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Stable Helical β^3-Peptides in Water via Covalent Bridging of Side Chains

Esther Vaz and Luc Brunsveld*

Max Planck Institute of Molecular Physiology, Otto-Hahn Strasse 11, D-44227, Dortmund, Germany, and Chemical Genomics Centre, Otto-Hahn Strasse 15, D-44227, Dortmund, Germany

luc.brunsveld@mpi-dortmund.mpg.de

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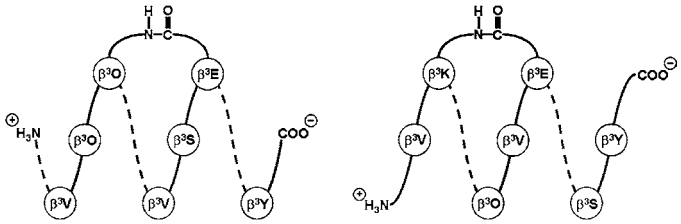
ABSTRACT



An on-bead cyclization protocol of β^3-peptides was developed, providing easy access to cyclic β^3-peptides. With this methodology, a small library of helical cyclic β^3-peptides was synthesized and investigated with CD spectroscopy. Covalent bridging of two side chains in β^3-peptides significantly stabilized their helical conformation in aqueous solutions and turned out to be superior to the previously described electrostatic interactions.

β-Amino acid based peptides belong to the class of oligoamide foldamers which fold in a varied set of secondary structures in a range of solvents.[1−3] One of the most important secondary structures is the 14-helix adopted by β^3-peptides, in which every third residue has the same orientation.[4] Due to its similarities in side chain positioning with certain biologically active helical α-peptides, the 14-helix of β^3-peptides has been successfully explored as a modulator of protein−protein interactions.[5−7]

Initially, stable secondary structures formed by β-peptides could only be obtained in organic solvents. Recently, incorporation of certain design elements enabled the construction of stable structures in aqueous solutions, with the 14-helix as a notable example. The synthesis of such water stable 14-helix structures was first addressed by Gellman[8] for β-peptides containing *trans*-aminocyclohexyl carboxylic acid residues combined with water-soluble building blocks. Seebach[9] and DeGrado[10] subsequently showed an increase of 14-helix stability in aqueous solutions by employing an alternating pattern of oppositely charged side chains at positions i and $i + 3$. These β^3-peptides featured charge−charge interactions at two of the three helical faces of a 14-helix. Subsequent work of Schepartz showed that incorporation of side chains stabilizing the 14-helix macrodipole can reduce the number of required electrostatic side chain interactions from two to only one face of the helix.[11,12] Optimization of these design paradigms has resulted in stable

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14-helical scaffolds, generally applicable for modulating protein interactions. Notwithstanding their strong stabilization of helicity and their proven utility for inhibiting protein−protein interactions does this approach still require the presence of charged side chains to form salt bridges, thus limiting the design of biologically active β^3-peptides to highly charged β^3-peptide scaffolds, with concomitant disadvantages for example in membrane passage and environment sensitivity (pH, salt concentration). Since helix stability contributes significantly to protein binding affinity and selectivity, general approaches for inducing stable secondary structures are still needed.

A frequently applied approach for stabilizing α-peptide helices is side chain cyclization.[13−16] Cyclic β^3-peptides from head-to-tail cyclizations have been studied previously focusing on the generation of stable scaffolds, nanotubes, and epitope display.[17−21] Up to now, cyclization of β^3-peptides for helix stabilization has only been explored in organic solvents.[22] We therefore decided to develop a solid-phase synthesis strategy that would give rapid access to side chain cyclized β^3-peptides for studying the effect of covalent cyclization on 14-helix stability in aqueous media.

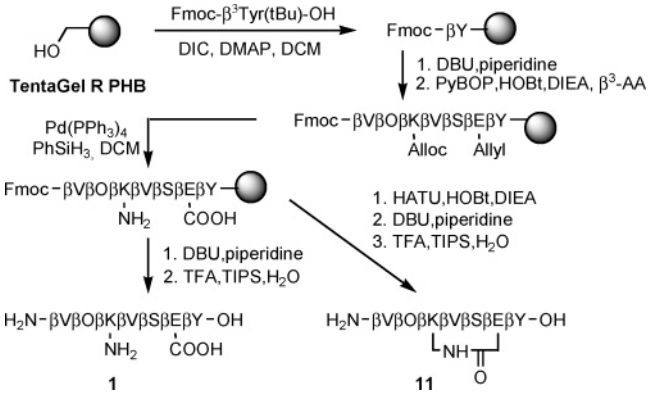
The development of an on-bead synthesis strategy including the essential side chain cyclization requires a set of orthogonally protected β^3-amino acids. The Allyl ester moiety and the allyloxycarbonyl carbamate (Alloc) group have proven to be reliable orthogonal protecting groups for carboxylic acids and amines, respectively, and are regularly employed in classical α-amino acid chemistry.[23] We therefore selected these moieties for protecting the side chains of the amino acids that were intended to be cyclized. The Arndt−Eistert homologation method has been successfully applied to the synthesis of various β^3-amino acids[4,24] and we consequently employed that approach for the preparation of Allyl and Alloc protected β^3-amino acid derivatives. The Fmoc-protected diazoketones were homologated by using a base-free, silver(I)-catalyzed, ultrasound promoted Wolff rearrangement protocol.[25] All β^3-amino acids were obtained in good yields (Table 1).

Table 1. Yields for the Preparation of Orthogonal Protected β^3-Amino Acids (β^3-Asp, β^3-Glu, β^3-Orn, and β^3-Lys) from the Corresponding α-Amino Acids

Fmoc-β^3-amino acid	yield (%)
Fmoc-β^3Orn(Alloc)-OH	72
Fmoc-β^3Lys(Alloc)-OH	72
Fmoc-β^3Glu(Allyl)-OH	70
Fmoc-β^3Asp(Allyl)-OH	83

Using this set of orthogonally protected building blocks with standard Fmoc-protected β^3-amino acids, we synthesized a library of 20 β^3-peptides by classical Fmoc solid-phase synthesis methods (Scheme 1 and Supporting Information).

Scheme 1. Synthetic Scheme for the Preparation of Linear and Cyclic β^3-Heptapeptides by Solid-Phase Synthesis and On-Bead Cyclization



After assembly of the complete sequence, the Allyl and Alloc protecting groups were cleaved selectively with tetrakis(triphenylphosphine)palladium(0) [$Pd(Ph_3)_4$] in dichloromethane and phenylsilane as a scavenger.[23] At this stage, the resin bound peptides were divided into two batches. One batch was used for the generation of linear analogues and the other for HATU-mediated on-bead cyclization for synthesis of the cyclic analogues. Cleavage from the resin and preparative RP-HPLC purification led to the corresponding fully deprotected linear (average yields ∼30%) and cyclized (average yields ∼10%) β^3-peptides in good yields with purities over 99% as determined via HPLC. Additionally, their sequences were confirmed by ESI mass spectrometry. It is difficult to compare yields of peptide synthesis and on-bead cyclization with previous studies on analogous oligomers, especially since purities between studies vary. Nevertheless, the yields of the purified cyclic β^3-peptides **11**−**20** compare favorable to other cyclic β^3-peptides[19] and cyclic α-peptides.[16] These encouraging yields might result from the intrinsic nature of β^3-peptides to fold into helices in organic solvents, possibly also under the cyclization conditions.

The aim of our β^3-peptide library design was the stabilization of the 14-helix in water, using only one constraining

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element. To allow a reasonable comparison of cyclic β^3-peptides with linear analogues, we also synthesized β^3-peptides of the same sequence but replacing the covalent bridge by a salt bridge, as this is the best helix stabilizing element known to date. The β^3-peptides were varied in length, position, and orientation of the constraining lactam bridge, which, however, was always positioned in the relative positions i and $i + 3$. Furthermore, two hydrophobic aliphatic substitutions (β^3-homovaline), mimicking a hydrophobic interaction surface, and two additional polar amino acids (β^3-homoserine and β^3-homoornithine), to allow for decent water solubility, were included in the heptameric β^3-peptides. Each peptide also contained a β^3-Tyr residue to facilitate concentration determination by spectrophotometric methods and HPLC purification. The secondary structure of the β^3-peptides was investigated by circular dichroism (CD) spectroscopy. The propensity of the β^3-peptides to form a 14-helix was judged by their characteristic CD signature using the ellipticity maxima and minima near 195−198 and 213−215 nm, respectively.[2,26] The 14-helix content of all β^3-peptides in methanol and aqueous buffer (pH 7.4, 25 °C) was determined at 215 nm.

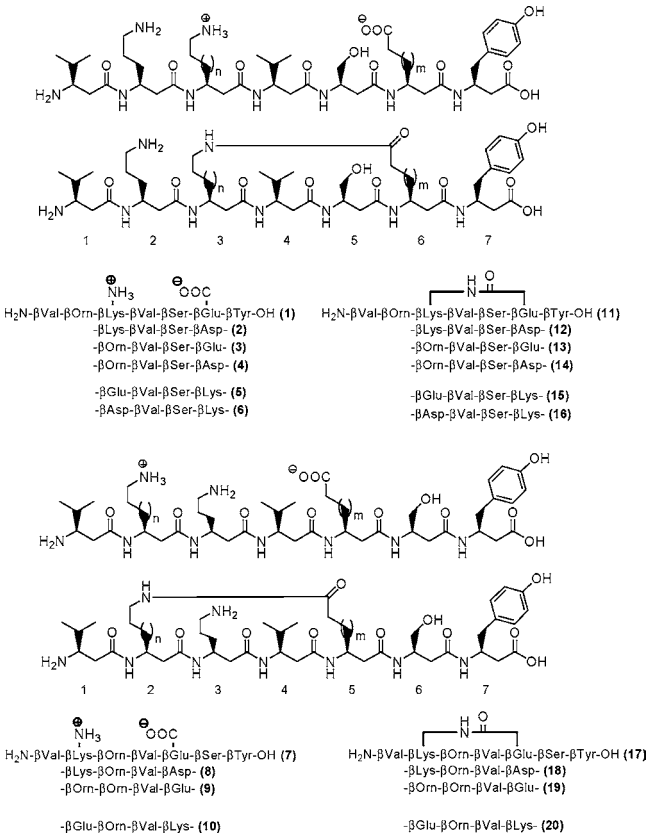

Figure 1. Library of heptameric β^3-peptides containing either electrostatic side chain interactions or a covalent bridge.

The CD spectra of all 20 β^3-peptides in methanol showed a characteristic minimum near 215 nm and a maximum near 195 nm, consistent with a 14-helix structure (Supporting Information). This indicates that salt bridges or lactam bridges in this organic solvent have only minor effects on the stabilization of the 14-helix.

On the other hand, measurements in aqueous phosphate buffer at pH 7.4 showed a much more dramatic effect of the side chain cyclization on the overall helical content. (Figure 2). We first studied the β^3-peptides with a stabilizing element at positions 3 and 6 (**1**−**4** and **11**−**14**). The linear peptides showed the highest 14-helix propensities when shorter side chains were used. The highest negative value of mean residue ellipticity (MRE) was observed for the β^3-peptides with a salt bridge via β^3-Asp (between −8000 and −7500 deg·cm^2·dmol^{-1}·residue^{-1}). These results are in agreement with observations by Guarracino et al.[27] In the case of the analogous cyclic peptides, however, the differences in helix stability were much more pronounced. The cyclization of the β^3-peptide seems to generate either more or less stable helices in comparison with the electrostatic stabilized β^3-peptides. The most stable helical structure was obtained via

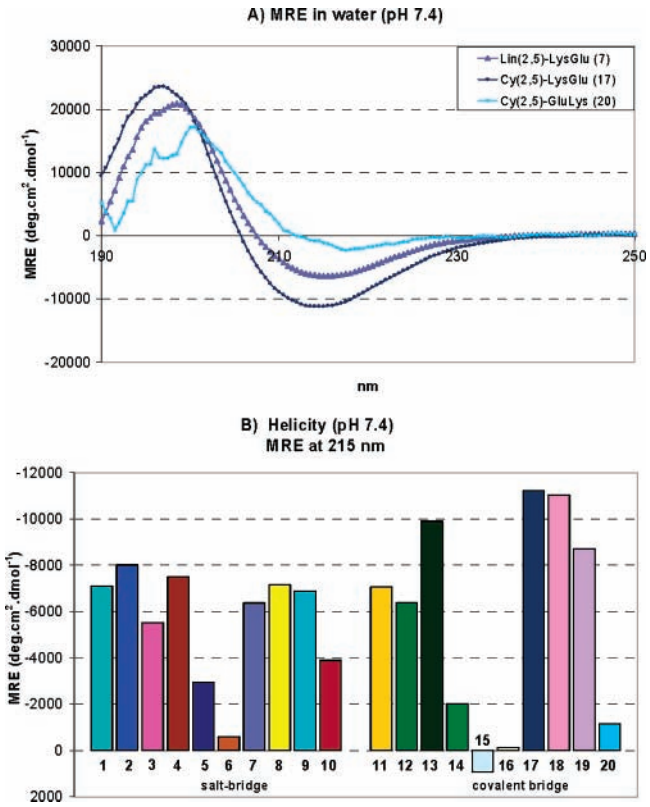

Figure 2. (A) Representative CD spectra of three β^3-heptapeptides at 150−200 μM (sodium phosphate buffer) at pH 7.4. (B) Mean residue ellipticities obtained at 215 nm and pH 7.4 (sodium phosphate buffer) for all β^3-heptapeptides.

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formation of a covalent bridge between β^3-Orn and β^3-Glu side chains, with a MRE of −9900 deg·cm^2·dmol^{-1} Å·residue^{-1}.

To study the position effect of the cyclization element within the β^3-sequence, we also placed the cyclizing residues at positions 2 and 5, respectively (**7**−**9** and **17**−**19**). The MRE observed for the linear peptides **7**−**9** showed similar values as the linear peptides with salt bridges at positions 3 and 6 (**1**−**4**). Analysis of these cyclized β^3-peptides showed that positioning the covalent bridge nearer to the *N*-terminus of the β^3-peptide greatly favored its folding into a 14-helix. The MRE values observed at 215 nm were strongly increased for **17** and **18** compared to **11** and **12** with MRE values in the range of −11000 deg·cm^2·dmol^{-1}·residue^{-1} and thus were the highest MRE of all studied β^3-peptides in these series in water. This pronounced stabilizing effect at positions near the *N*-terminus is in line with recent computational studies that revealed a lower population of hydrogen bonding at the *N*-terminus than at the *C*-terminus.[12]

Interactions of side chains with the helix macrodipole are important in 14-helix stabilization,[11] analogously for α-peptides.[28] Because of its *H*-bonding pattern, the 14-helix macrodipole is oriented in the opposite direction than in the α-helix, and has a partial positive charge at the *C*-terminus and a partial negative charge at the *N*-terminus.[2] It is therefore reasonable to expect an influence of 14-helix stabilization via cyclic lactam bridges by the orientation of the linking amide bond. Consequently, we synthesized peptides in which the relative positions of the two interacting side chains were switched, thus modifying the overall macrodipole while retaining the covalent bridges at the same length (**5**, **6**, **10** and **15**, **16**, and **20**). The CD spectra of these β^3-peptides revealed an almost complete lack of the 14-helix secondary structure, in which the destabilizing effect was most pronounced for the cyclic peptides, thus confirming the influence of orientation on lactam bridge induced helix stabilization.

Dramatic differences in helix stability as observed for the cyclic β^3-peptides **11**−**20** have also frequently been noted for α-helices.[16] The direction of the dipole of the lactam bridge, hydrogen bonding of the newly introduced amide bond, and hydrophobic interactions between the bridge and the helix have all been shown to influence α-helix stability. These factors will presumably also govern the 14-helix stability of the β^3-peptides **11**−**20**.

To explore the environment stability of the 14-helix, CD measurements of the β^3-peptides **7** and **17** at different pH were performed. Although the cyclic β^3-peptide **17** also showed an appreciable pH sensitivity, the 14-helix of **17** was in all cases significantly more stable than that for the corresponding electrostatically stabilized β^3-peptide **7**. The influence of pH on the helicity of **17** might indicate that the protonation state of the termini of the cyclic β^3-peptides has an effect on helix stability, similar to that for linear peptides.

Table 2. MRE Values (deg·cm^2·dmol^{-1}·residue^{-1}) at 215 nm of **7** and **17** at Different pH Values

pH	Lin(2,5)-LysGlu **7**	Cy(2,5)-LysGlu **17**
1.75	−3747	−6316
3.6	−5522	−8821
7.5	−6388	−11237
9.6	−1526	−5043

Temperature-dependent ^{1}H NMR experiments of β^3-peptides **16** and **18** revealed a stronger exchange of the amide protons of **16** with water, compared to **18**. Whereas at 20 °C all amide protons of **18** can be clearly detected, for **16** especially the *N*-terminal amide protons are hardly detectable, thus indicating a higher level of secondary structure stability for **18** (see the Supporting Information).

In summary, we have developed a robust and rapid solid-phase approach to cyclic β^3-peptides. A spatial constraining of β^3-peptides with an appropriate covalent side chain to side chain linkage yields stable 14-helices in aqueous media, which surpasses the well-known electrostatic stabilization approach. Further work will be directed to the understanding of the best positioning of the covalent linkage and the biological application of this stabilizing motif to protein−protein modulators.

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Supporting Information Available: Characterization and synthesis data of all new β^3-amino acids and β^3-peptides **1**−**20**, CD spectra in methanol, and ^{1}H NMR spectra of **16** and **18** in water. This material is available free of charge via the Internet at http://pubs.acs.org.

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