), 1.38–1.46 (m, 1H; CH_2), 1.57 (br, 3H; CH_2), 1.73 (t, J(H,H) = 6.5 Hz, 2H; CH_2), 2.07 (s, 3H; Me), *2.30*, 2.52–2.54 (m, 2H; CH_2CO), 2.52 (s, 3H; Me), 2.55 (s, 3H; Me), 3.17 (br, 1H; CH_2N), *3.57*, 3.93 (br, 1H; CHN), 4.09–4.16 (m, 1H; C*H*CH_2O), 4.31–4.33, *4.49* (br, 2H; CHCH_2O), 5.75 (d, J(H,H) = 9.0 Hz, 1H; NH), 6.27 (br, 3H; NH), 7.20–7.26 (m, 2H; arom.), 7.33 (t, J(H,H) = 7.5 Hz, 2H; arom.), 7.52 (d, J(H,H) = 7.5 Hz, 2H; arom.), 7.69 (d, J(H,H) = 7.5 Hz, 2H; arom.); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 12.24, 17.6, 18.6, 21.5, 25.5, 26.8, 29.8, 31.4, 32.7, 39.51, 40.8, 47.2, 66.7, 73.7, 117.9, 119.8, 124.1, 125.0, 126.9, 127.5, 132.7, 134.8, 135.4, 141.1, 143.5, 143.7, 153.6, 156.2, 156.3, 174.7; IR ($CHCl_3$): $\tilde{\nu}$ = 3348 (w), 2944 (w), 1713 (m), 1622 (m), 1651 (s), 1450 (m), 1385 (w), 1299 (m), 1248 (w), 1166 (w), 1110 (s), 1014 (w), 658 cm^{-1} (w); MS (MALDI): *m*/*z* (%): 715 (5) $[M+K]^+$, 7.1 (12), 700 (43), 699 (100) $[M+Na]^+$, 412 (20), 411 (86) $[M-Pmc+2H]^+$, 393 (17), 393 (77) $[M-Pmc-NH_2]^+$, 215 (19); elemental analysis calcd (%) for $C_{36}H_{44}N_4O_7S$ (676.3): C 63.89, H 6.55, N 8.28; found C 63.77, H 6.68, N 8.12.

Compound 5: TFA (12 mL) was added at 0°C to a solution of Fmoc-(*S*)-β^{3}hOrn(Boc)-OH (2.92 g, 6.23 mmol) in CH_2Cl_2 (12 mL), and the mixture allowed to warm to RT while stirring. After 4 h the solvent was removed under reduced pressure, coevaporated with CH_2Cl_2 and the residue dried under high vacuum to yield the TFA salt. The TFA salt was used as is. (iPr)$_2$NEt (3.4 mL, 20.14 mmol) was added to a suspension of the TFA salt in formamide followed by a solution of **4** (2.9 g, 9.34 mmol) in dioxane (9 mL). After stirring the mixture for 48 h, 1N HCl (40 mL) was added and extracted with EtOAc (3×100 mL). The organic phase was washed with brine and concentrated under reduced pressure. FC (EtOAc/pentane/AcOH 4:6:0.2) afforded **5** (2.67 g, 70%) as a white foam. R_f = 0.43 (EtOAc/pentane/AcOH 5:5:0.2); $[\alpha]_D^{RT} = +1.88$ (c = 0.96 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$, a mixture of slowly interconverting rotamers); ^{1}H NMR (75 MHz, $CDCl_3$): δ = 1.48 (s, 9H; *t*Bu), 1.49 (s, 9H; *t*Bu), 1.62–1.69 (br, 4H; CH_2), *2.35*, 2.59–2.68 (m, 2H; CH_2CO), 3.25–3.40, *3.63* (br, 2H; CH_2N), 4.00 (brs, 1H; CHN), 4.20 (t, J(H,H) = 6.7, 1H; C*H*CH_2O), 4.39, *4.55* (d, J(H,H) = 9.3, 2H; CHCH_2O), 5.70 (d, J(H,H) = 9.3, 1H; NH), 7.27–7.41 (m, 5H; Ph), 7.59, *7.60* (d, J(H,H) = 7.2, *7.2* Hz, 2H; arom.), 7.75 (d, J(H,H) = 7.5, 2H; arom.), 8.38 (br, 1H; NH), 11.46 (br, 1H; NH); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 26.2, 28.2, 28.4, 31.1, 39.1, 40.5, 47.4, 48.1, 66.6, 79.5, 83.3, 119.9, 125.0, 126.9, 127.6, 141.2, 143.7, 153.1, 156.1, 163.1, 174.3; IR ($CHCl_3$): $\tilde{\nu}$ = 3325 (w), 2984 (w), 1720 (s), 1616 (s), 1511 (w), 1450 (w), 1416 (m), 1333 (m), 1248 (m), 1135 (s), 1054 (w), 1028 (w), 622 cm^{-1} (w); MS (MALDI): *m*/*z* (%): 633.3 (3) $[M+Na]^+$, 412.2 (23), 411.2 (100); elemental analysis calcd (%) for $C_{32}H_{42}N_4O_8$ (610.7): C62.94, H 6.93, N 9.17; found: C 62.92, H 6.95, N 9.13.

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