DOI: 10.1002/chem.200500249

Cyclic β-Tetra- and Pentapeptides: Synthesis through On-Resin Cyclization and Conformational Studies by X-Ray, NMR and CD Spectroscopy and Theoretical Calculations

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Abstract: The solution-phase synthesis of the simplest cyclic β-tetrapeptide, cyclo(β-Ala)$_4$ (**4**), as well as the solid-phase syntheses through side chain anchoring and on-resin cyclization of the cyclic β^3-tetrapeptide cyclo(-β^{3}hPhe-β^{3}hLeu-β^{3}hLys-β^{3}hGln-) (**14**) and the first cyclic β^3-pentapeptide cyclo(-β^{3}hVal-β^{3}hPhe-β^{3}hLeu-β^{3}hLys-β^{3}hLys-) (**19**) are reported. Extensive computational as well as spectroscopic studies, including X-ray and NMR spectroscopy, were undertaken to determine the preferred conformations of these unnatural oligomers in solution and in the solid state. cyclo(β-Ala)$_4$ (**4**) with no chiral side chains is shown to exist as a mixture of rapidly interchanging conformers in solution, whereas inclusion of chiral side chains in the cyclo-β^3-tetrapeptide causes stabilization of one dominating conformer. The cyclic β^3-pentapeptide on the other hand shows larger conformational freedom. The X-ray structure of achiral cyclo(β-Ala)$_4$ (**4**) displays a C_i-symmetrical 16-membered ring with adjacent C=O and N-H atoms pointing pair wise up and down with respect to the ring plane. CD spectroscopic examinations of all cyclic β-peptides were undertaken and revealed results valuable as starting point for further structural investigations of these entities.

Keywords: conformation analysis · cyclization · NMR spectroscopy · peptides

Introduction

The investigation of β-peptides is one fundamental part of the growing research field of "foldamers" (oligomers capable to form well-defined secondary structures) that has attracted the attention of several research groups during the last years.[1] On the one hand, structural investigations have revealed interesting results that these artificial oligomers are capable of forming secondary structures such as helices, β-sheetlike strands and other structures in solution and in the solid state.[1a,2] On the other hand, β-peptides have also been shown to display various biological activities, such as antibacterial activity[3] and inhibition of cholesterol absorption.[4]

In contrast to the detailed work on linear β-peptides, cyclic β-peptides have gained less interest, possibly because of their challenging synthesis. The main reason of the small number of reports is in our opinion the low solubility of the protected intermediates involved in the solution-phase synthesis. Usually laborious procedures, for example, the addition of the chaotropic salts, are necessary to dissolve the cyclic peptide for final deprotection and to obtain water soluble derivatives.[5] Progress in the synthesis of cyclic β-peptides seems to be possible by on-resin cyclization. By carrying out this crucial step on solid phase one can take advantage of the pseudo-dilution effect of a resin with a low level of substitution. One publication reported several examples where the synthesis was executed through backbone anchoring.[6] In contrast to this, we have focused on the methodology through side chain anchoring and subsequent cleavage from the resin/side chain deprotection at the same time.[7] In this synthetic approach we can reduce the num-

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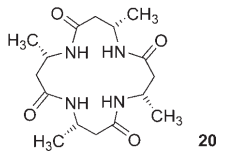
Supporting information for this article is available on the WWW under http://www.chemeurj.org/ or from the author: Theoretical calculations of **20**, NMR data and Monte Carlo conformational sampling of **14** an **19**.

bers of the orthogonal protecting groups needed to three and obtain water soluble peptides.

Motivation for the synthesis of cyclic β-peptide arises not only from a fundamental chemical point of view, but also from the interesting biological activities of these new entities found up to now. One cyclic β^3-tetrapeptide was shown to act as a somatostatin analogue,[5b,8] and a number of cyclic β-tripeptides were found to display antiproliferative activity and growth inhibition of human cancer cell lines.[9] Additionally, it was reported that cyclic β-peptides can form hollow, tubular structures, so called nanotubes, in the solid state[10] and inside biomembranes, causing formation of transmembrane ion channels.[5a]

Considering that the majority of the few hitherto synthesized cyclic β-peptides display biological activities, these results imply that cyclic β-peptides may share the property of cyclic α-peptides to act as privileged structures due to their inherent reduced conformational flexibility.[11] This constrained geometry is the main reason for the display of increased and manifold biologic activities of cyclo-α-peptides as for example, hormones, antibiotics, antimycotics, and toxins. Conformational and molecular modeling studies on key secondary structural elements, that is, β-turns, are also widely undertaken with cyclic peptides, especially in early stages of drug discovery.[12] Additionally, β-peptides show an innate and total stability towards proteolytic degradation caused by the unnatural building blocks.[13] For these reasons we are interested in further detailed physicochemical investigations on cyclic β-peptides in order to rationalize the structural aspects and open this field for more studies in the fields of medicinal chemistry, material science, and catalysis.

In previous work it was shown by Gademann and Seebach,[5c] and by us,[7] that cyclic β^3-tripeptides exist in aqueous solution in one predominant conformation with uniformly oriented amide bonds and all side chains in lateral positions. This predominant conformation was also found in the structure of cyclo(β-Ala)$_3$, the only high resolution X-ray structure reported so far for cyclic β-tripeptides.[14] Now we have extended the scope of our experiments and want to report herein the solution phase synthesis, NMR structure, and the X-ray structure of cyclo(β-Ala)$_4$ (**4**) which has no side chains. A theoretical study on the model peptide cyclo-(β^3hAla)$_4$ (**20**) with four methyl substituents will provide a basis for understanding the conformational behavior of the cyclic β-tetrapeptides. Additionally, we will describe the syntheses with on-resin cyclization and the NMR-solution structures of a cyclic β^3-tetrapeptide and of the first cyclic β^3-pentapeptide, both with proteinogenic side chains. CD spectroscopical data for all cyclic β-peptides are reported as well.



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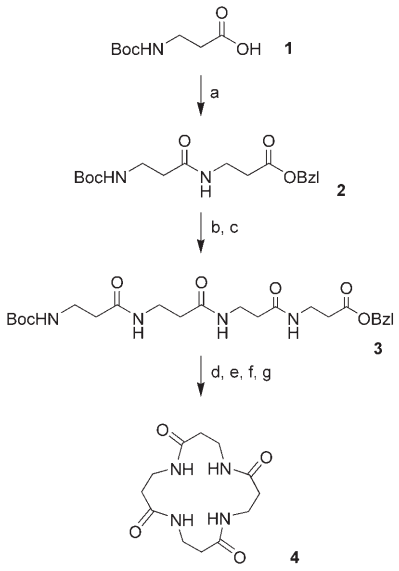
Results and Discussion

Synthesis: To gain a starting point in the structural investigations of cyclic β-tetrapeptides, we decided to begin our studies with the simplest derivative cyclo(β-Ala)$_4$ (**4**), that is, the derivative without chiral side chain. The synthesis of **4** was reported previously in the literature: one procedure included solution-phase synthesis with nowadays uncommon reagents;[15] a recent method used backbone amide anchoring and on-resin cyclization,[6] but no additional data such as NMR- or X-ray structural data have been published so far. For reasons of convenience we developed a simple solution-phase method, using the standard protecting groups Boc and Bzl and conventional coupling reagents (Scheme 1).



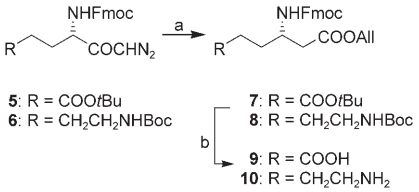
Scheme 1. a) TEA, isobutyl chloroformate, H-β-Ala-OBzl·HCl, THF, 3 d, RT, 81 %; b) 10 % Pd/C, H_2, THF/MeOH 1:1, 12 h, RT; c) TEA, isobutyl chloroformate, THF, −15 °C, 15 min, then H-β-Ala-β-Ala-OBzl·TFA, 3 d, RT, 63 %; d) 10 % Pd/C, H_2, THF/H_2O 2:1, 12 h, RT; e) pentafluorophenol, DIEA, DMF, RT for 16 h; f) TFA/ CH_2Cl_2 1:1; g) DIEA, THF, 32 h, 60 °C, 58 %.

Thus, the linear precursor Boc-(β-Ala)$_4$-OBzl (**3**) was synthesized starting from the suitable protected monomer **1** and the dimer **2**, respectively, by coupling via the mixed anhydride formed with isobutyl chloroformate. The Bzl group of Boc-(β-Ala)$_4$-OBzl (**3**) was removed by catalytic hydrogenation; the active pentafluorophenyl ester was formed, which after Boc deprotection by TFA underwent cyclization to **4** under high dilution conditions (2.5 mM) in a good yield of 58 % (conditions not optimized).

In the syntheses of the cyclic β-peptides with proteinogenic amino acid side chains through on-resin cyclization we utilized the method of side chain anchoring to gain the desired products. A water soluble β-tripeptide was recently obtained by us in excellent yield applying this technique.[7] To extend the scope of this methodology we attempted to anchor the side chains of two different amino acids utilizing

two different linkers. For the synthesis of the cyclic β^3-tetrapeptide **14** we chose attachment of a β^3-glutamic acid derivative to the Rink amide linker, whereas the cyclic β^3-pentapeptide **19** was originally anchored via a β^3-lysine residue to an activated carbonate resin. A three dimensional orthogonal protecting group strategy, including Fmoc, Boc and All groups, was utilized as general synthetic route, based on the pioneering work of Trzeciak and Bannwarth,[16] and Albericio and co-workers.[17]

The required building blocks were synthesized by Arndt–Eistert homologation of the corresponding Fmoc-protected α-L-amino acid derivatives according to procedures published by Seebach and co-workers,[18] and later Sewald and co-workers.[19] For the side chain anchoring to the resin, and the final cyclization, it was necessary to synthesize new Fmoc-β^3-amino acid derivatives with unprotected side chains and allyl protected carboxyl functions. Thus, the diazoketones Fmoc-Glu(O*t*Bu)-CHN_2 (**5**)[18] and Fmoc-Lys-(Boc)-CHN_2 (**6**)[18] were subjected to the conditions of the Wolff rearrangement with allyl alcohol and *N*-methylmorpholine (NMM)[20] to give the corresponding allyl esters **7** and **8** in good yields of 67 and 77%, respectively (Scheme 2). Cleavage of the Boc and the *t*Bu protecting

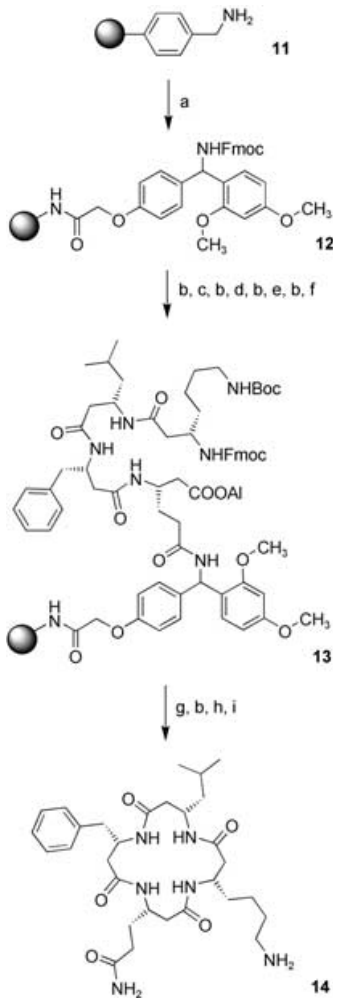


Scheme 2. a) Allyl alcohol, silver benzoate, NMM, THF, −15°C to RT, 5 h; 67% for **7**, 77% for **8**; b) TFA, TIPS, CH_2Cl_2, RT, 91% for **9**, > 95% for **10**.

groups of both derivatives was accomplished with TFA and TIPS in CH_2Cl_2[21] and the special protected amino acid derivatives Fmoc-β^3-hGlu-OAll (**9**) and Fmoc-β^3hLys-OAll (**10**) were obtained in 91% and almost quantitative yield, respectively.

Two types of TentaGel S resins were chosen for the solid-phase synthesis of the peptides, as these resins combine good swelling properties and a low level of substitution. The synthesis of cyclo-β^3-tetrapeptide **14** (Scheme 3) started with attachment of Rink amide linker to TentaGel NH_2 resin **11** to give **12**.[22] The fully protected resin bound linear tetrapeptide **13** was obtained by standard Fmoc solid-phase peptides synthesis protocols[23] and included side chain anchoring of Fmoc-β^3hGlu-OAll (**9**) and successive coupling of the other three β^3-amino acid derivatives.

The following allyl deprotection was accomplished with $[Pd(PPh_3)_4]$ following a method reported by Bloomberg et al.[24] The final Fmoc deprotection yielded the precursor for on-resin cyclization, which was accomplished with HBTU/HOBt and DIEA in DMF at room temperature. After the reaction mixture was stirred over night the TNBS



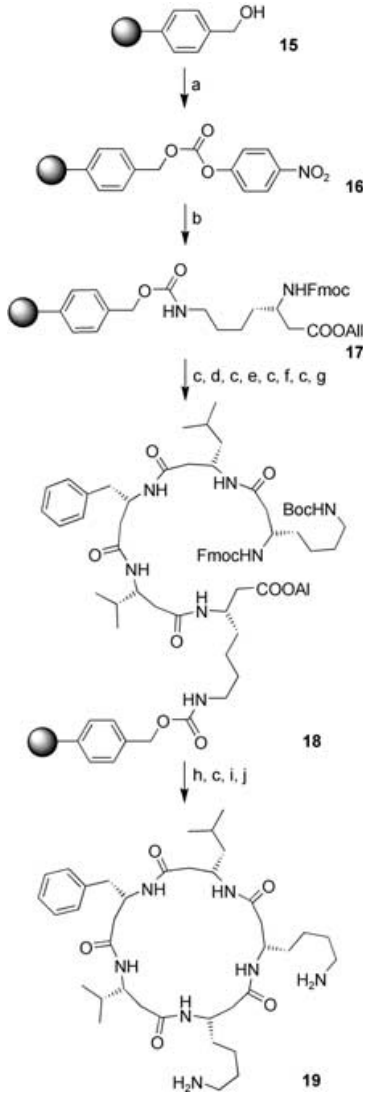
Scheme 3. a) Fmoc-Rink linker, HBTU, HOBt, DIEA, CH_2Cl_2, RT, 3 h, 81–92%; b) 2% DBU/2% piperidine in DMF, 5×5 min; c) Fmoc-β^3hGlu-OAll (**9**), HBTU, HOBt, DIEA, DMF, RT, 3 h; d) Fmoc-β^3hPhe-OH, HBTU, HOBt, DIEA, DMF, RT, 3 h; e) Fmoc-β^3hLeu-OH, HBTU, HOBt, DIEA, DMF, RT, 3 h; f) Fmoc-β^3hLys(Boc)-OH, HBTU, HOBt, DIEA, DMF, RT, 3 h; g) $[Pd(PPh_3)_4]$, DMSO/THF/0.5 M HCl/NMM 4:4:2:1, RT, 4.5 h; h) HBTU, HOBt, DMF, RT, 17 h; i) TFA/H_2O/TIPS 95:2.5:2.5, RT, 2.5 h.

test showed completeness of the reaction. The resin was treated with TFA, which resulted in the cleavage from the resin and Boc deprotection at the same time. The crude mixture of products had to be purified extensively by reversed-phase HPLC, as a variety of unidentified products was formed. No known side products, which usual can occur during cyclization with HBTU/HOBt (e.g. oligomers or tetramethylguanidinium derivatives[25]), could be identified through LC-MS analysis. The cyclic β^3-tetrapeptide **14** was obtained in 10% yield (based on anchored Rink amide linker, conditions not optimized).

TentaGel S PHB resin **15**, which includes a Wang linker, was used for the synthesis of the cyclo-β^3-pentapeptide **19** (Scheme 4). Reaction of the solid support with *p*-nitrophenyl chloroformate gave the active carbonate resin **16**[26] and

afterwards the ε-amino group of Fmoc-β^{3}hLys-OAll·TFA (**10**) was anchored to the resin in 60% yield.[27]

Just as in the case of tetrapeptide **14**, the assembly of the linear, resin bound fully protected β-peptide **18** was carried out following standard peptide synthesis procedures.[23] The subsequent allyl deprotection was executed with [Pd(PPh$_3$)$_4$] following a methodology of Guibé and Albericio and co-workers who introduced phenylsilane as an allyl group scavenger into the solid-phase peptide synthesis.[28] The final Fmoc deprotection yielded the precursor for on-resin cyclization. Two cyclization experiments with different coupling reagents were carried out. It was found that, based on HPLC chromatograms, the reaction with PyAOP proceeded with slight better conversions to the desired cyclic product and with full consumption of the starting material after 12 h in contrast to the reaction executed with PyBOP/HOBt where substantial amounts of the linear precursor could be detected after the same time. Cleavage from the resin and simultaneous Boc deprotection gave the cyclic product **19** in crude form, which after extensive purification by reversed-phase HPLC was obtained in pure form in only 9% yield.



Scheme 4. a) *p*-Nitrophenyl chloroformate, NMM, CH_2Cl_2, RT, 14 h; b) Fmoc-β^{3}hLys-OAll·TFA (**10**), DIEA, DMF, RT, 14 h, 60%; c) 2% DBU/2% piperidine in DMF, 5×5 min; d) Fmoc-β^{3}hVal-OH, HBTU, HOBt, DIEA, DMF, RT, 3 h; e) Fmoc-β^{3}hPhe-OH, HBTU, HOBt, DIEA, DMF, RT, 3 h; f) Fmoc-β^{3}hLeu-OH, HBTU, HOBt, DIEA, DMF, RT, 3 h; g) Fmoc-β^{3}hLys(Boc)-OH, HBTU, HOBt, DIEA, DMF, RT, 3 h; h) [Pd(PPh$_3$)$_4$], phenylsilane, CH_2Cl_2, RT, 2×10 min; i) PyAOP, DIEA, DMF, RT, 12 h; j) TFA/H_2O/TIPS 95:2.5:2.5, RT, 3 h.

NMR spectroscopy of cyclo(β-Ala)$_4$ (4): Both proton and carbon NMR spectra of the cyclic β-tetrapeptide **4** in water were found to be very simple, as expected, due to the lack of side chains and the symmetry of the molecule. Three signals were recorded in the ^{1}H NMR spectrum (two triplets for the eight methylene groups and one broad singlet for all NH resonances) and also three signals in the ^{13}C NMR spectrum. These data clearly indicate the occurrence of rapid interchanging conformers in solution; therefore mean values of the signals were measured, as it is very unlikely that only one fixed, symmetrical conformation of **4** in solution would occur.

X-ray structure of cyclo(β-Ala)$_4$ (4): Colorless crystals suitable for X-ray analysis were grown by slow evaporation from a MeOH/H_2O solution at room temperature. The crystal structure of the 16-membered ring of **4** (Figure 1) forms a flat C_i-symmetrical rhombic-shaped ring of dimensions 4.05×6.57 Å and does not adopt a hollow tube as in other cyclic β-tripeptides and cyclic β-tetrapeptides.[10a,14]

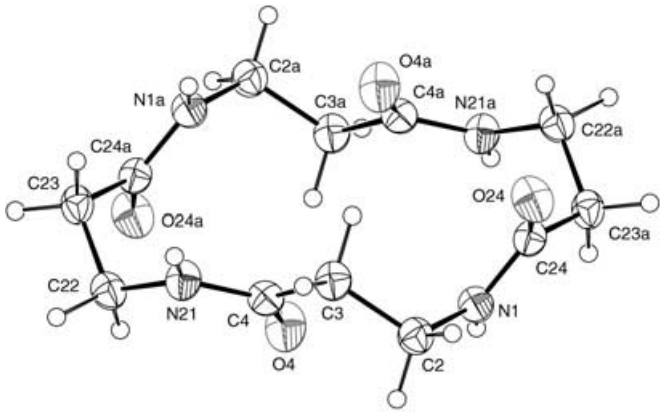


Figure 1. An ORTEP drawing of the crystal structure of **4**.

Instead only four of the eight intermolecular hydrogen bonds of one molecule stabilize a "column-like" supramolecular structure which is "filled" with hydrogen bonds (Figure 2a and b). The other four intermolecular hydrogen bonds connect the four neighboring columns. The planes of the cyclic subunits are not perpendicular to the column axis.

All the amide planes are in *trans* geometry; all four C=O bonds are aligned almost in a parallel fashion relative to the ring plane with two adjacent amide groups pointing up and the other two pointing in the opposite direction, similarly

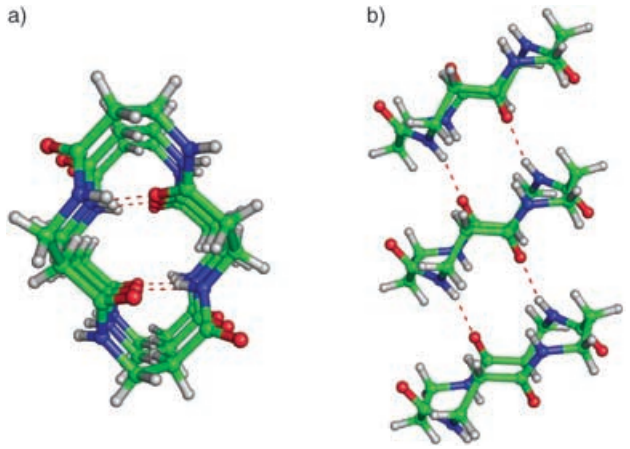


Figure 2. Crystal packing of **4**: a) view from the top of one column of molecules, b) side view of one column. Three cyclic subunits of one column are presented. Four of eight intermolecular H-bonds from one molecule **4** are shown, and the others are omitted for clarity.

observed in a structure of (*R*,*R*,*S*,*S*)-cyclotetra-β-homoalanine determined from powder diffraction data.[10a] The observed torsional angles suggest that a β-alanine unit in the crystal structure of **4** adopts two distinct conformations. The first conformation, with ϕ, θ and ψ dihedral angles of 101.46, −78.04 and 106.37°, orients the C=O and the N-H groups of the same monomer pointing to the opposite directions whereas the second conformation with ϕ, θ and ψ dihedral angles of 80.03, 78.46 and 153.97°, orients the two groups into the same direction (where ϕ is defined as CO-N-C-C, θ as N-C-C-CO and ψ as C-C-CO-N torsional angle[29]). With no side chains, the two methylene carbons in each monomer, not restricted by the $A^{1,3}$ strains,[30] contribute to the ring flexibility and thus allows more densely packing to maximize favorable van der Waals interactions. In addition, the crystal packing is stabilized through intermolecular hydrogen-bonding patterns similar to an antiparallel β-sheetlike strand with an intersubunit N–O distance of 2.88 and 2.92 Å.

Theoretical studies of all-(*S*)-cyclo(β^3hAla)$_4$ (20): Prior to the NMR spectroscopic analysis of the cyclo-β-peptides synthesized, we initiated a theoretical investigation of cyclo(β^3-hAla)$_4$ (**20**) as a simplified model for β^3-tetrapeptide **14**. The objective of this study was to gain more quantitative information on the conformers expected for this peptide than provided by the conformational averaged structure which would be derived from the NMR spectroscopic measurements.

Initially, the conformational space of this model peptide was sampled through an unrestrained Monte Carlo simulation and the resulting approximately 2000 structures were clustered into five families. One member from each family was extracted and optimized with a density functional method (B3LYP/6-31G(d,p)). The optimized structures, as shown in Figure 3, are characterized by different backbone conformations. Two conformers, **A** and **B**, have a uniform orientation of all amide groups, but differ in backbone and side chain conformations. Conformer **B** is highly symmetrical with all side chains in equatorial positions on the peptide ring. Conformer **A**, on the other hand, is approximately C_2 symmetrical with two of the side chains bent upwards, relative to the plane of the ring. Conformer **C** is characterized by three uniformly oriented amide groups, while one amide NH now is pointing in the opposite orientation. This leads to an energetically disfavored NH–CH(β) staggered interaction, but allows one intramolecular hydrogen bond to be formed.

Conformers **D** and **E** have two amide groups oriented "up" and two "down" relative to the plane of the ring. The orientation of the amide groups in conformer **D** is alternating, while the orientation in conformer **E** is non-alternating (cf. the orientation of the amide groups in the crystal structure of the unsubstituted cyclo(β-Ala)$_4$ **4**). Both conformers are destabilized by two staggered NH–CH(β) interactions, which forces the side chains to adopt an axial orientation with resulting 1,3-strain to CH$_2$(α) and the carbonyl oxygen of the following residue. Conformer **D** is stabilized by two intramolecular hydrogen bonds, while conformer **E** forms only one hydrogen bond between the neighboring amide groups.

The relative energies of these conformers in the gas phase may be readily explained by the stabilizing hydrogen bonds and the destabilizing steric interactions discussed above. In the gas phase, conformer **D** is most stable due to the lack of a net dipole moment, while conformers **A** and **B** are strongly disfavored by their large macrodipole moments. This situation is completely reversed when the relative energies are calculated in water using the SCRF solvent model. Now, the symmetrical conformer **B** is the most stable due to a minimum of repulsive steric interactions, and an efficient stabilization of the peptides macrodipole by the solvent. According to this DFT calculation, conformer **B** is about 10 kcal mol^{-1} more stable than the other low energy conformations investigated. These calculations suggest that all the conformers investigated are populated at room temperature, assuming a low activation enthalpy for rotation of the amide groups, but that conformer **B** should be the most predominant conformation in solution.

The five theoretical conformers found for cyclo(β^3hAla)$_4$ (**20**) were evaluated one at a time in order to get a higher comprehension of which NOE is arising from which conformer. With this information we can more easily determine the major conformer of the cyclic β^3-tetrapeptide when looking at a recorded ROESY spectrum of **14**.

Starting with the two conformers, where all the amide bonds are directed in the same orientation, the residues would either adopt a structure with completely symmetrical shape **B**, or one that gives an unsymmetrical oval cyclic peptide **A**. The four amino acid residues in conformer **B** would give rise to identical NOE signals; all NH- and axial α-CH protons would show NOE signals to neighboring axial α-protons. The NOE signals expected for conformer **A** would be a special case of conformer **B** in which some effects would be stronger, while others would be weaker.

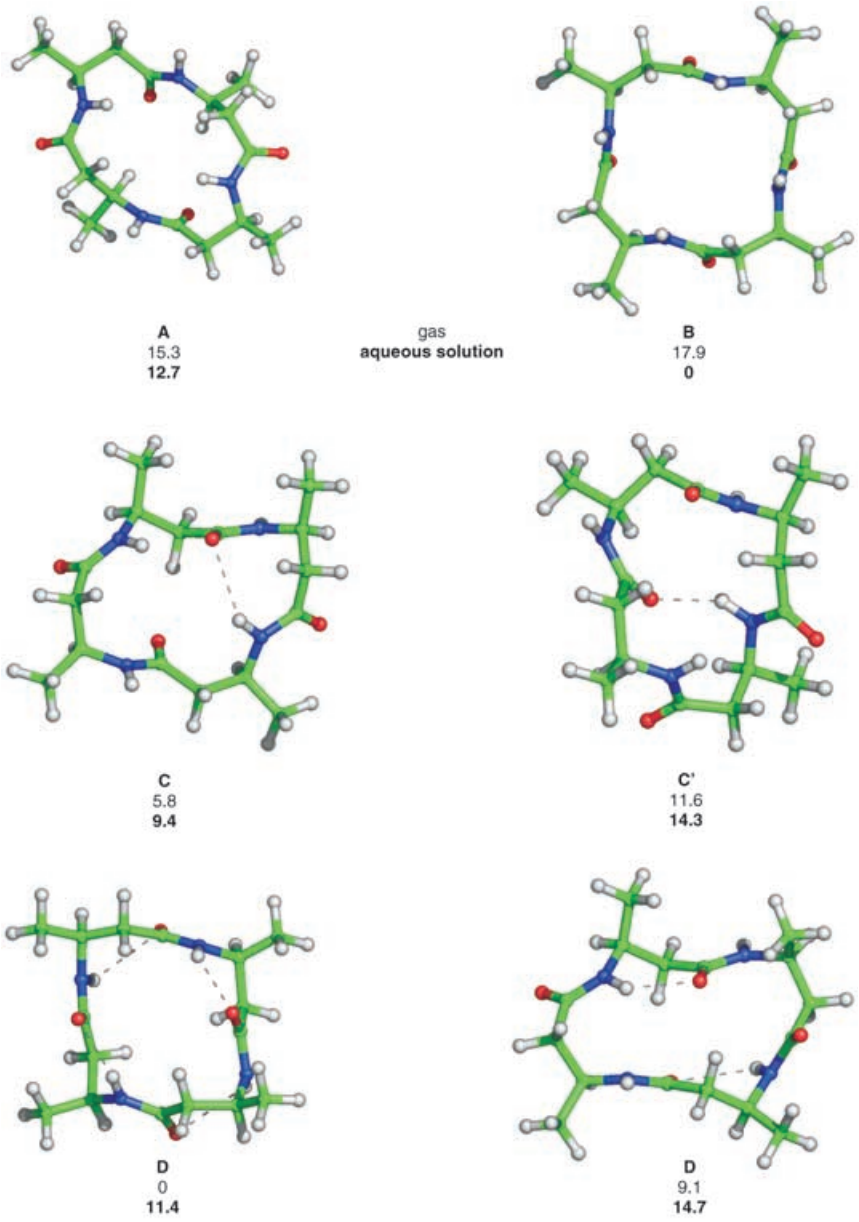


Figure 3. Calculated relative energies [kcal mol^{-1}] for the model peptide cyclo(β^3hAla)$_4$ (**20**) in the gas phase (B3LYP/6-31G(d,p)) and in aqueous solution (B3LYP/6-311+G(d,p); SCRF solvation model).

Regarding the amide bonds in these two conformers, they are all positioned in an almost 180° angle relative to the β-proton, differing slightly between the four amino acids. Conformer **B** would give rise to symmetrical coupling constant values, whereas the coupling constants of conformer **A** would fluctuate within a small range.

One of the amino acid residues in conformer **C** has one of its amide bonds in a different orientation, compared to the other three. The four NH–CH(β) angles would be significantly different in this case, since one of the angles is close to 90°, which would lead to one amide proton having a coupling constant close to 0 Hz.

Two of the amide protons are rather close, which should give an NOE signal between those two. NH–CH$_2$(α) NOE signals would arise between the same residues. For the β-protons, only one of these would be expected to have a NOE to an α-proton positioned on another residue. Further, since one of the NH–CH(β) angles reaches an angle close to 0°, the NOE arising between these two protons are expected to be strong. The other residues would be expected to give rise to weak inter-residue NOE signals.

The discussion regarding conformer **D** and **E** are quite similar each other, since both conformers have two β^3hAla residue amide bonds in an up-orientation and two in a down-orientation. The NOE signals arising between the CH(β) and the two α-protons on the neighboring carbon are expected to be different whether they are positioned on the up- or down-oriented residues. The first scenario will show stronger NOE between CH$_2$(α) *pro-S* and CH(β) than between CH$_2$(α) *pro-R* and CH(β), while the latter one gives rise to two NOE signals of similar strength. The NH–CH(β) protons on the down-oriented residues are in almost 0° relative each other, and the NOE signals arising are therefore expected to be strong. As a last observation, the possible NH–CH(β) angles of conformation **D** and **E** are either close to 180 or 0°, which both would result in large coupling constants.

Also included in Figure 3 is a structure resembling that found in solution by Seebach and co-workers for a somatostatin mimicking cyclic β^3-tetrapeptide,[5b] that is, **C**′. This molecule was not found in the original molecular mechanics based Monte Carlo sampling, but was included for comparison. The optimized geometry shown in Figure 3 is of comparable energy as the other conformers investigated (by DFT calculation); all attempts to build a "flat" structure, more similar to the one reported, resulted in the structure shown or structures similar to **C**, that is, with hydrogen bonds between neighboring residues, after unrestrained optimization. The structures **C** and **C**′ are also expected to have similar NOE signals.

NMR Spectroscopic investigations: A detailed 2D-NMR spectroscopic study was undertaken to obtain high-resolution data on the conformational performance of the cyclic β-peptides **14** and **19**. The studies were made in methanol for both peptides, whereas only the conformation of the pentapeptide **19** could be established in water, due to solubility problems of **14** in this solvent.

All spectra were recorded at 500 MHz by using 80% non-deuterated solvent and solvent suppression by using the WET pulse sequence.[31] A complete assignment of all ^{1}H resonances was possible by using TOCSY,[31] P.E. COSY,[32] and ROESY[33] experiments. The assignment of ^{13}C spectra was either fully or partially established by using gHMBC[34] technique. The sequence of **19** could also be established by the use of this experiment, distinguishing β^{3}hLys4 and β^{3}hLys5 from each other.

Solution structure of cyclic β^3-tetrapeptide 14: The amide region of the ^{1}H NMR spectrum of **14** in methanol is shown in Figure 4a. Traces of extraneous signals are visible which probably stem from alternative conformers, indicating the presence of one major conformer. But we can not completely exclude the option that impurities are causing these signals, although the peptide was pure according to LC-MS analysis. All ^{3}J(NH,CH(β)) coupling constants show high values (7.6, 8.4, 8.6 and 9.4 Hz, respectively), which suggests an *anti* arrangement between the amide and the β-protons of the amino acid residues. The coupling constants also indicate an unsymmetrical shape of **14**, as the size of the coupling constants and thereby the torsional angles differ significantly.

The diastereotopic α-protons could be assigned from the P.E. COSY, assuming that the coupling constants between the β-proton and the *pro-R* (equatorial) α-protons are small and that the *pro-S* (axial) α-protons have a large coupling constant to CH(β). The cross-peaks in the P.E. COSY are thus assigned to be the *pro-S* protons. Even though the *pro-S* and *pro-R* protons could be distinguished from each other, the coupling constants for CH(β)–CH$_2$(α) could not be determined, neither from the 1D ^{1}H NMR nor from the P.E. COSY spectra.

The indication that **14** possesses an unsymmetrical shape was not only assumed from the NH–CH(β) coupling constants mentioned above, but also emerged when analyzing the ROESY spectra. The observed NOE signals differ too much from the ones expected for a symmetric molecule (see above); this leads to the exclusion of conformer **B** as the major conformer, even though this is lowest in energy according to the theoretical calculations. The requirement to have not only different but also large values of the NH–CH(β) coupling constants make conformer **C** a non-likely option as the major conformer, since this would require an NH–CH(β) coupling constant close to 0 Hz.

That the major conformer would have all amide bonds pointing at the same directions seems most likely when analyzing the ROESY spectrum of **14** (Figure 4b). In the spectrum, β^{3}hGln in position 4 gives rise to weak NOE signals

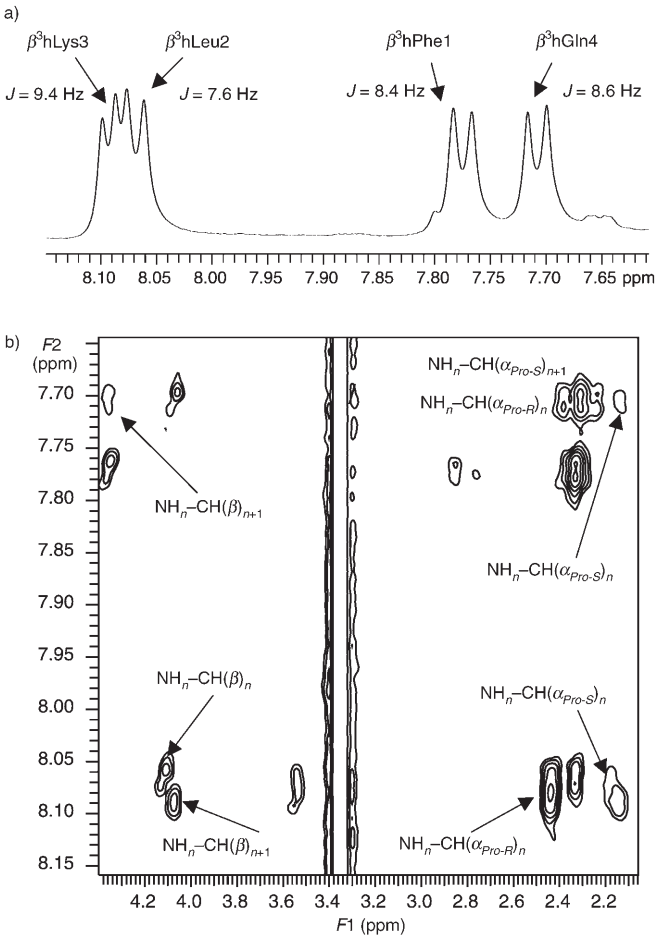


Figure 4. a) Expansion of the amide region of the ^{1}H NMR spectrum of **14** recorded in methanol. b) Expansion of the amide region of the ROESY spectrum of **14** recorded in methanol. Some of the NOE signals discussed in the text are highlighted.

between NH and *pro-S* α-proton, and strong NOE signals between NH and *pro-R* α-proton. This, in combination with the opposite observations for β^{3}hLys in position 3, indicates that one conformation possesses an unsymmetrical shape. The region of NH–CH(β) on the other hand shows signs of dynamics, as NOE signals are observed between two amide protons and their neighboring β-protons.

The conclusions made from the comparison of the five theoretically calculated conformers and the observed NOE signals of **14** are that the β^3-tetrapeptide most likely adopts an oval cyclic conformer having all amide bonds pointing in the same direction, that is, similar to conformer **A**. Of course, it should be noted that these five possible conformations of cyclo(β^{3}hAla)$_4$ (**20**) do not differ significantly in energy, and some observed intra-residue NOE signals contradict the conformer supported by the other NOE signals.

A total of 29 NOE signals were classified into three categories with the following upper bound distance limits: strong 3.0 ± 1 Å, medium 4.0 ± 1 Å and weak 5.0 ± 1 Å, and used as such together with four (NH, CH(β)) dihedral

angle restraints, for a restrained Monte Carlo conformational search in Macromodel 7.0 (Figure 5).[35] The four H-N-CH(β) torsional angles were constrained according to a modified Karplus curve,[36] the β^{3}hPhe in position 1 and the β^{3}hGln in position 4 were assigned the angle of 151 ± 20°, while the β^{3}hLeu in position 2 and the β^{3}hLys in position 3 were assigned 144 ± 20° and 162 ± 20°, respectively.

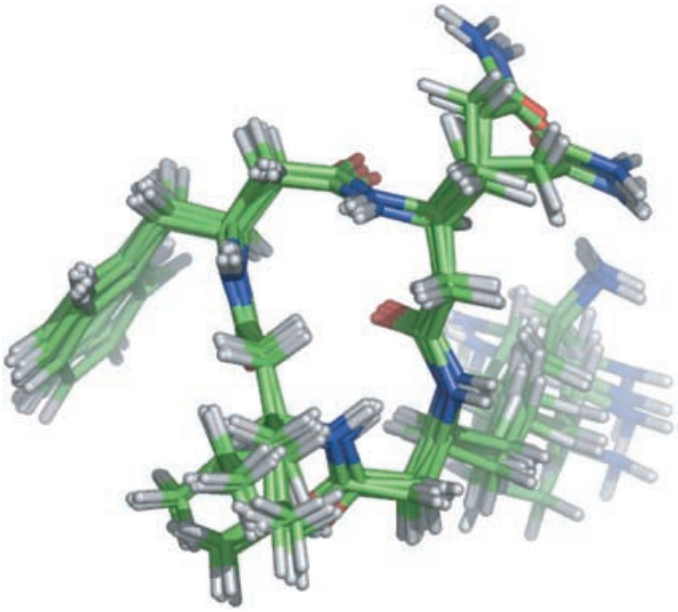


Figure 5. Solution structure of the cyclic β^3-tetrapeptide **14** in MeOH represented as a bundle of six lowest-energy structures obtained from Monte Carlo search, using NMR dihedral-angles and NOE distance restraints. View from the top.

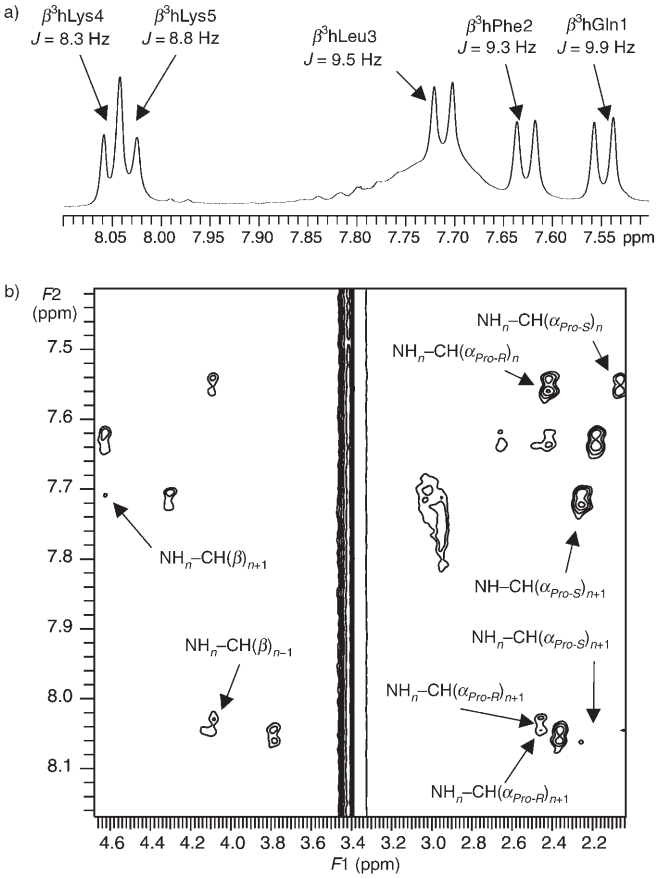


Figure 6. a) Expansion of the amide region of the ^{1}H NMR spectrum of **19** recorded in methanol. b) Expansion of the amide region of the ROESY spectrum of **19** recorded in methanol. Some of the NOE signals discussed in the text are highlighted.

From this conformational search it could be concluded that even though **14** most probably fluctuate between oval and very symmetrical conformations, it preferably reaches the boat-shaped oval structure (Figure 5). We could also conclude that even though the peptide possesses a relatively high flexibility, the amide bonds do not have such degree of free rotation to form the conformation where the amide bonds are pointing in different directions. This finding is also supported by a preliminary molecular dynamics simulation, which shows that the molecule eventually adopts a parallel orientation of the amide groups independently of the starting conformation.

Solution structure of cyclic β^3-pentapeptide 19: An expansion of the amide region of the ^{1}H NMR spectrum of **19** in methanol is shown in Figure 6a. Broad unresolved signals are seen in the amide region similar to the spectrum of **14**. The major signals are caused by one conformer or conformational family, the minor ones either by disfavored conformers or by impurities (although the peptide was pure according to LC-MS data). The size of the coupling constants (8.3–9.9 Hz) indicates an *anti* arrangement of the NH–CH(β) protons. The difference in size of the coupling constants for the amide protons is similar to those of cyclopeptide **14**. This indicates, as expected, that **19** possesses an unsymmetrical shape as well.

The assignment of the protons of **19** was made using the same experiments as for **14**; a full assignment of the carbons could not be done. By using the ROESY spectra obtained in methanol (Figure 6b), the first Monte Carlo conformational search of **19** was made. A total of 32 NOE signals were extracted and classified in the same way as the NOE signals of **14**.

The amide region of **19** is shown in Figure 7 as an expansion of the recorded ^{1}H NMR spectrum in water. The chemical shifts have changed somewhat for all amino acids except for β^{3}hPhe and β^{3}hLeu; the chemical shift of the signal belonging to β^{3}hVal slightly shifted to lower field, while the signals for β^{3}hLys4 and β^{3}hLys5 shifted to higher field. Not only chemical shifts but also the coupling constants are different in water compared with methanol. Except for the coupling constant of β^{3}hLeu, which has decreased in size, all amino acids have an increased value. Similar to the amide region of **19** recorded in methanol, the

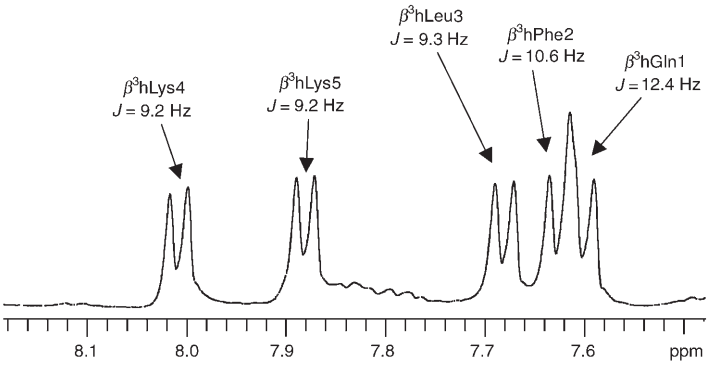


Figure 7. Expansion of the amide region of the ^{1}H NMR spectrum of **19** recorded in water.

broad baseline close to the signal of β^{3}hLys5 indicates the existence of more than one major conformer.

The assignment of the protons of **19** in water was also made. The flexibility observed for the ROESY spectrum in methanol could be seen in water as well. Again, a number of NOE signals (47) were extracted from the ROESY spectrum, this time obtained in water. Running the Monte Carlo conformational search according to the same principles used earlier, a possible representative structure of the mean conformation of **19** in water was found (Figure 8c).

From the Monte Carlo search of the cyclic β^3-pentapeptide in methanol, three different families of conformations were found (Figure 8a and b). All these families possess high flexibility in their backbone structure, and the amide bonds do no longer have a unidirectional arrangement, as was the case for the β^3-tetrapeptide **14**. Between the two amino acids β^{3}hVal in position 1 and β^{3}hLys in position 5 a turn similar to the β-turn arisen from a β^2/β^3-sequence[37] is observed. Although it seems likely that such turns could occur between any two consecutive amino acids in the ring, the data only showed a turn between the β^{3}hVal and the β^{3}hLys; this suggests that the α-branching at the side chain β^{3}hVal may contribute somehow.[38]

In water, the number of different conformational families has been reduced by one. These structures also seem to be more similar to each other in the backbone conformation. The high flexibility observed in methanol remains, which is clearly indicated by the completely opposite direction of the amide bonds at one position (Figure 8c, position marked by an arrow). Reflecting on the amide bonds in the two families, one has them in rather defined down- and up-orientations, whereas the other has its bonds in more irregular orientations.

CD Spectroscopy: CD spectra (Figure 9) were measured for all cyclo-β-peptides synthesized. Although circular dichroism is a powerful tool for the determination of secondary structures of β-peptides, it is still an empirical method and assured data exist only for linear β-peptides capable to form helices[1a] and turns.[39] The only reports on CD spectra of

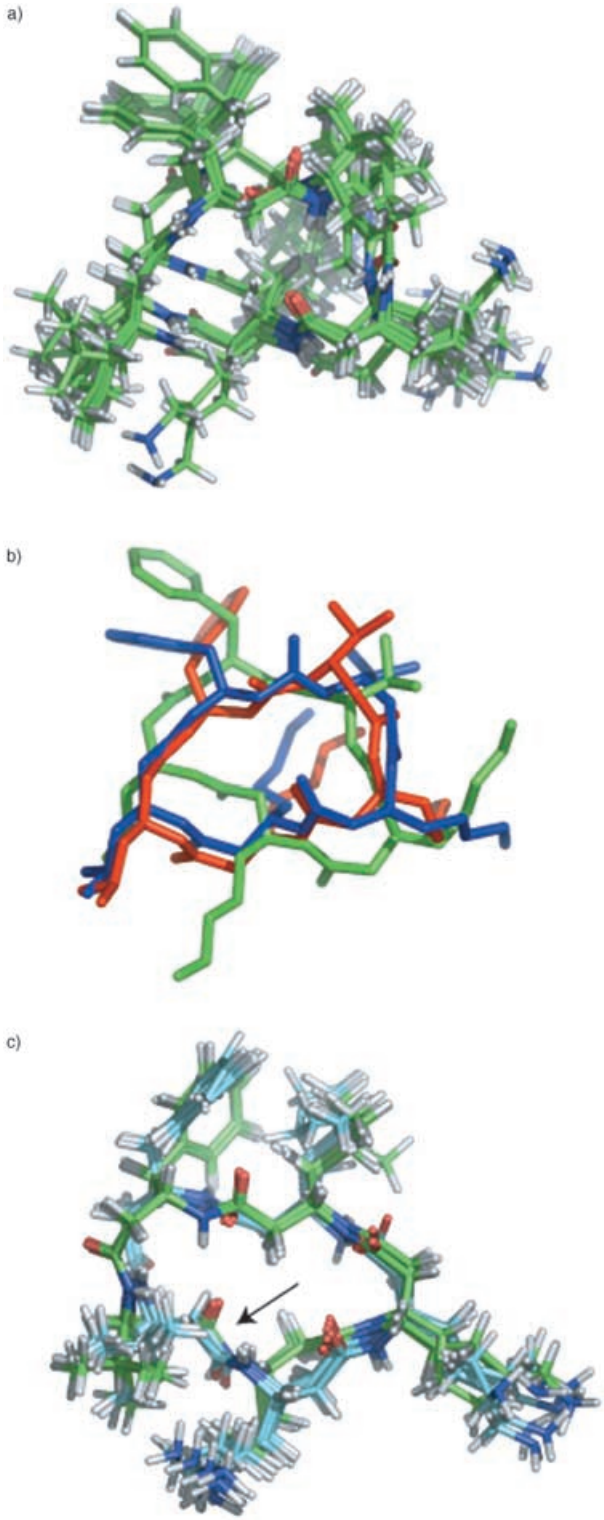


Figure 8. Solution structure of the cyclic β^3-pentapeptide **19** in MeOH (a and b) and in H_2O (c). a) The result of the conformational search of the β-pentapeptide in methanol as a bundle of 12 low-energy structures. Three different families were detected. b) Superposition of the backbones of the three conformational families. c) The results of the investigation in water as a bundle of six low-energy structures, where two different conformational families were found. The arrow indicates the position of a completely reversed direction of one amide bond.

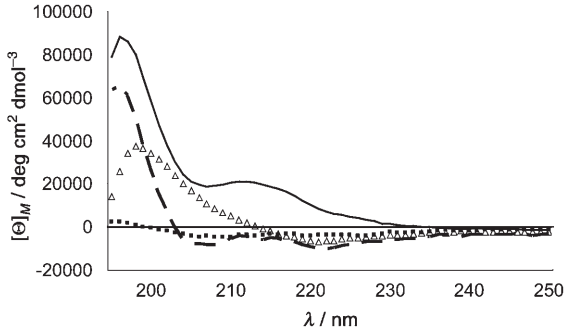


Figure 9. CD spectra of cyclic β-peptides (0.1 mM, 24 °C), ——: cyclic β^3-pentapeptide **19** in MeOH, -----: cyclic β^3-pentapeptide **19** in water, △: cyclic β^3-tetrapeptide **14** in MeOH, •••••: cyclo(β-Ala)$_4$ (**4**) in water.

some cyclic β-peptides are that of Seebach and co-workers.[5b,40,41]

The cyclic β^3-pentapeptide **19** displays distinct Cotton effects in both methanol and water. One intense maximum at 196 nm and an additional maximum at 211 nm are observed in methanol. But a drastic change in the spectrum of **19** in water is noted: the first maximum at 196 nm is sustained, but after one zero crossing at 203 nm two minima occur: one at 208 nm and the other at 222 nm. The differences in the spectra clearly indicate a major change in the secondary structure of **19** dependent on the solvent and may reflect the reduction from three to two conformational families when switching from methanol to water as found in the NMR studies.

The cyclic β^3-tetrapeptide **14** shows one maximum at 198 nm which is less pronounced than for **19**, and one weak minimum at 221 nm after a zero crossing at 213 nm. This pattern is quite similar to that of the tetrapeptide cyclo-(β^3hAla)$_4$ (**20**).[41] Both spectra resemble that obtained from linear β^3-peptides forming (*M*)-3$_{14}$ helices.[42] Contrarily, the CD pattern of cyclo(-β^3hPhe-β^3hThr-β^3hLys-β^3hTrp-),[5b] shows a CD spectrum similar to that observed for the 12/10-helix,[1a,3d] or for the β-peptide hairpin,[39] because of an intramolecular hydrogen bond which divides the ring[5b] (cf. structure **C′** in Figure 3). These observed differences suggest that cyclo-β^3-tetrapeptides can fold in different conformations, maybe depending if their side chains are branched or not.

Cyclo(β-Ala)$_4$ (**4**) exhibits almost no Cotton effect in agreement with the lack of both chiral groups and a preferred secondary structure, which supports our findings by NMR spectroscopy.

Conclusion

The synthesis of cyclo(β-Ala)$_4$ (**4**) including conventional solution-phase synthesis and cyclization in solution is reported. Both the NMR and CD spectra of this simple cyclic β-peptide points to a mixture of conformers in solution, whereas the X-ray structure of **4** shows the formation of column-like supermolecular structures in the solid state, due to extensive intermolecular hydrogen bonding. One molecule of cyclo(β-Ala)$_4$ adopts in the crystal state a conformation with two C=O bonds pointing down and two up with respect to the average ring plane, similar to the structure of (*R*,*R*,*S*,*S*)-cyclo-(β^3hAla)$_4$. We also demonstrate that the scope of the on-resin cyclization protocol could be broadened to obtain cyclic β-peptides by anchoring the side chains of Fmoc-β^3hGlu-OAll and Fmoc-β^3hLys-OAll to the solid support via Rink-amide linker and an active carbonate linker, respectively. The target compounds cyclo(-β^3Phe-β^3hLeu-β^3hLys-β^3hGln-) (**14**) and cyclo(-β^3hVal-β^3hPhe-β^3hLeu-β^3hLys-β^3hLys-) (**19**) could be obtained in overall yields of 10 and 9 %, respectively. The conformationally averaged structures of the synthesized cyclic β^3-tetrapeptide **14** in aqueous solution were calculated based on NMR investigations. The conformational search resulted in a set of predominant similar structures with predictable up-orientation of the amide bonds and a pseudo-equatorial orientation of the side chains. Although **14** shows flexibility, it seems limited enough to render it potentially useful as a molecular scaffold. In contrast to this, the cyclic β^3-pentapeptide **19** displays a more flexible backbone ring structure including a β-turn both in water and in methanol.

Experimental Section

Abbreviations: All: allyl, Boc: *tert*-butoxycarbonyl, Bzl: benzyl, DBU: 1,8-diazabicyclo[5.4.0]undec-7-ene, DIEA: *N*,*N*-diisopropylethylamine, DMAP: 4-(dimethylamino)pyridine, EDC: 1-[3-(dimethylamino)propyl]-3-ethylcabodiimide hydrochloride, Fmoc: (9*H*-fluoren-9-yl)methoxycarbonyl, HBTU: 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, HOBt: 1-hydroxy-1*H*-benzotriazole, NMM: 4-methylmorpholine, PyAOP: 7-azabenzotriazol-1-yl-oxytris(pyrrolidino)phosphonium hexafluorophosphate, PyBOP: benzotriazole-1-yl-oxytris(pyrrolidono)phosphonium hexafluorophosphate, TFA: trifluoroacetic acid, TFE: trifluoroethanol, TIPS: triisopropyl silane, TNBS: 2,4,6-trinitrobenzenesulfonic acid.

Solvents and reagents: All α-amino acid derivatives, HOBt and HBTU were purchased from Senn Chemicals AG, Switzerland. PyAOP was from Applied Biosystems (USA). DMF (peptide synthesis grade) was used from Scharlau Chemie S.A. Dichloromethane was distilled under nitrogen from powdered CaH_2. Methanol and acetonitrile were HPLC grade and were purchased from VWR International Ltd., England. TentaGel NH_2 resin (0.24 mmol g^{-1}) and TentaGel S PHB resin (0.29 mmol g^{-1}) were purchased from Rapp Polymere GmbH, Germany.

Instruments: Solid-phase peptide synthesis was performed on a Quest 210 synthesizer, Argonaut Technologies (USA). HPLC purifications of the peptides were run on a Gilson system (Gilson 215 Liquid Handler, Gilson UV/VIS-152, Gilson 322 Pump). Analytical runs were performed on a Phenomenex Luna C8(2) column (100 × 4.60 mm, 5 μm), preparative runs on a Phenomenex Luna C8(2) column (250 × 21.20 mm, 10 μm). HPLC-MS runs were made on the same Gilson system coupled to a Finnigan AQA Thermo Quest mass spectrometer with electrospray ionisation. Solvents for HPLC: A = 0.1 % TFA in water, B = 0.1 % TFA in acetonitrile. NMR spectra were recorded on a Varian Unity 500 (^{1}H at 500 MHz, ^{13}C at 125.8 MHz) or a Varian Unity 400 (^{1}H at 400 MHz, ^{13}C at 100.5 MHz) or a Varian Mercury plus (^{1}H at 300 MHz, ^{13}C at 75.4 MHz) spectrometer. Measurements were made at ambient temperature (unless otherwise stated) using the residual solvent signal as internal reference. Peptides were analyzed by Matrix-assisted Laser Desorption

(MALDI) with an Ultraflex Tof/Tof instrument (Bruker, Germany) operated in positive and reflectron mode. α-Cyano-4-hydroxycinnamic acid was used as matrix and the instrument was calibrated using matrix ions. All electrospray mass spectra were acquired by using a Bruker Daltonics BioAPEX-94e superconducting 9.4 T FTICR mass spectrometer (Bruker Daltonics, Billerica, MA) in broadband mode. A home-built apparatus controlled the direct infusion of sample. The sample was delivered using a helium gas container at a pressure of 1.3 bar, pushing the sample through a 30 cm fused silica capillary of inner diameter 20 μm. The sample end of the capillary was lowered into the sample tube inside the pressurized container and the electrospray end was coated by a conducting graphite/polymer layer and connected to ground.[43,44] No sheath flow or nebulizing gas was used and the flow rate was approximately 100 nL min^{-1}. The ion source was coupled to an Analytica atmosphere/vacuum interface (Analytica of Branford, CT) and a potential difference of 2–4 kV was applied across a distance of approximately 5 mm between the spraying needle and the inlet capillary. Infrared spectra were obtained from a Perkin–Elmer 1760 IR FT spectrometer. CD spectra were measured with a Jasco J-810 spectropolarimeter ($c=0.1$ mM, 24 °C). UV spectra were recorded on a Varian Cary 3 Bio spectrophotometer. X-ray Crystallographic Analysis was carried out on a Bruker-Nonius kappaCCD diffractometer. For TLC, Alugram SIL G/UV$_{254}$ silica sheets from Macherey–Nagel were used. Flash Chromatography was carried out on silica gel (35–70 μm) from Millipore Corp. Melting points were determined with a melting point apparatus SMP 10 from Stuart Scientific/Bibby Sterilin Ltd. and are uncorrected.

The Fmoc-β^3-amino acid derivatives Fmoc-β^{3}hGlu(O*t*Bu)-OAll, Fmoc-β^{3}hLeu-OH, Fmoc-β^{3}hLys(Boc)-OAll, Fmoc-β^{3}hPhe-OH, Fmoc-β^{3}hVal-OH were prepared via Arndt–Eistert homologation as described in references.[18,19] The Wolff rearrangement to the carboxylic acids was facilitated with a High Intensity Ultrasonic Processor VCX 500 from Sonics Materials Inc.

H-β-Ala-OBzl·HCl: H-β-Ala-OH (8.90 g, 100 mmol), benzyl alcohol (50 mL) and chlorotrimethylsilane (20 mL) were heated to 100 °C for 4 h. The reaction mixture was cooled to room temperature and was poured into diethyl ether (2 L) and subsequently cooled in an ice bath for 24 h. The resulting precipitate was isolated by filtration. The benzyl protected β-alanine was obtained as hydrochloric salt (20.5 g, 95 mmol, 95 %) and used without further purification.

Boc-β-Ala-β-Ala-OBzl (2): Boc-β-Ala-OH (**1**) (7.57 g, 40.0 mmol) was dissolved in dry THF (400 mL) under an argon atmosphere. The solution was cooled to −15 °C and TEA (11.7 mL, 83 mmol) and isobutyl chloroformate (5.2 mL, 40.0 mmol) were added. The mixture was stirred for 15 min and H-β-Ala-OBzl (8.62 g, 40.0 mmol) was added. The reaction mixture was allowed to warm to room temperature and was stirred for 3 d. The solvent was evaporated and the residue was dissolved in ethyl acetate (800 mL). The organic phase was washed successively with H_2O, 1 N citric acid, H_2O, sat. $NaHCO_3$ and brine, subsequently dried with anhydrous $MgSO_4$ and the solvent was evaporated. The residue was recrystallized from ethyl acetate and heptane to give dipeptide **2** as colorless crystals (11.4 g, 3.24 mmol, 81 %). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.45 (s, 9H; Boc), 2.35 (t, 2H, $J=7.5$ Hz; CH_2), 2.61 (t, 2H, $J=7.5$ Hz; CH_2), 3.40 (q, 2H, $J=7.5$ Hz; CH_2), 3.56 (q, 2H, $J=7.5$ Hz; CH_2), 5.16 (s, 3H; CH_2-Bzl, NH), 6.08 (s, 1H; NH), 7.2–7.5 (m, 5H; Bzl).

Boc-(β-Ala)$_4$-OBzl (3): This compound was prepared previously by a similar method.[45] Boc-β-Ala-β-Ala-OBzl (**2**) (3.52 g, 10.0 mmol) was added to a mixture of TFA and CH_2Cl_2 (1:1, 10 mL) at 0 °C and the reaction mixture was stirred for 1 h at 0 °C and additionally for 1 h at room temperature. The solvent was evaporated and β-Ala-β-Ala-OBzl·TFA was obtained and used without further purification. Boc-β-Ala-β-Ala-OBzl (**2**) (3.52 g, 10.0 mmol) and 10 % Pd/C (0.35 g) were added to a mixture of THF and CH_3OH (1:1, 200 mL) and hydrogenated for 12 h at atmospheric pressure. Control of the reaction by TLC (Boc-β-Ala-β-Ala-OBzl, $R_f=0.73$, Boc-β-Ala-β-Ala-OH, $R_f=0.38$, $CHCl_3/CH_3OH/AcOH$ 90:8:2) revealed the completeness of the deprotection. Pd/C was removed by filtration and the solvent was evaporated. The residue was dissolved in dry THF (100 mL) under an argon atmosphere and the solution was cooled to −15 °C. TEA (4.68 mL, 33 mmol) and isobutyl chloroformate (1.3 mL, 10 mmol) were added and stirring was continued for 15 min. All of the H-β-Ala-β-Ala-OBzl·TFA obtained before was added and the reaction mixture was stirred for 3 d at room temperature. The solvent was evaporated and the residue was purified by flash chromatography ($CH_2Cl_2/CH_3OH/AcOH$ 90:8:2). The linear tetrapeptide **3** was obtained as colorless solid (3.12 g, 6.3 mmol, 63 %). $R_f=0.36$ ($CHCl_3/CH_3OH/AcOH$ 90:8:2); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.42 (s, 9H, Boc), 1.86 (br s, 4H, CH_2), 2.34 (br s, 6H, CH_2), 3.51 (m, 6H, CH_2), 5.13 (s, 2H, CH_2-Bzl), 5.33 (br s, 1H, NH), 6.52 (br s, 1H, NH), 6.68 (m, 2H, NH), 7.32–7.39 (m, 5H, Bzl).

Cyclo(β-Ala)$_4$ (4): Boc-(β-Ala)$_4$-OBzl (**3**) (1.23 g, 2.50 mmol) and 10 % Pd/C (120 mg) were added to a mixture of THF and H_2O (2:1, 150 mL) and hydrogenated for 12 h at atmospheric pressure. Control of the reaction by TLC (Boc-(β-Ala)$_4$-OBzl, $R_f=0.6$, Boc-(β-Ala)$_4$-OH, $R_f=0.3$, $CHCl_3/CH_3OH/AcOH$ 20:4:1) revealed the completeness of the deprotection. Pd/C was removed by filtration and the solvent was evaporated. Boc-(β-Ala)$_4$-OH was obtained as a colorless solid and further used without purification (991 mg, 2.46 mmol, 98 %).

Boc-(β-Ala)$_4$-OH (402 mg, 1.0 mmol) was dissolved in DMF (100 mL) and the solution was cooled under an atmosphere of argon to 0 °C. Pentafluorophenol (193 mg, 1.05 mmol) and DIEA (18 μL, 1.1 mmol) were added. The reaction mixture was allowed to warm to room temperature and stirred for further 16 h. The DMF was evaporated and the residue was taken up in dichloromethane and washed with 1 M HCl and brine. The solvent was evaporated to give the activated ester as a colorless solid.

This intermediate was deprotected by stirring for 1 h at 0 °C and subsequently for 1 h at room temperature in a mixture of CH_2Cl_2 and TFA (1:1, 2 mL). After evaporation the residue was dissolved in dry THF (20 mL) and was slowly added via syringe pump over 8 h to a mixture of DIEA (260 μL, 1.6 mmol) in dry THF (400 mL) at 60 °C. The stirring was continued for further 24 h at room temperature. The resulting precipitate was filtered and washed with THF to give cyclic β-tetrapeptide **4** (164 mg, 0.58 mmol, 58 %). ^{1}H NMR (500 MHz, D_2O): δ = 2.28 (dd, 8H, $^3J=5.5$ Hz; CH_2), 3.32 (br q, 8H, $^3J=5.5$ Hz; CH_2), 7.92 (br s, 4H; NH); ^{13}C NMR (125 MHz, D_2O): δ = 174.5 (C=O), 35.8 (CH_2), 35.6 (CH_2).

Colorless crystals of **4** suitable for X-ray crystallography were grown from a mixture of MeOH/H_2O at room temperature (Table 1).

CCDC-264 893 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

(*S*)-3-(9*H*-Fluoren-9-ylmethoxycarbonylamino)-hexanedioic acid 1-allyl ester 6-*tert*-butyl ester (Fmoc-β^{3}hGlu(O*t*Bu)-OAll) (7): Fmoc-Glu(O*t*Bu)-CHN_2 (**5**)[18] (660 mg, 1.47 mmol) was dissolved in dry THF (10 mL) and allyl alcohol (0.15 mL, 2.25 mmol) was added. The mixture was cooled to −15 °C and a solution of silver benzoate (34 mg, 0.15 mmol) in NMM (0.33 mL, 3 mmol) was added. The reaction mixture was stirred and warmed to room temperature. After 5 h the mixture was filtered through a pad of Celite and the THF was removed under reduced pressure. The residue was dissolved in ethyl acetate and successively washed with saturated $NaHCO_3$, water and 5 % HCl. After drying with $MgSO_4$ the solvent was evaporated under reduced pressure. The residue was purified by flash chromatography (pentane/ethyl acetate 4:1) and yielded allyl ester **7** (456 mg, 0.98 mmol, 67 %). $R_f=0.68$ (pentane/ethyl acetate 3:1); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.44 (s, 9H; *t*Bu), 1.86 (m, 2H; CH_2), 2.31 (m, 2H; CH_2COOAll), 2.61 (d, 2H, $^2J=5$ Hz; CH_2COO*t*Bu), 4.00 (m, 1H; C*H*NH), 4.20 (t, 1H, $^3J=7.5$ Hz; C*H*CH_2O), 4.37 (m, 2H; CHCH_2O), 4.60 (d, 2H, $^2J=6$ Hz; CHOCH_2), 5.23 (dd, 1H, $J=10.5$, 1 Hz; CH=CH_2), 5.30–5.35 (m, 3H; CH=CH_2, NH), 5.90 (m, 1H; C*H*=CH_2), 7.31 (dt, 2H, $^3J=7.5$, $^4J=1$ Hz; Fmoc), 7.40 (t, 2H, $^3J=7.5$ Hz; Fmoc), 7.59 (d, 2H, $^3J=8$ Hz; Fmoc), 7.76 (d, 2H, $^3J=7.5$ Hz; Fmoc); ^{13}C NMR (100.5 MHz, $CDCl_3$): δ = 28.1 (*t*Bu), 29.2, 32.3, 39.1, 47.3 (CH), 47.9 (CH), 65.4 (CH_2), 66.7 (CH_2), 80.6 (*t*Bu), 118.6 (CH_2 allyl), 119.9, 125.1, 127.0, 127.7 (4×CH Fmoc), 131.8 (CH allyl), 141.3, 143.9 (2×C Fmoc), 155.8 (OCONH), 171.0 (CO), 172.5 (CO); IR (KBr): $\tilde{\nu}$ = 3363 (m), 2975 (m), 1734 (s), 1689 (s), 1521 (s), 1452 (m), 1368 (m), 1246 (m), 1155 (s), 761 (m), 739 cm^{-1} (m); HR ESI-MS: *m*/*z*: calcd for $C_{28}H_{33}NO_6$: 479.231; found: 480.238 $[M+H]^+$, 502.220 $[M+Na]^+$.

Table 1. Crystallographic data for **4**.

empirical formula	$C_{12}H_{20}N_4O_4$
M_r	284.316
radiation	$Mo_{K\alpha}$
crystal system	monoclinic, colorless prism
λ [Å]	0.71073
space group	$P2_1/c$
a [Å]	5.291(2)
b [Å]	10.4820(7)
c [Å]	12.6910(8)
α [°]	90.00
β [°]	106.391(4)
γ [°]	90.00
θ [°]	0.998–29.57
V [Å^3]	675.24(7)
Z	2
T [K]	298
μ [mm^{-1}]	0.106
ρ_{calcd} [g cm^{-3}]	1.3982
θ_{max} [°]	29.57
cell parameters	1843
(1485 with $I > 4\sigma(I)$)	
parameters refined	92
final R	0.0455
R_w	0.1325
GOF	1.045
data collection:	KappaCCD
absorption correction	none
θ_{max}	29.57
	$-7 \le h \le 7$
	$-14 \le k \le 14$
	$-17 \le l \le 16$
measured reflections	7397
independent reflections	1869
observed reflections	1485
refinement on F^2	full-matrix least squares
calcd weights Δ/σ_{max}	0.000
$\Delta\rho_{max}$ [e Å^3]	0.120
$\Delta\rho_{min}$ [e Å^3]	−0.138
R(all)	0.0580
R(gt)	0.0455
wR(ref)	0.1325
wR(gt)	0.1201
S(ref)	1.0045
extinction correction	none

(*S*)-3-(9*H*-Fluoren-9-ylmethoxycarbonylamino)-hexanedioic acid 1-allyl ester (Fmoc-β^{3}hGlu-OAll) (9): Fmoc-β^{3}hGlu(O*t*Bu)-OAll (**7**) (2.50 g, 5.2 mmol) was dissolved in dry CH_2Cl_2 (30 mL). After addition of TFA (5.1 mL, 67.8 mmol) and TIPS (2.7 mL, 13 mmol) the reaction mixture was stirred at room temperature. After 2 h much starting material remained and additional TFA (5.1 mL) was added. After stirring over night the solvent was removed under reduced pressure and the residue was purified by flash chromatography (ethyl acetate/pentane 1:2 → 1:0). The product obtained (2.0 g, 4.72 mmol, 91%) was re-crystallized from ethyl acetate/pentane. R_f = 0.64 (ethyl acetate); m.p. 105 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.88 (m, 2H; CH_2), 2.41 (t, 2H, ^2J = 5 Hz; CH_2COOAll), 2.60 (m, 2H; CH_2COO*t*Bu), 4.03 (m, 1H, C*H*NH), 4.20 (t, 1H, ^3J = 7 Hz; C*H*CH_2O), 4.35–4.44 (m, 2H, CHCH_2O), 4.59 (d, 2H, ^2J = 5.5 Hz, CHOCH_2), 5.23 (dd, 1H, J = 10.5, 1 Hz, CH=CH_2), 5.31 (d, 1H, J = 17.5 Hz; CH=CH_2), 5.36 (d, 1H, ^3J = 9.5 H; N*H*), 5.89 (m, 1H, C*H*=CH_2), 7.31 (dt, 2H, ^3J = 7.5, ^4J = 1.5 Hz; Fmoc), 7.39 (t, 2H, ^3J = 7.5 Hz; Fmoc), 7.58 (d, 2H, ^3J = 7.5 Hz; Fmoc), 7.75 (d, 2H, ^3J = 8 Hz; Fmoc); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ = 29.1, 30.7, 39.0 (3×CH_2), 47.2 (CH), 47.6 (CH), 65.4 (CH_2), 66.7(CH_2), 118.7 (CH_2 allyl), 120.0, 125.0, 127.0, 127.7 (4×CH Fmoc), 131.7 (CH allyl), 141.3, 143.8 (2×C Fmoc), 156.0 (CONH), 171.0 (*C*OOAll), 177.8 (COOH); IR (KBr): ν = 3321 (m), 3067 (m), 2939 (m), 1732 (s), 1699 (s), 1540 (s), 1451 (m), 1418 (m), 1278 (s), 1141 (m), 1087 (m), 1051 (m), 985 (m), 931 (m), 759 (m), 738 cm^{-1} (s); HR ESI-MS: m/z: calcd for $C_{24}H_{25}NO_6$: 423.168; found: 424.175 $[M+H]^+$, 446.157 $[M+Na]^+$.

(*S*)-7-*tert*-Butoxycarbonylamino-3-(9*H*-fluoren-9-ylmethoxycarbonylamino)-heptanoic acid allyl ester (Fmoc-β^{3}hLys(Boc)-OAll) (8): Fmoc-Lys(Boc)-CHN_2 (**6**)[18] (980 mg, 2.0 mmol) was dissolved in dry THF (10 mL) and allyl alcohol (0.2 mL, 3.0 mmol) was added. The mixture was cooled to −15 °C and a solution of silver benzoate (48 mg, 0.2 mmol) in NMM (0.44 mL, 4.0 mmol) was added. The reaction mixture was stirred and warmed to room temperature. After 5 h the mixture was filtered through a pad of Celite and the THF was removed under reduced pressure. The residue was dissolved in ethyl acetate and successively washed with saturated $NaHCO_3$, water and 5% HCl. After drying with $MgSO_4$ the solvent was evaporated under reduced pressure. The residue was purified by flash chromatography (pentane/ethyl acetate 3:1 → 2:1) and yielded allyl ester **8** (805 mg, 1.54 mmol, 77%), which was recrystallized from CH_2Cl_2/pentane to obtain colorless crystals. M.p. 94 °C; R_f = 0.18 (pentane/ethyl acetate 3:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.21–1.66 (m, 15H; 3×CH_2, Boc), 2.58 (d, 2H, ^2J = 7 Hz; CH_2CO), 3.10 (d, 2H, ^2J = 10 Hz; CH_2NHBoc), 3.97 (m, 1H; C*H*NHFmoc), 4.21 (t, 1H, ^3J = 11.5 Hz; C*H*CH_2O), 4.37 (d, 2H, ^2J = 11.5 Hz; CH_2O), 4.58 (d, 2H, ^2J = 9.5 Hz; CHOCH_2), 5.22–5.34 (m, 3H; CH=CH_2, NH), 5.90 (m, 1H; C*H*=CH_2), 7.31 (dt, 2H, ^3J = 12.5, ^4J = 2 Hz; Fmoc), 7.40 (t, 2H, ^3J = 12 Hz; Fmoc), 7.59 (d, 2H, ^3J = 12.5 Hz; Fmoc), 7.76 (d, 2H, ^3J = 12.5 Hz; Fmoc); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ = 23.2 (CH_2), 28.4 (Boc), 29.6 (CH_2), 33.9 (CH_2), 39.0 (CH_2), 40.1 (CH_2), 47.2 (CH), 48.0 (CH), 65.3 (CH_2), 66.6 (CH_2), 79.1 (Boc), 118.6 (CH_2 allyl), 119.9, 125.0, 127.0, 127.6 (4×CH Fmoc), 131.8 (CH allyl), 141.3, 143.9 (2×C Fmoc), 155.9 (OCONH), 156.0 (OCONH), 171.2 (COOAll); IR (KBr): ν = 3362 (s), 3338 (s), 2939 (m), 1735 (s), 1684 (s), 1534 (s), 1451 (m), 1369 (m), 1281 (s), 1253 (s), 1173 (m), 1090 (m), 987 (m), 736 cm^{-1} (m); HR ESI-MS: m/z: calcd for $C_{30}H_{38}N_2O_6$: 522.273; found: 523.280 $[M+H]^+$, 545.262 $[M+Na]^+$.

(*S*)-7-Amino-3-(9*H*-fluoren-9-ylmethoxycarbonylamino)-heptanoic acid allyl ester trifluoroacetic acid salt (Fmoc-β^{3}hLys-OAll·TFA) (10): Fmoc-β^{3}hLys(Boc)-OAll (**8**) (164 mg, 0.31 mmol) was dissolved in dry CH_2Cl_2 (10 mL). After addition of TFA (3 mL, 67.8 mmol) and TIPS (0.2 mL, 1 mmol) the reaction mixture was stirred at room temperature. After 2 h stirring at room temperature (no more starting material detectable by TLC) the solvent was removed under reduced pressure. The residue was purified by flash chromatography (CH_2Cl_2/methanol 9:1) if necessary. The primary amine was obtained as the trifluoroacetic acid salt **10** and appeared as yellowish oil, which turned solid after several weeks. R_f = 0.56 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.35–1.49 (m, 4H; 2CH_2), 1.64 (m, 2H; CH_2), 2.45–2.54 (m, CH_2CO), 2.90 (m, 2H; CH_2), 3.89 (m, 1H, CHN), 4.15 (t, 1H, ^3J = 7 Hz; C*H*CH_2O), 4.32 (d, 2H, J = 6.5 Hz; CH_2), 4.54 (d, 2H, J = 5.5 Hz; CH_2), 5.20 (d, 1H, ^2J = 10.5 Hz; CH=CH_2), 5.27 (m, 1H; CH=CH_2), 5.37 (d, 1H, ^3J = 9 Hz; NH), 5.87 (m, 1H; C*H*=CH_2), 7.27 (t, 2H, ^3J = 7.5 Hz; Fmoc), 7.36 (t, 2H, ^3J = 7.5 Hz; Fmoc), 7.54 (d, 2H, ^3J = 7.5 Hz; Fmoc), 7.72 (d, 2H, ^3J = 7.5 Hz; Fmoc), 7.92 (br s, 3H, NH_3); ^{13}C NMR (100.5 MHz, $CDCl_3$): δ = 22.6 (CH_2), 26.7 (CH_2), 33.5 (CH_2), 39.0 (CH_2), 39.6 (CH_2), 47.1 (CH), 47.7 (CH), 65.4 (CH_2), 66.7 (CH_2), 118.6 (CH_2 allyl), 119.9, 125.0, 127.0, 127.7 (4×CH Fmoc), 131.7 (CH allyl), 141.2, 143.7 (2×C Fmoc), 156.2 (OCONH), 161.9 (q, ^2J(C,F) = 36 Hz, CF_3COOH), 171.3 (COOAll); IR (KBr): ν = 3070 (s), 2946 (s), 1685 (s), 1540 (m), 1451 (m), 1203 (s), 1133 (s), 987 (m), 836 (m), 799 (m), 761 (m), 739 (m), 721 cm^{-1} (m); HR ESI-MS: m/z: calcd for $C_{25}H_{30}N_2O_4$: 422.221; found: 423.228 $[M+H]^+$, 445.2100 $[M+Na]^+$.

Cyclo(β^{3}hGln-β^{3}hPhe-β^{3}hLeu-β^{3}hLys) (14)

Attachment of the Rink linker: TentaGel NH_2 resin **11** (0.24 mmol g^{-1}, 720 mg, 0.17 mmol) was swollen in dry CH_2Cl_2 for 30 min. A solution of Fmoc-Rink linker (466 mg, 0.86 mmol), HBTU (328 mg, 0.86 mmol), HOBt (117 mg, 0.86 mmol) and DIEA (0.15 mL, 0.86 mmol) in dry CH_2Cl_2 (6 mL) were added and the suspension was stirred at room temperature for 3 h (TNBS test negative). The resin was washed with CH_2Cl_2 (5×6 mL), DMF (5×6 mL) and was finally shrunk down with diethyl

ether (5×6 mL). Fmoc/UV spectrophotometry with a small sample of the resin resulted in a calculated substitution level of 82%. Unreacted sites of the resin were capped with a mixture of acetic anhydride (3 mL) and CH_2Cl_2 (4 mL) and stirring for 3 h at room temperature. The resin was subsequently washed with CH_2Cl_2 (6×6 mL).

Synthesis of the linear peptide: The Fmoc-Rink amide TentaGel resin **12** was swollen in DMF and Fmoc-deprotected with 2% DBU/2% piperidine in DMF (5×5 min). The resin was washed with DMF (5×6 mL). A solution of Fmoc-β^{3}hGlu-OAll (**9**) (148 mg, 0.35 mmol), HBTU (128 mg, 0.34 mmol), HOBt (48 mg, 0.35 mmol) and DIEA (0.12 mL, 0.7 mmol) in DMF (6 mL) was added and the suspension was stirred for 3 h at room temperature (TNBS test negative). The resin was subsequently washed with DMF (5×6 mL). The Fmoc-deprotection/coupling procedure was repeated three times with 2.5 equiv Fmoc-β^{3}hPhe-OH, Fmoc-β^{3}hLeu-OH and Fmoc-β^{3}hLys(Boc)-OH. After the last coupling the resin was additionally washed with CH_2Cl_2 (5×6 mL) and shrunk down with diethyl ether (5×6 mL).

Allyl deprotection: The resin with the linear, fully protected peptide **13** was dried under high vacuum at 40°C for 1 h and a solution of [Pd(PPh_3)$_4$] (81 mg, 0.07 mmol) in a mixture of DMSO/THF/0.5 M HCl/NMM (4:4:2:1, 38.5 mL) was added. The reaction mixture was incubated at room temperature with occasional shaking for 4.5 h. The resin was washed consecutively with THF (5×6 mL), 0.5% DIEA in DMF (5×6 mL), 0.5% sodium diethyl dithiocarbamate in DMF (5×6 mL), CH_2Cl_2 (5×6 mL) and shrunk down with diethyl ether (5×6 mL).

Cyclization and cleavage: After Fmoc deprotection (2% DBU/2% piperidine in DMF, 5×5 min) and washing (5×6 mL of DMF) a solution of HBTU (127 mg, 0.34 mmol), HOBt (47 mg, 0.35 mmol) and DIEA (0.12 mL, 0.35 mmol) in DMF (6 mL) was added to the resin. The suspension was stirred for 17 h at room temperature (TNBS test negative) and finally washed with DMF (5×6 mL), CH_2Cl_2 (5×6 mL) and shrunk down with diethyl ether (5×6 mL). To the resin were added a mixture of TFA/H_2O/TIPS (95:2.5:2.5, 6 mL). After stirring for 2.5 h at room temperature the resin was filtered, washed two times with the cleavage mixture (6 mL, 2 min) and the collected filtrates were combined. This mixture was concentrated under reduced pressure and the residue was treated with cold ether. The crude peptide precipitated and the solvent was decanted. This step was repeated two times and yielded the crude peptide as a white solid. The mixture was purified by RP-HPLC (15–45% of B over 44 min, t_r=23.1 min) to give cyclic β^3-tetrapeptide **14** in pure form as white powder (8 mg, 10%). Analytical HPLC (10–70% B over 14 min): t_r=8.68 min. MALDI-TOF MS: *m*/*z*: calcd for $C_{30}H_{48}N_6O_5$: 572.369; found 573.306 $[M+H]^+$, 595.249 $[M+Na]^+$, 611.231 $[M+K]^+$.

Cyclo(β^{3}hLys-β^{3}hVal-β^{3}hPhe-β^{3}hLeu-β^{3}hLys) (19)

Attachment of the first amino acid: TentaGel S PHB resin **15** (0.29 mmol g^{-1}, 600 mg, 0.17 mmol) was suspended in dry CH_2Cl_2 (10 mL) and cooled to 0°C. 4-Nitrophenyl chloroformate (117 mg, 0.58 mmol) and NMM (0.06 mL, 0.58 mmol) were added. The suspension was warmed to room temperature and stirred over night. After washing the resin with CH_2Cl_2 (5×6 mL) and DMF (2×6 mL) a solution of Fmoc-β^{3}hLys-OAll (**10**) (613 mg, 1.45 mmol) and DIEA (0.76 mL, 4.35 mmol) in DMF (6 mL) was added. The suspension was stirred at room temperature for 14 h. The resin was washed with DMF (5×6 mL) and the coupling step was repeated once. The resin was finally washed with DMF (5×6 mL), CH_2Cl_2 (5×6 mL) and shrunk down with diethyl ether (5×6 mL).

Assembly of the linear peptide: The Fmoc-β^{3}hLys-OAll TentaGel resin **17** was swollen in DMF and Fmoc-deprotected with 2% DBU/2% piperidine in DMF (5×5 min). The resin was washed with DMF (5×6 mL). A solution of Fmoc-β^{3}hVal-OH (150 mg, 0.42 mmol), HBTU (155 mg, 0.41 mmol), HOBt (57 mg, 0.42 mmol) and DIEA (0.15 mL, 0.85 mmol) in DMF (6 mL) was added and the suspension was stirred for 3 h at room temperature (TNBS test negative). The resin was subsequently washed with DMF (5×6 mL), CH_2Cl_2 (5×6 mL) and shrunk down with diethyl ether (5×6 mL). Fmoc/UV spectrophotometry with a small sample of the resin resulted in a calculated substitution level of 60%. The Fmoc-deprotection/coupling procedure was repeated three times with 2.5 equiv Fmoc-β^{3}hPhe-OH, Fmoc-β^{3}hLeu-OH and Fmoc-β^{3}hLys-(Boc)-OH. After the last coupling the resin was additionally washed with CH_2Cl_2 (5×6 mL) and shrunk down with diethyl ether (5×6 mL).

Allyl deprotection: The resin with the linear, fully protected peptide **18** was dried under high vacuum at 40°C for 1 h and swollen in CH_2Cl_2. To the damp resin were added CH_2Cl_2 (1 mL), phenylsilane (0.3 mL, 2.4 mmol) and a solution of [Pd(PPh_3)$_4$] (12 mg, 0.01 mmol) in CH_2Cl_2 (4 mL). Argon was bubbled through the suspension for 10 min and the resin was filtered and washed with CH_2Cl_2 (5×6 mL). The deprotection procedure was repeated once and the resin was washed with CH_2Cl_2 (5×6 mL) and shrunk down with diethyl ether (5×6 mL).

Cyclization and cleavage: After Fmoc deprotection (2% DBU/2% piperidine in DMF, 5×5 min) and washing (5×6 mL of DMF) a solution of PyAOP (209 mg, 0.4 mmol) and DIEA (0.14 mL, 0.8 mmol) in DMF (6 mL) was added to the resin. The suspension was stirred for 12 h at room temperature (TNBS test negative) and finally washed with DMF (5×6 mL), CH_2Cl_2 (5×6 mL) and shrunk down with diethyl ether (5×6 mL). To the resin were added a mixture of TFA/H_2O/TIPS (95:2.5:2.5, 6 mL). After stirring for 3 h at room temperature the resin was filtered, washed two times with the cleavage mixture (6 mL, 2 min) and the collected filtrates were combined. This mixture was concentrated under reduced pressure and the residue was treated with cold ether. The crude peptide precipitated and the solvent was decanted. This step was repeated two times and yielded the crude peptide as a white solid. The mixture was purified by RP-HPLC (10–50% B over 29 min, t_r=19.4 min) to yield cyclic β^3-pentapeptide **19** after freeze-drying (6 mg, 9%). Analytical HPLC (10–70% B over 14 min): t_r=9.15 min. MALDI-TOF MS: *m*/*z*: calcd for $C_{37}H_{63}N_7O_5$: 685.49; found 686.145 $[M+H]^+$, 708.089 $[M+Na]^+$, 724.042 $[M+K]^+$.

Acknowledgements

This work was supported by Vetenskapsrådet (The Swedish Research Council), the Carl Trygger Foundation, and Magnus Bergvall's Foundation. F.B. acknowledges the Carl Trygger Foundation for a postdoctoral fellowship during 2003, and S.Z. is grateful to the Wenner-Gren foundation for a fellowship 2003–2004. P.K. acknowledges the Thailand Research Fund (TRF4780020) for a financial support and S.P. thanks the Postgraduate Education and Research Program in Chemistry for a scholarship. MALDI-Tof MS analysis was done at the Expression Proteomics Laboratory at Uppsala University supported by the Wallenberg foundation. We are grateful to Jonas Bergquist (Uppsala University, Department of Analytical Chemistry) for performing the ESI-MS analyses. The CD instrument was purchased with funding from the Wallenberg foundation.

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Received: March 4, 2005
Published online: July 29, 2005

