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Design and Synthesis of Novel Peptides Bearing a Host and a Guest Side Chains

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Abstract: The synthesis of model heptapeptides bearing a host and a guest side chains at different positions is reported. These peptidic structures were designed in such a way that specific backbone conformations could be induced and stabilized by cooperative side chain interactions. Although circular dichroism studies demonstrated that intra- and intermolecular host guest interactions are involved in the stabilization of the peptidic conformation, they are not strong enough to induce a complete conformational reorganization.

The construction of peptidic molecule with predictable well defined conformation is a fundamental prerequisite for the preparation of *de novo* designed proteins,[1] functional molecular devices,[2] and peptide based therapeutics.[3] In addition, these types of compounds represent useful and accessible structural models of many biological systems.[4] To prepare such rigidified peptidic structures, chemists usually synthesize cyclic analogues[5] or introduce conformationally constrained amino acids[6] such as α,α-dialkylglycines. However, these strategies often have the disadvantage of requiring considerable synthetic efforts.

On the other hand, in nature protein and enzyme structural stability results mainly from specific non-covalent side chain interactions[7] such as hydrophobic, electrostatic, and hydrogen bonding interactions. Following this guideline, several *de novo* designed peptides and proteins have been prepared successfully using favourable side chain interactions between natural amino acids to confer structural stability.[8] By contrast, few reports use specific interactions between ligand modified side chains to induce and stabilize desired peptidic conformations.[9] This latter strategy has two major advantages over the conventional method using proteogenic amino acids. First, the conformational behavior can possibly be modulated or regulated by remote reversible complexation processes as illustrated in Figure 1.

Secondly, different (man made) interactions, stronger than the ones encountered in natural systems, could infer a greater structural stability. Furthermore, the peptidic backbone would still be subjected to the conformational folding-unfolding equilibrium. This equilibrium could eventually served to regulate the function of molecular devices.

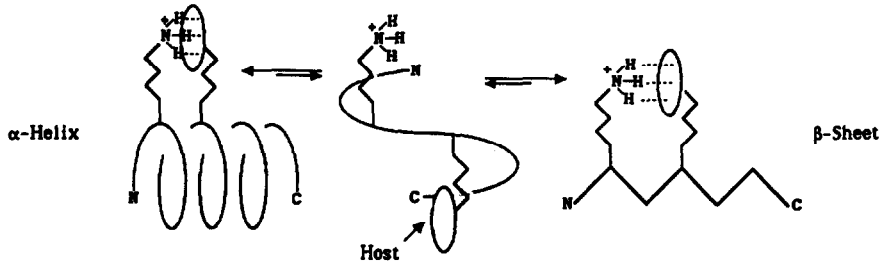


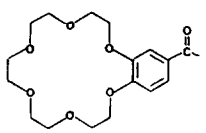
Figure 1. Present working hypothesis: the formation of favourable intramolecular complexes between a host and a guest side chains can occur under specific conformations. These cooperative recognition processes should displace the conformational equilibrium towards the more ordered structures.

Here we report our results on the synthesis of model heptapeptides, **1-3**, designed to adopt a specific conformation that could be induced and stabilized by favourable intramolecular recognition phenomena between an alkylammonium and a crown ether side chains.[10]

$$\overset{NH_3^+}{\underset{\sim}{|}} \quad \overset{CE}{\underset{\sim}{|}}$$
BOC-Ala-Ala-Ala-**Lys**-Ala-**Lys**-Ala-OMe **1**

$$\overset{NH_3^+}{\underset{\sim}{|}} \quad \overset{CE}{\underset{\sim}{|}}$$
BOC-Ala-Ala-**Lys**-Ala-Ala-**Lys**-Ala-OMe **2**

$$\overset{NH_3^+}{\underset{\sim}{|}} \quad \overset{CE}{\underset{\sim}{|}}$$
BOC-Ala-**Lys**-Ala-Ala-Ala-**Lys**-Ala-OMe **3**

CE= 

Lys= -HN-CH(-$(CH_2)_4$-NH_2)-CO- Ala= -HN-CH(CH_3)-CO-

RESULTS AND DISCUSSION

Design

Heptapeptides **1-3** were designed using CPK models and molecular modelling techniques. They are composed of five L-alanines and two L-lysines. One of the lysine side chain NH_3^+ group serves as a guest while the other is used to link a benzo-18-crown-6 derivative. Thus, the host and the guest moieties are remote and approximately at the same distance from the peptidic backbone. Alanine was chosen because it can fit easily in several secondary structures due to its small side chain CH_3 group. Also, in addition to the biological importance of alanine rich peptides,[11] alanine based model peptides have been used extensively in structural studies.[12] In the primary sequence of peptides **1-3**, the host and the guest residues are separated respectively by one, two, and three alanines. Hence, favourable cooperative intramolecular recognition processes can occur only under specific backbone conformations. This is illustrated in Figure 2 with the schematic representations of the two most biologically important conformations, the ß-sheet and the α-helix.

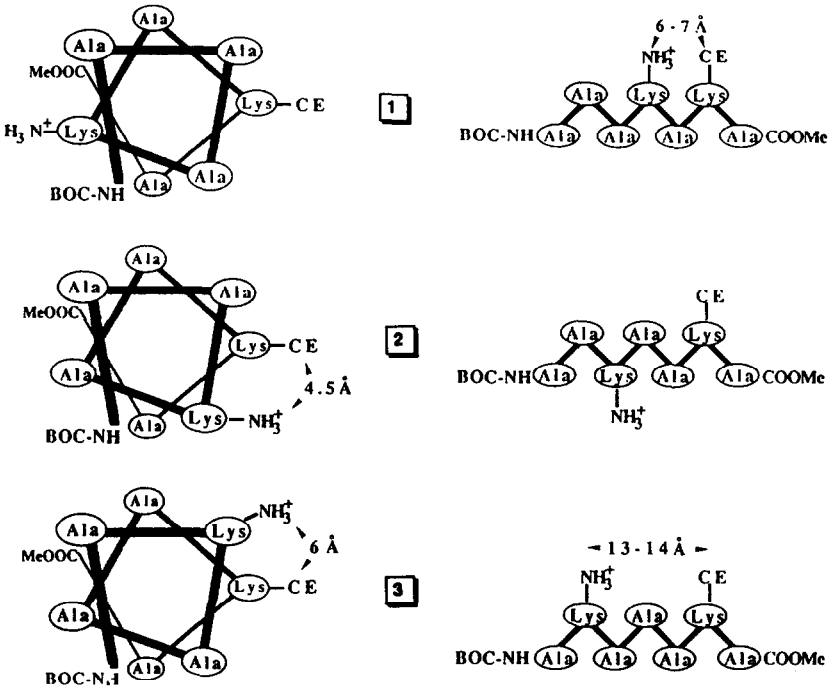


Figure 2. Schematic representations of the α-helix (axial projection) and the ß-sheet conformations of peptides **1-3**.

In addition to these conformations, the ligand and the guest side chains can be well organized for complexation when the backbone of **1-3** adopts several other conformations such as reverse turns and 3_{10}-helix structures.

One notes that the host and guest side chains of peptide **1** (see Figure 2) are prone to induce a ß-sheet conformation. Presumably, such conformation would be stabilized by the side chain interactions. While the two residues are 6-7Å apart, the several methylene units of the side chains allow enough motional freedom for the binding partners to adopt the required orientation. By contrast, with peptide **2**, only in the α-helical conformation does cooperative complexation occur. In this case, the host and guest side chains are separated by 4.5Å. Finally, peptide **3** is an interesting case since both α-helix and ß-sheet conformations can allow the guest and the host side chains to form an intramolecular complex. However, as the two residues are 6Å and 13-14Å apart respectively in these conformations, the α-helix form should be favoured.

Synthesis

Peptides **1-3** were synthesized by conventional solution phase peptide synthesis using a convergent strategy illustrated in Figure 3. This strategy simplifies advantageously the synthesis since common intermediates can be used for different target compounds. Also, it allows for the preparation of fully protected peptides necessary to study the complexation and conformational behavior of the model peptides in organic solvents prior to aqueous studies. Furthermore, the synthesis can be done on a larger scale than in a solid phase peptide synthesis.

The orthogonal protection scheme used the t-butyloxycarbonyl (BOC) group to protect the α-amino group, the methyl ester for the C-terminal group, and the benzyloxycarbonyl (Z) group for the lysine ε-amino function. The protecting groups were selectively cleaved using 4N HCl in dioxane, 1N NaOH in methanol, and H_2 with 10% palladium on charcoal, respectively. All coupling reactions were performed using the DCC/HOBT method,[13] except for the segment coupling reactions to produce peptides **2** and **3**, where BOP reagent[14] gave higher coupling yields. The fully protected peptides **16**, **20**, and **25** were hydrogenolyzed in the presence of 10% Pd on charcoal in a 1:1 mixture of methanol and acetic acid to obtain the desired peptides **1-3** in their acetate form with overall yields of 20, 39, and 11% respectively. Separation and purification of **1-3** were accomplished with size exclusion chromatography in acetic acid using Sephadex® G-10-120. All were characterized with standard analytical HPLC, FAB mass spectrometry, and high resolution ^1H NMR.

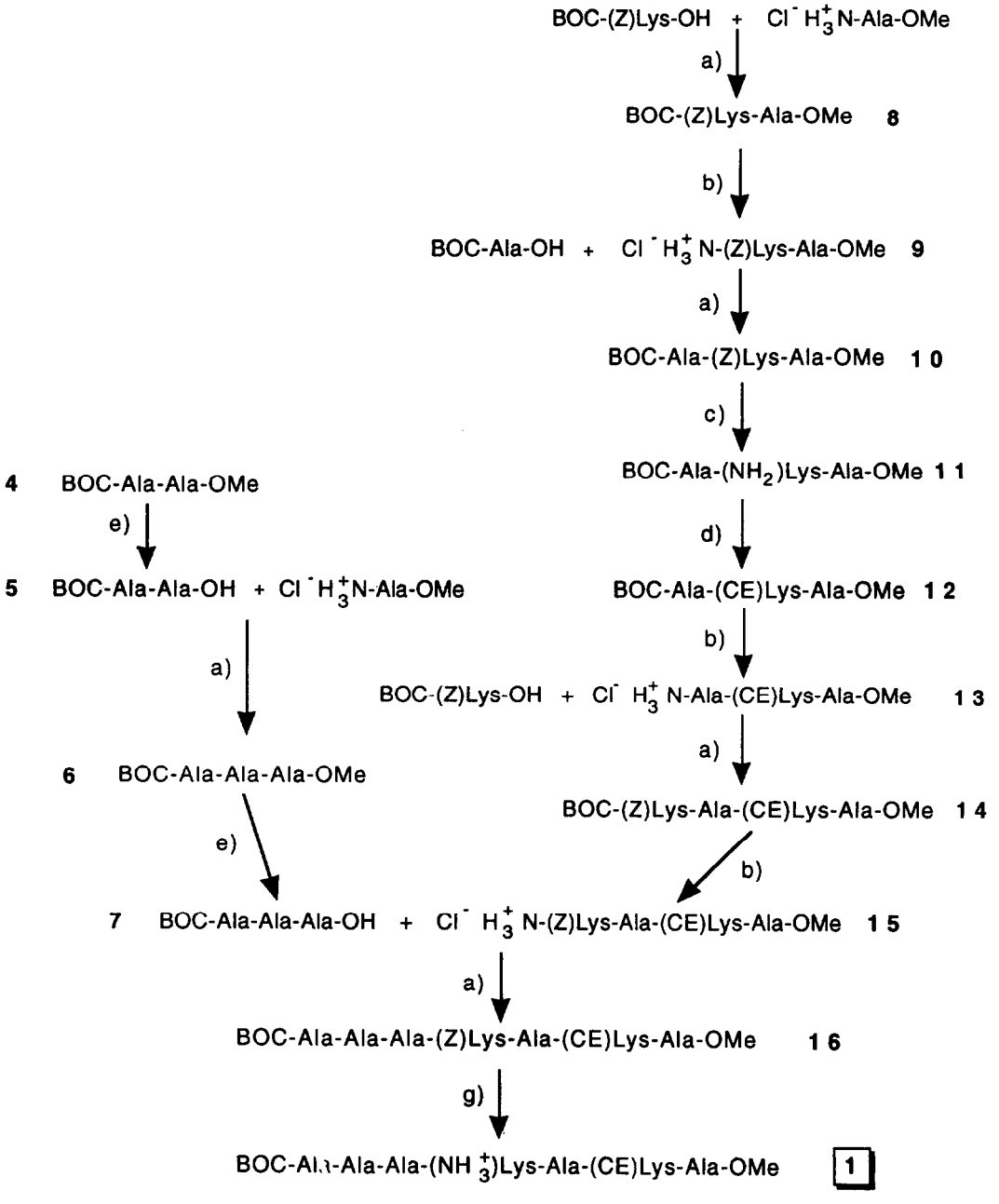


Figure 3. Synthesis of the crown ether modified peptides **1-3**. (Continued on next page)

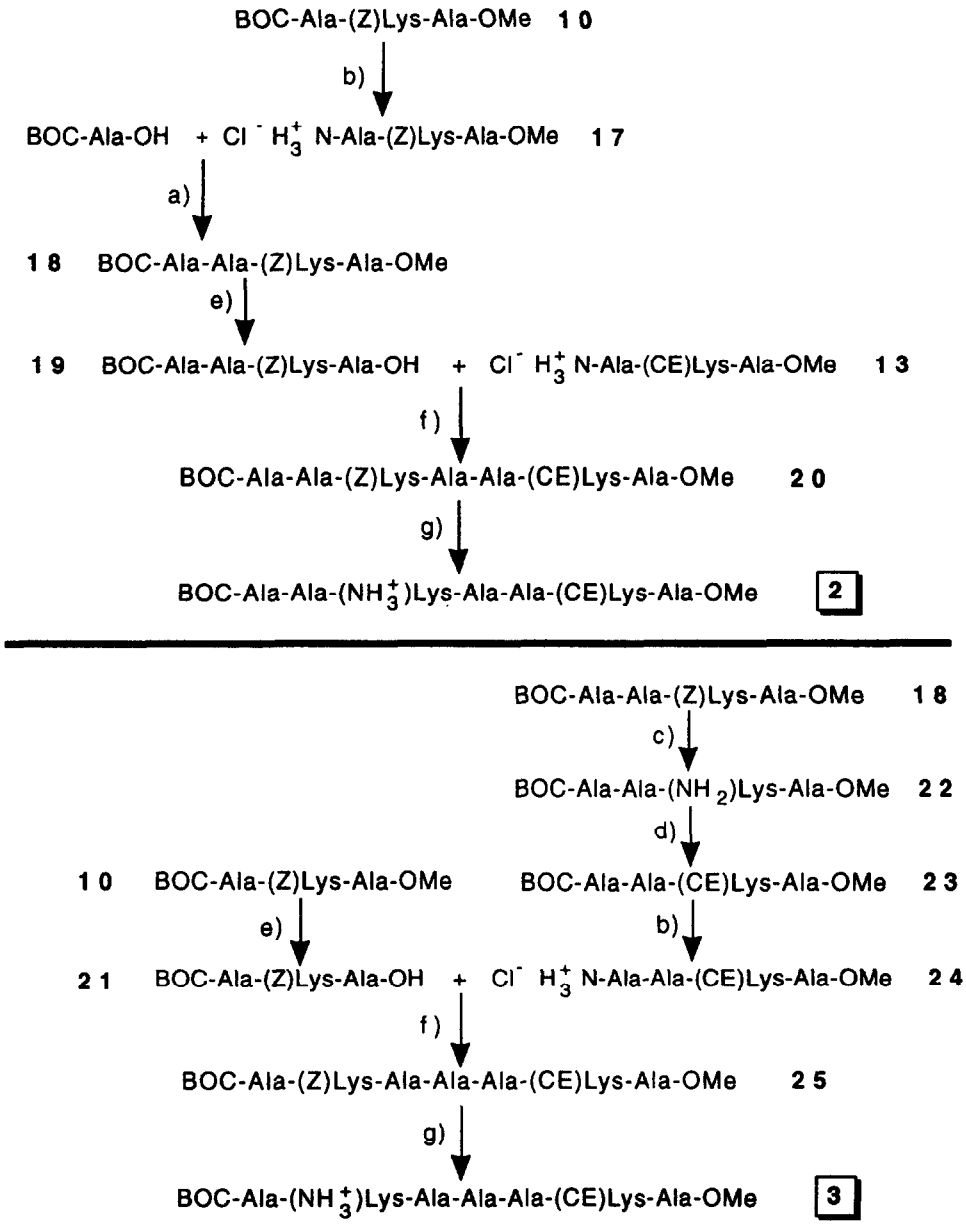


Figure 3. (Continued).Synthesis of the crown ether modified peptides **1-3**. General conditions: a) DCC/HOBT, CH_2Cl_2 or DMF; b) 4N HCl in Dioxane, 2h, 25°C; c) H_2, 10% Pd/C, MeOH; d) (Benzo-18-crown-6)-4-carboxylic acid[15], DCC/HOBT, CH_2Cl_2; e) 1N NaOH, MeOH, 25°C; f) BOP, NEt_3, DMF; g) H_2, 10% Pd/C, AcOH:MeOH (1:1); see experimental section for details.

Conformational Studies

The conformational behavior of the crown peptides **1-3**, as well as their Z-protected analogues **16**, **20**, and **25**, was investigated with circular dichroism spectropolarimetry in a strong hydrogen bond donor solvent, 2,2,2-trifluoroethanol (TFE), and in a weak hydrogen bond acceptor solvent, acetonitrile.

At a concentration of 10^{-5} M in TFE, peptides **1-3** and their protected analogues are largely structureless and monomeric as indicated by a large negative band at around 195-200 nm (Figure 4). The model peptides **1-3** show slightly more order than their protected analogues. This is seen in Figure 4 with the smaller values of ellipticities for the 195-200 nm band. This phenomenon is not due to the smaller molar extinction coefficient (ε) of **1-3**. Indeed, upon warming to 55 °C, the ellipticities increased almost to the intensity of the protected analogues. Since **1-3** exist as monomers in TFE, these observations seem to suggest that intramolecular host-guest

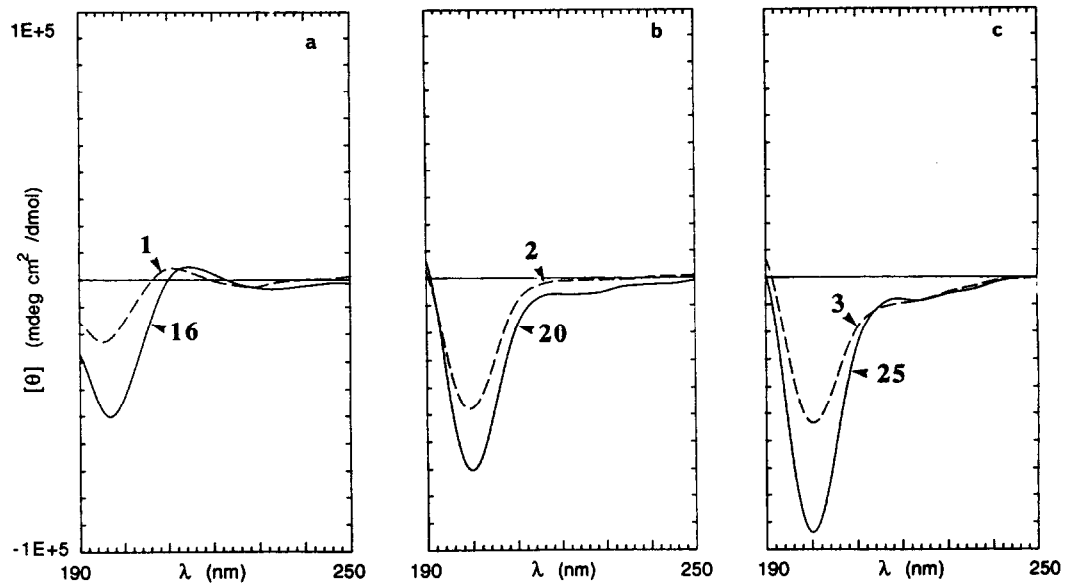


Figure 4. Circular dichroism spectrum of crown ether peptides **1** (a), **2** (b), **3** (c) (dashed curves) and their protected analogues **16**, **20**, and **25** (solid curves) in TFE at 25°C.

interactions provide some conformational stabilization. However, this stabilization is rather weak in a strong hydrogen bonding solvent like TFE. It was therefore anticipated that the conformational stabilization induced by the side chains recognition interactions should be more important in a less competitive solvent such

as acetonitrile. Studies in this solvent show that peptides **1** and **2** are again slightly more ordered than their protected analogues, but not as much as expected (see Figure 5). No changes are observed in the case of **3**. Crown peptides **1** and **3** are partially ordered as demonstrated by a negative band at 195 and 202 nm respectively. On the other hand, peptide **2** exists mainly under a ß-sheet conformation at 25°C whereas its protected analogue **20** has only a partial ß-sheet character (see Figure 5). Indeed, the spectrum of **2** has a minimum at 217 nm and a maximum at around 190 nm typical of ß-sheet peptides.[16]

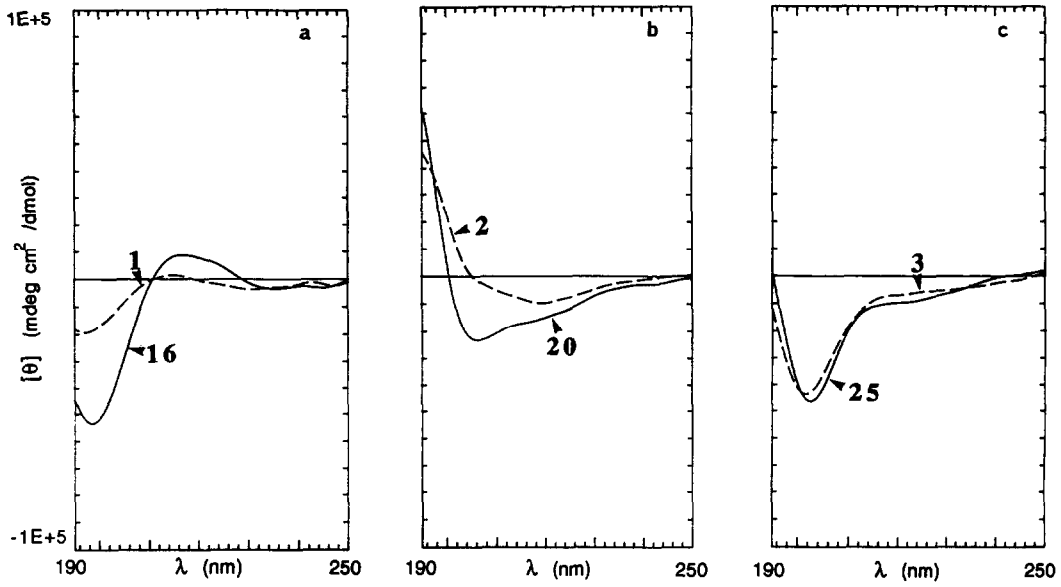


Figure 5. Circular dichroism spectrum of crown ether peptides **1** (a), **2** (b), **3** (c) (dashed curves) and their protected analogues **16**, **20**, and **25** (solid curves) in acetonitrile at 25°C.

This observation contradicts our initial hypothesis that peptide **2** should be stabilized by an intramolecular host guest interaction under an α-helix form but not under a ß-sheet structure (Figure 2). In the ß-sheet conformation, the host and the guest side chains are on opposite sides of the backbone, and intramolecular stabilizing interactions are therefore impossible. However, the ß-sheet form of **2** is more stable than the one of its protected counterpart **20**. This is demonstrated by variable temperature experiments with these two peptides. As seen in Figure 6, the ß-sheet structure of **20** is destroyed at a lower temperature than peptide **2**. This observation indicates that some other host-guest interactions are involved in the stabilization of

the secondary structure of **2**. This is also supported by other results: (**i**) the ß-sheet of **2** is less stable in the presence of K^+ ions which competes with the ammonium ion, (**ii**) neutralization of the ammonium side group by the addition of quinuclidine rapidly destroys all ordered structure in **2** but not in **20**. These results can be rationalized by invoking *intermolecular* host-guest interactions, instead of intramolecular, between the ammonium of one strand and the crown ring of another

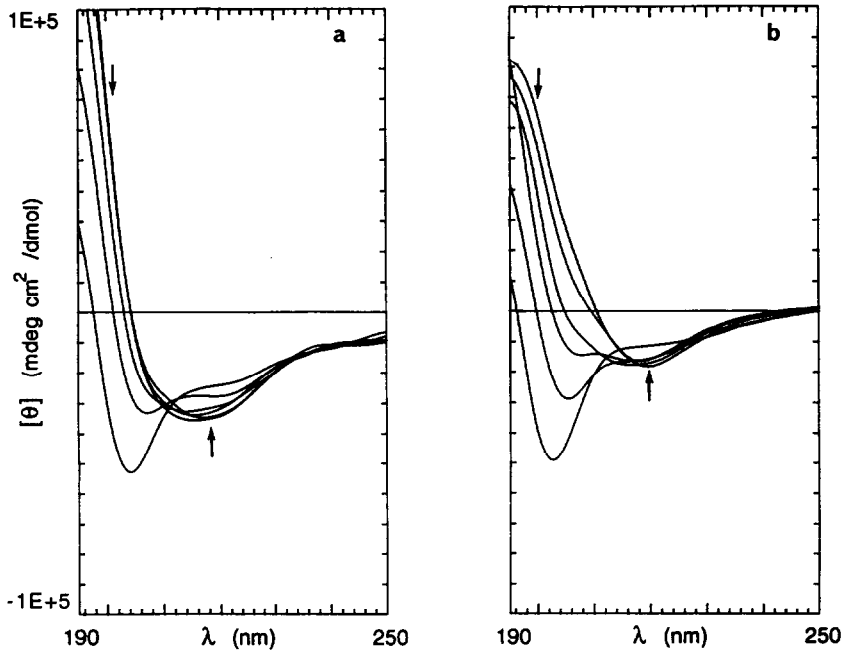


Figure 6. Variable temperature (5-55°C) circular dichroism studies on crown peptides **2** (a) and **20** (b) in acetonitrile. The arrows indicate the changes in ellipticities upon increasing the temperature.

strand in a supramolecular anti-parallel ß-sheet aggregated structure of **2** as depicted in Figure 7. The ß-sheet conformation being the most stable for short hydrophobic peptides in aprotic low polarity solvents,[17] this conformation is adopted by both **2** and **20**, but stabilized by host-guest interactions only in **2**. Variable temperature control experiments with **20** in the presence of K^+ or quinuclidine showed that these additives have little or no effects on its conformational stability. Furthermore, concentration dependence studies using circular dichroism also support the proposed stabilized structure of **2** in acetonitrile. Indeed, the ß-structure of **2** is more stable and persisted at a lower concentration (10^{-6}M) than its protected analogue **20** (see

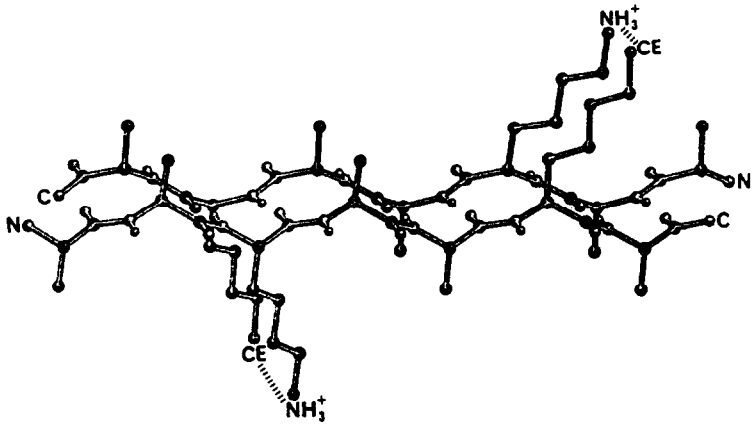


Figure 7. Portion of the anti-parallel ß-sheet aggregated structure stabilized by intermolecular recognition interactions proposed for crown peptide **2** in acetonitrile (N- and C-terminal protecting groups not shown).

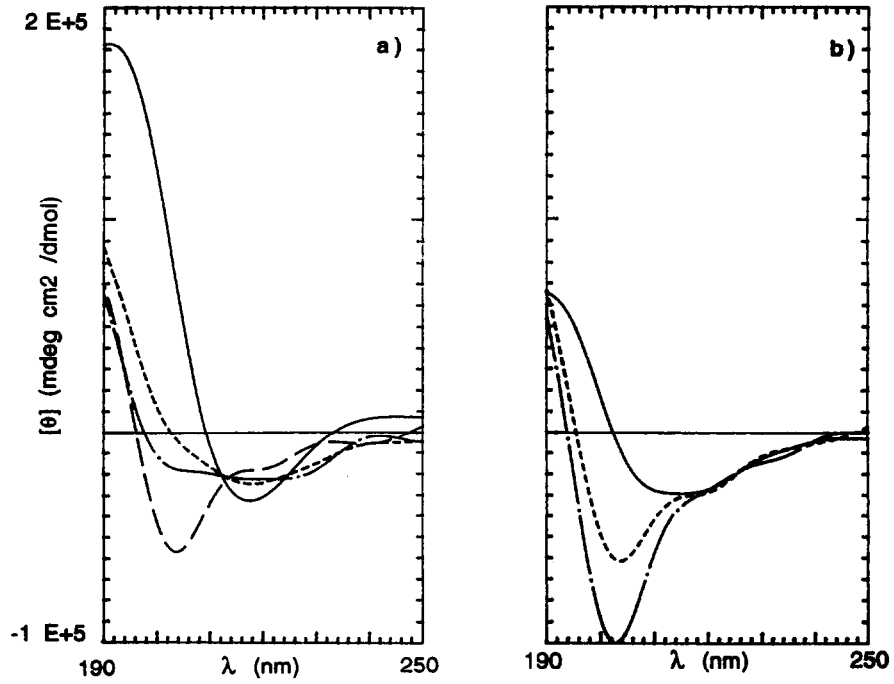


Figure 8. Circular dichroism spectra at 25 °C of peptides (a) **2** in 50% TFE/acetonitrile and (b) **20** in acetonitrile at different concentrations: solid curves= 1.75 X 10^{-4} M, dashed curves= 1.75 X 10^{-5} M, dotted curves= 3.5 X 10^{-6} M, broken curve= 3.5 X 10^{-6} M at 50 °C.

Figure 8). In fact, to observe its ß-sheet-random coil equilibrium, the concentration study had to be performed in a 50% TFE/acetonitrile mixture (Figure 8a). On the other hand, the anti-parallel nature of the ß-sheet structure of **2** is supported by infrared spectroscopy (Figure 9). The IR spectrum in acetonitrile shows the amide I absorptions at 1626 and 1692 cm^{-1} highly characteristic of this type of structure.[18]

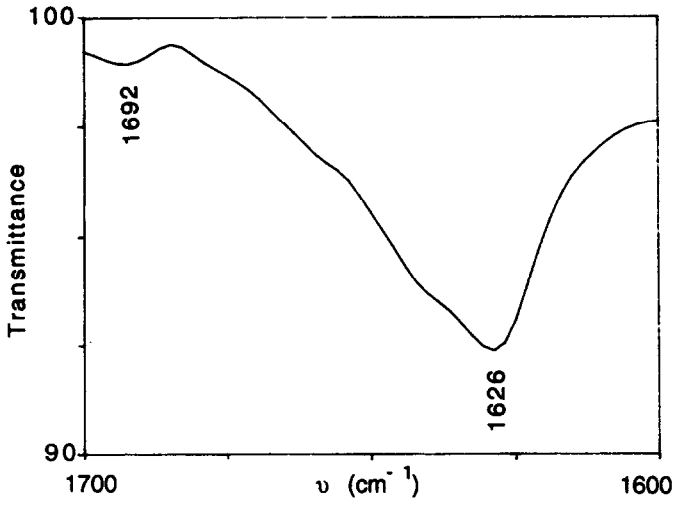


Figure 9. FTIR spectrum of crown peptide **2** at a concentration of 1 X 10^{-3} M in acetonitrile.

In summary, the results reported demonstrate the possibility of using recognition interactions to stabilize specific peptidic conformations. However, the type of interaction studied in this work, namely the interactions between an ammonium group and a crown ether, does not appear strong enough to rigidify strongly or to induce an important conformational reorganization of the peptidic chain. In addition, the findings that both intra- and intermolecular host guest interactions could be involved in the conformational stabilization of peptides **1-3** suggest that very subtle changes, like the relative position of the residues bearing the host and guest side chains, can have important different consequences and that more model studies are required to be able to predict them accurately. Work is in progress to determine the solution conformation of the free and bound model peptides and to improve this novel non-covalent strategy of conformational restriction.

EXPERIMENTAL SECTION

Melting points were determined on a Reichert Hot Stage apparatus and are uncorrected. Mass spectral assays (ms, m/e) were performed using a VG Micromass ZAB-1F spectrometer. FAB mass spectra (glycerol matrix) were recorded on a VG-Autospec Q spectrometer and were performed at the Regional Center of Mass Spectrometry, Université de Montréal. Optical rotations were measured at 25°C on a Perkin Elmer 241 polarimeter. Proton NMR spectra were recorded on a 250 MHz Bruker WM-250 instrument. Chemical shifts are reported in δ values (ppm) relative to DMSO or chloroform as internal standard. Abbreviations used are m: multiplet, s: singulet, d: doublet, t: triplet, q: quartet, qn: quintuplet. Circular dichroism studies were performed using a Jasco J-710 spectropolarimeter. FTIR spectra were recorded on a Bomem Michelson 100 instrument.

Chromatographic separations were made using Merck Kieselgel 60 silica gel (230-400 mesh ASTM). Analytical and preparative reversed phase HPLC were performed on Vydac C_4 columns with gradients of H_2O/0.1% TFA and CH_3CN/0.1% TFA solutions. Gel permeation chromatography was performed in acetic acid using Sephadex G-10-120 from Pharmacia. The column used was 1 meter long with a diameter of 2.5cm. The absorption of the fractions was read at 254 nm and the desired products were recovered by lyophilisation. Thin layer chromatography (TLC) was performed using silica gel Kieselgel 60 plates. The developed plates were visualized directly with ninhydrin or with ninhydrin after exposure to HCl in the case of BOC-protected peptides. The following solvent mixtures (v/v) were used: chloroform-methanol-acetic acid 85:10:5 (A); chloroform: methanol 1:1 (B). Compounds **4**, **5**, **8**, **9**, and **10** were prepared according to described procedures.[19,20]

N$^{\alpha}$-*tert*-Butoxycarbonyl-L-alanyl-L-alanyl-L-alanine methyl ester 6. To a stirred solution of BOC-Ala-Ala-OH[19] (0.10 g, 0.38 mmol) in a minimum of dichloromethane at 0°C was added hydroxybenzotriazole (HOBT•H_2O) (0.064 g, 0.47 mmol) and dicyclohexylcarbodiimide (DCC) (0.08 g, 0.47 mmol). After 20 min, the white precipitate (dicyclohexylurea, DCU) was filtered off. Then, a solution of HCl•Ala-OMe (0.053 g, o.38 mmol) in dichloromethane neutralized with triethylamine (65 µL, 0.80 mmol) was added to the filtrate. After 6h the mixture was washed (3 x 15 mL) with 0.5N HCl, water, 5% sodium bicarbonate solution, and water. The dichloromethane layer was dried over magnesium sulfate and evaporated to give an oil. After trituration with ether, 0.11 g (89%) of a white solid, **6**, was obtained; TLC, R_f: 0.68 (A); mp=173-175°C; ^{1}H NMR (DMSO-D_6) δ: 8.28 (1H, d, C-term. Ala NH), 7.79 (1H, d, NH), 6.94 (1H, d, BOC NH), 4.21-4.30 (2H, qn, 2Ala αCH), 3.91-3.96 (1H, qn, N-term. Ala αCH), 3.60 (3H, s, -OCH_3), 1.36 (9H, s, BOC CH_3) 1.25 (3H, d, C-term. Ala CH_3), 1.18 (3H, d, Ala CH_3), 1.14 (3H, d, N-term. Ala CH_3).

N$^{\alpha}$-*tert*-Butoxycarbonyl-L-alanyl-L-alanyl-L-alanine 7. Peptide ester **6** (0.11 g, 0.34 mmol) was treated with 1.5 mL of 1N NaOH solution in 2 mL of methanol for 4h. After removal of methanol, the resulting solution was diluted with 10 mL of water and washed twice with dichloromethane. The aqueous layer was cooled and acidified to pH ≈ 3 with 1N HCl and extracted with dichloromethane (3 x 25 mL). The organic phase was dried with anhydrous magnesium sulfate, filtered, and evaporated to give the desired peptide acid **7**. Yield: 0.095 g (90%); TLC, R_f: 0.125 (B); mp = 178-179°C (lit.[21] 177.5-178°C); ^{1}H NMR (DMSO-D_6) δ: 8.09 (1H, d, C-term. Ala amide NH), 7.82 (1H, d, amide NH), 6.95 (1H, d, BOC NH), 4.27 (1H, qn, C-term. Ala αCH), 4.14 (1H, qn, Ala αCH), 3.91 (1H, qn, N-term. Ala αCH), 1.36 (9H, s, BOC CH_3), 1.23 (3H, d, C-term. Ala CH_3), 1.18 (3H, d, Ala CH_3), 1.13 (3H, d, Ala CH_3).

N$^{\alpha}$-*tert*-Butoxycarbonyl-L-alanyl-L-lysyl-L-alanine methyl ester 11. The protected peptide **10**[20] (0.36 g, 0.7 mmol) was dissolved in 25 mL of methanol and 50 mg of 10% Pd on charcoal was added. The solution was treated with hydrogen at 50 psi of pressure for 4h, filtred on celite, and evaporated to yield **11**; 0.29 g (98%), colorless oil; TLC, R_f: 0.41 (B); $[\alpha]_D$ = +1° (c = 1, 1N HCl), $[\alpha]_{546}$ = -41° (c = 1, 1N HCl); ^{1}H NMR (DMSO-D_6) δ: 8.42 (1H, d, C-term. Ala amide NH), 7.64 (1H, d, amide NH), 6.98 (1H, d, BOC NH), 4.23-4.27 (2H, m, Lys and Ala C-term. αCH), 3.92-3.98 (1H, qn, N-term Ala αCH), 3.70 (2H, m, Lys εNH_2), 3.59 (3H, s, -OCH_3), 2.54 (2H, m, Lys εCH_2), 1.36 (9H, s, BOC CH_3), 1.25 (3H, d, C-term. Ala CH_3), 1.22-1.72 (6H, m, Lys β, δ, and γCH_2), 1.13(3H, d, N-term. Ala CH_3).

N$^{\alpha}$-*tert*-Butoxycarbonyl-L-alanyl-(N$^{\varepsilon}$-(benzo-18-crown-6)-4-carbonyl)-L-lysyl-L-alanine methyl ester 12. To a stirred solution of (benzo-18-crown-6)-4-carboxylic acid[15] (0.25 g, 0.70 mmol) in a minimum of dichloromethane at 0°C, HOBT•H_2O (0.10 g, 0.77 mmol) and DCC (0.16 g, 0.77 mmol) were added. After 20 min the DCU was filtered off. A solution of amino peptide **11** (0.032 g, 0.70 mmol) in dichloromethane was then added to the filtrate. After 15h stirring at room temperature, the reaction was stopped and worked up by the usual procedure described above. The product was precipitated from dichloromethane with ether to yield 0.44 g (93%) of a white hygroscopic solid. TLC, R_f: 0.18 (B); $[\alpha]_D$ = -23° (c = 1, AcOH); ^{1}H NMR (DMSO-D_6) δ: 8.35 (1H, d, C-term. amide NH) 8.27 (1H, t, Lys εNH), 7.69 (1H, d, amide NH), 7.41-7.44 (2H, m, benzo H_3 and H_5), 6.92-7.00 (2H, m, benzo H_6 and BOC NH), 4.24-4.29 (2H, m, Lys and C-term. Ala αCH), 3.93-3.95 (1H, qn, N-term. Ala αCH), 3.59 (3H, s, -OCH_3), 3.51-4.11 (20H, m, crown CH_2), 3.24 (2H, m, Lys εCH_2), 1.36 (9H, s, BOC CH_3), 1.35-1.60 (6H, m, Lys β, δ, and γCH_2), 1.26 (3H, d, Ala CH_3), 1.25 (3H, d, N-term. Ala CH_3).

L-alanyl-(N$^{\varepsilon}$-(benzo-18-crown-6)-4-carbonyl)-L-lysyl-L-alanine methyl ester hydrochloride 13. Peptide **12** (0.36 g, 0.5 mmol) was treated for 3h with 10 mL of a 4N HCl solution in dioxane at room temperature. The evaporation of dioxane was followed by addition of ether and the resulting precipitate (very hygroscopic) was filtered off and washed with ether to yield 0.31 g (96%) of the deprotected ester **13**; TLC, R_f: 0.08 (B); $[\alpha]_D$ = -10° (c = 1, AcOH); ^{1}H NMR (DMSO-D_6) δ: 8.55 (1H, d, amide NH), 8.48 (1H, d, amide NH), 8.41 (1H, t, Lys εNH), 8.11-8.14 (3H, m, NH_3^+), 7.43-7.46 (2H, m, benzo H_3 and H_5), 6.97-7.01 (1H, m, benzo H_6), 4.28-4.32 (3H, m, Lys, Ala C-term., and Ala N-term. αCH), 3.59 (3H, s, -OCH_3), 3.51-4.11 (20H, m, crown CH_2), 3.22-3.24 (2H, m, Lys εCH_2), 1.35-1.60 (6H, m, Lys β, δ, and γCH_2), 1.32 (3H, d, Ala C-term. CH_3), 1.26 (3H, d, Ala CH_3).

N$^{\alpha}$-*tert*-Butoxycarbonyl-(N$^{\varepsilon}$-benzyloxycarbonyl)-L-lysyl-L-alanyl(N$^{\varepsilon}$-(benzo-18-crown-6)-4-carbonyl)-L-lysyl-L-alanine methyl ester 14. BOC-(Z)Lys-OH (223 mg, 0.58 mmol) was activated in a minimum of dichloromethane at 0°C with HOBT•H_2O (86 mg, 0.63 mmol) and DCC (132 mg, 0.63 mmol). After 30 min, the DCU was filtered off and a solution of the ester **13** (360 mg, 0.58 mmol) and 200 µL of triethylamine was added. The reaction mixture was worked up 3h later by the usual procedure. The product was precipitated from dichloromethane with ether to yield 442 mg, (78%) of product **14**. TLC, R_f: 0.13 (B); mp 148-150°C; ^{1}H NMR (DMSO-D_6) δ: 8.30 (1H, d, C-term. amide NH), 7.85 (2H, m, Lys and Ala amide NH), 7.32-7.35 (7H, m, benzo H_3 and H_5 and benzyl 5 arom. H), 7.30 (2H, m, 2 Lys εNH), 6.96-7.00 (1H, d, benzo H_6) 6.89 (1H, d, BOC NH), 4.98 (2H, s, benzyl CH_2), 4.23 (3H, m, Lys, Ala and C-term. Ala αCH), 3.84 (1H, q, N-term. Lys αCH), 3.58 (3H, s, -OCH_3), 3.50-4.11 (20H, m, crown CH_2), 3.20 (2H, m, Lys εCH_2), 2.95 (2H, m, N-term. Lys εCH_2), 1.35 (9H, s, BOC CH_3), 1.26 (3H, d, C-term. Ala CH_3), 1.22-1.60 (12H, m, 2 Lys β, δ, and γCH_2), 1.16 (3H, d, Ala CH_3).

(N$^{\varepsilon}$-benzyloxycarbonyl)-L-lysyl-L-alanyl-(N$^{\varepsilon}$-(benzo-18-crown-6)-4-carbonyl)-L-lysyl-L-alanine methyl ester hydrochloride 15. The peptide **14** (241 mg, 0.24 mmol) was dissolved in 15 mL of 4N HCl in dioxane and stirred 2h at room temperature. Evaporation of dioxane followed by addition of ether yielded a very hygroscopic white solid which was recovered and washed with ether. Yield: 302 mg (92%). TLC, R_f: 0.13 (B); ^{1}H NMR (DMSO-D_6) δ: 8.64 (1H, d, C-term. Ala amide NH), 8.37 (2H, d, Lys and Ala amide NH), 8.20 (3H, m, NH_3^+), 8.04 (1H, m, Lys εNH), 7.30-7.50 (8H, m, benzo H_3 and H_5, benzyl arom. H, and N-term. Lys εNH), 7.00 (1H, m, benzo H_6), 4.99 (2H, s, benzyl CH_2), 4.36 (1H, qn, C-term. Ala αCH), 4.24 (2H, m, Lys and Ala αCH), 3.59 (3H, s, -OCH_3), 3.51-4.11 (21H, m, crown CH_2 and N-term. Lys αCH), 3.21 (2H, m, Lys εCH_2), 2.97 (2H, m, N-term. Lys εCH_2), 1.31-1.71 (12H, m, 2 Lys β, δ, and γCH_2), 1.22-1.28 (6H, m, 2 Ala CH_3).

N^{α}-*tert*-Butoxycarbonyl-L-alanyl-L-alanyl-L-alanyl-(N^{ε}-benzyloxy carbonyl)-L-lysyl-L-alanyl-(N^{ε}-(benzo-18-crown-6)-4-carbonyl)-L-lysyl-L-alanine methyl ester 16. To a stirred solution of peptide **7** (112 mg, 0.36 mmol) in dichloromethane at 0°C was added HOBT•H_2O (53 mg, 0.39 mmol) and DCC (81 mg, 0.39 mmol). The DCU was filtered off after 30 min and a solution of ester **15** (383 mg, 0.47 mmol) in dichloromethane, neutralized with 150 µL of triethylamine, was added. After 40h, the dichloromethane was evaporated. The mixture was dissolved in acetic acid and loaded directly on a Sephadex G-10-120 column. Elution with acetic acid followed by lyophilization gave the desired product **16**, as a white hygroscopic powder; 72.5 mg (17%); TLC, R_f; 0.31 (B); ^{1}H NMR (DMSO-D_6) δ: 8.30 (2H, m, 2 amide NH), 7.75-7.80 (5H, m, 4 amide NH and crown Lys εNH), 7.33-7.50 (8H, m, benzo H_3 and H_5, benzyl arom. H, and (Z)-Lys εNH), 6.96-7.01 (2H, m, benzo H_6 and BOC NH), 4.98 (2H, s, benzyl CH_2), 4.24 (6H, m, 6 αCH), 3.91 (1H, qn, N-term. Ala αCH), 3.58 (3H, s, -OCH_3), 3.50-4.11 (20H, m, crown CH_2), 3.22 (2H, m, crown Lys εCH_2), 2.97 (2H, m, (Z)-Lys εCH_2), 1.36 (9H, s, BOC CH_3), 1.31-1.71 (12H, m, 2 Lys β, δ, and γCH_2), 1.17-1.31 (15H, m, 5 Ala CH_3).

L-alanyl-(N^{ε}-benzyloxycarbonyl)-L-lysyl-L-alanine methyl ester hydro-chloride 17. The peptide **10**[20] (0.50 g, 0.9 mmol) was stirred in a 15 mL of 4N HCl in dioxane for 2h at room temperature. The solvent was evaporated and the residue triturated with ether. The solid obtained was filtrered and washed with ether to yield 0.43 g (96%) of deprotected ester **17**. TLC, R_f: 0.17 (B); $[\alpha]_D$ = -7° (c = 1, AcOH); ^{1}H NMR (DMSO-D_6) δ: 8.49 (1H, d, amide NH), 8.47 (1H, d, amide NH), 8.07-8.10 (3H, m, NH_3^+), 7.33-7.36 (5H, m, benzyl arom. H), 7.24-7.29 (1H, t, Lys εNH), 4.98 (2H, s, benzyl CH_2), 4.21-4.27 (2H, m, Lys and C-term. Ala αCH), 3.92-3.94 (1H, m, N-term. Ala αCH), 3.59 (3H, s, -OCH_3), 2.96-2.98 (2H, m, Lys εCH_2), 1.31 (3H, d, C-term. Ala CH_3), 1.27-1.44 (6H Lys β, δ, and γCH_2), 1.26 (3H, d, Ala CH_3).

N^{α}-*tert*-Butoxycarbonyl-L-alanyl-L-alanyl-(N^{ε}-benzyloxy-carbonyl)-L-lysyl-alanine methyl ester 18. To a stirred solution of BOC-Ala-OH (0.45 g, 2.4 mmol) in dichloromethane at 0°C was added HOBT•H_2O (0.35 g, 2.6 mmol) and DCC (0.54 g, 2.6 mmol). After 20 min. the precipitated DCU was removed and a solution of the ester **17** (1.12 g, 2.4 mmol) and triethylamine (0.66 mL, 4.8 mmol) in dichloromethane was added. After 4h, the mixture was worked up by the standard procedure and the crude mixture was purified by flash chromatography (dichloromethane/methanol 99:1→70:30) to yield 0.98 g (68%) of product **18**. TLC, R_f: 0.67 (A); mp 166-168°C; $[\alpha]_D$ = -42° (c = 1, AcOH), $[\alpha]_{546}$ = -62° (c = 1, AcOH); ^{1}H NMR (DMSO-D_6) δ: 8.29 (1H, d, amide NH), 7.85 (1H, d, Lys amide NH), 7.82 (1H, d, Ala amide NH), 7.33 (5H, m, benzyl arom. H), 7.21 (1H, t, Lys εNH), 6.97 (1H, d, BOC NH),

4.98 (2H, s, benzyl CH_2), 4.27 (1H, m, Lys αCH), 4.23 (1H, m, C-term. Ala αCH), 4.21 (1H, m, Ala αCH), 3.93 (1H, qn, N-term. Ala αCH), 3.58 (3H, s, $-OCH_3$), 2.95 (2H, m, Lys εCH_2), 1.63 (2H, m, Lys βCH_2), 1.47 (2H, m, Lys δCH_2), 1.35 (11H, m and s, Lys γCH_2 and BOC CH_3), 1.26 (3H, d, C-term. Ala CH_3), 1.17 (3H, d, Ala CH_3), 1.14 (3H, d, N-term. Ala CH_3).

N^{α}-*tert*-Butoxycarbonyl-L-alanyl-L-alanyl-(N^{ε}-benzyloxycarbonyl)-L-lysyl-L-alanine 19. Peptide ester **18** (0.61 g, 1 mol) was saponified with 1.5 mL of 1N aqueous NaOH in 2 mL methanol for 4h. Methanol was evaporated and 10 mL of water was added. The unreacted starting material was recovered by extraction into dichloromethane (2 x 25 mL). The water layer was then cooled to 0°C, acidified to pH=3 with a 1N HCl solution and extracted with dichloromethane (3 x 25 mL). The organic layer was dried with anhydrous magnesium sulfate, filtered, and evaporated to give **19** as a white powder. Yield 0.39 g (95%); TLC, R_f: 0.70 (B); mp: 96-99°C, $[\alpha]_D$ = -48° (c = 1, AcOH), $[\alpha]_{546}$ = -61° (c = 1, AcOH); ^1H NMR (DMSO-D_6) δ: 8.15 (1H, d, C-term. Ala amide NH), 7.85 (2H, m, 2 amide NH), 7.33 (5H, m, benzyl arom. H), 7.22 (1H, t, Lys εNH), 6.99 (1H, d, BOC NH), 4.98 (2H, s, benzyl CH_2), 4.22-4.26 (3H, m, 3 αCH), 4.15-4.18 (1H, qn, N-term. Ala αCH), 2.93-2.95 (2H, m, Lys εCH_2), 1.35 (9H, s, BOC CH_3), 1.24 (3H, d, C-term. Ala CH_3), 1.17 (3H, d, Ala CH_3), 1.12-1.70 (6H, m, Lys β, δ, and γCH_2), 1.11 (3H, d, N-term. Ala CH_3).

N^{α}-*tert*-Butoxycarbonyl-L-alanyl-L-alanyl-(N^{ε}-benzyloxycarbonyl)-L-lysyl-L-alanyl-L-alanyl-(N^{ε}-(benzo-18-crown-6)-4-carbonyl)-L-lysyl-L-alanine methyl ester 20. To a stirred solution of peptide acid **19** (104 mg, 0.17 mmol) in dimethylformamide (DMF) was added BOP[14] (Castro's reagent) (75 mg, 0.17 mmol), peptide **13** (100 mg, o.17 mmol) and 50 µL of triethylamine. After 18h, the crude mixture was chromatographed twice on a Sephadex G-10-120 using acetic acid as eluent. The desired protected heptapeptide **20** was obtained as a white hygroscopic powder after lyophilization. Yield: 152 mg (71%); TLC, R_f: 0.88 (B); $[\alpha]_{546}$ = -32° (c = 0.5, AcOH), $[\alpha]_{436}$ = -55° (c = 0.5, AcOH); ^1H NMR (DMSO-D_6) δ: 8.31 (1H, m, C-term. Ala NH amide), 7.80-8.07 (6H, m, 5 amide NH and crown Lys εNH), 7.34-7.43 (7H, m, benzo H_3 and H_5, benzyl arom. H), 7.23 (1H, t, (Z)-Lys εNH), 7.00 (2H, m, benzo H_6 and BOC NH), 4.98 (2H, s, benzyl CH_2), 4.23 (6H, m, 6 αCH), 3.95 (1H, qn, N-term. Ala αCH), 3.58 (3H, s, $-OCH_3$), 3.51-4.11 (20H, m, crown CH_2), 3.21 (2H, m, crown Lys εCH_2), 2.97 (2H, m, (Z)-Lys εCH_2), 1.36 (9H, s, BOC CH_3), 1.31-1.71 (12H, m, 2 Lys β, δ, and γCH_2), 1.13-1.28 (15H, m, 5 Ala CH_3).

N^{α}-*tert*-Butoxycarbonyl-L-alanyl-(N^{ε}-benzyloxycarbonyl)-L-lysyl-L-alanine 21. Peptide ester **10**[20] (0.40 g, 0.74 mmol) was saponified for 4h with 1.5 mL of 1N NaOH solution in 2 mL of methanol. The methanol was then evaporated and

10 mL of water was added. The unreacted ester **10** was recovered by extraction with ethyl acetate. After cooling to 0°C, the aqueous layer was acidified to pH = 3 with 0.1N HCl and the crude acid **21** was extracted with ethyl acetate. The organic layer was dried with anhydrous $MgSO_4$, filtrered, and evaporated to give the peptide acid **21**. Yield 0.34 g (88%); mp: 93-5°C (lit.[20] 94-7); $[\alpha]_D$ = -35° (c = 1, AcOH), $[\alpha]_{546}$ = -43° (c = 1, AcOH); ^1H NMR (DMSO-D_6) δ: 12.4-12.6 (1H, broad s, COOH), 8.18 (1H, d, C-term. amide NH), 7.68 (1H, d, amide NH), 7.31-7.36 (5H, m, arom. H), 7.19 (1H, t, Lys εNH), 6.96 (1H, d, BOC NH), 4.98 (2H, s, benzyl CH_2), 4.23-4.26 (1H, q, Lys αCH), 4.16-4.19 (1H, qn, C-term. Ala αCH), 3.94 (1H, qn, N-term. αCH), 2.93-2.95 (2H, m, Lys εCH_2), 1.35 (9H, s, BOC CH_3), 1.24 (3H, d, C-term. Ala CH_3), 1.12-1.70 (6H, m, Lys β, δ, and γCH_2), 1.13 (3H, d, N-term. Ala CH_3).

N$^{\alpha}$-*tert*-Butoxycarbonyl-L-alanyl-L-alanyl-L-lysyl-L-alanine methyl ester 22. Peptide **18** (1.16 g, 2 mmol) was solubilized in 25 mL of methanol and 50 mg of 10% Pd on charcoal was added. The solution was treated with hydrogen at 40 psi of pressure for 4h, filtered on celite, and evaporated to dryness. The desired product was precipitated from dichloromethane with ether to yield 0.81 g (90%) of **22**. TLC, R_f: 0.07 (B); mp 132-133°C; $[\alpha]_D$ = -38° (c = 1, AcOH), $[\alpha]_{546}$ = -54° (c = 1, AcOH),; ^1H NMR (DMSO-D_6) δ: 8.44 (1H, d, C-term. Ala amide NH), 7.90-7.95 (1H, m, amide NH), 7.64 (1H, d, amide NH), 7.01 (1H, d, BOC NH), 4.22-4.27 (3H, m, Lys and 2 Ala αCH), 3.90-3.92 (1H, qn, N-term. Ala αCH), 3.59 (3H, s, -OCH_3), 2.54 (2H, m, Lys εCH_2), 1.35 (9H, s, BOC CH_3), 1.25 (3H, d, C-term. Ala CH_3), 1.22-1.72 (6H, m, Lys β, δ, and γCH_2), 1.16 (3H, d, Ala CH_3), 1.13 (3H, d, N-term. Ala CH_3). Lys εNH_2 is screened.

N$^{\alpha}$-*tert*-Butoxycarbonyl-L-alanyl-L-alanyl-(N$^{\varepsilon}$-(benzo-18-crown-6)-4-carbonyl))-L-lysyl-L-alanine methyl ester 23. HOBT•H_2O (20 mg, 0.15 mmol) and DCC (31 mg, 0.15 mmol) were added to a stirred solution of (benzo-18-crown-6)-4-carboxylic acid[15] (48.5 mg, 0.14 mmol) in dichloromethane at 0°C. Twenty minutes later, the DCU was filtered off and a solution of ester **22** (72 mg, 0.14 mmol) in dichloromethane with triethylamine(41 μL, 0.30 mmol) was added. The mixture was stirred overnight and worked up in the usual manner. The desired peptide **23** was crystallized from dichloromethane and ether. Yield: 64 mg (59%). TLC, R_f: 0.90 (B); mp 127-128°C; $[\alpha]_D$ = -19° (c = 1, AcOH), $[\alpha]_{546}$ = -28° (c = 1, AcOH); ^1H NMR (DMSO-D_6) δ: 8.30 (1H, d, C-term. Ala amide NH), 7.85 (1H, d, Lys amide NH), 7.67 (1H, d, Ala amide NH), 7.42 (2H, m, benzo H_3 and H_5), 7.33 (2H, m, benzo H_6 and Lys εNH), 6.95 (1H, d, BOC NH), 4.24 (3H, m, Lys, Ala, and C-term. Ala αCH), 3.94 (1H, qn, N-term. Ala αCH), 3.59 (3H, s, -OCH_3), 3.51-4.11 (20H, m, crown CH_2), 3.21 (2H, m, Lys εCH_2), 1.35 (9H, s, BOC CH_3), 1.25 (3H, d, C-term. Ala CH_3), 1.22-1.60 (6H, m, Lys β, δ, and γCH_2), 1.17 (3H, d, Ala CH_3), 1.14 (1H, d, N-term. Ala CH_3).

L-alanyl-L-alanyl-(N$^{\varepsilon}$-(benzo-18-crown-6)-4-carbonyl))-L-lysyl-L-alanine methyl ester hydrochloride 24. The BOC protected peptide **23** (500 mg, 0.62 mmol) was stirred in 10 mL of 4N HCl in dioxane for 2h at room temperature. Evaporation of dioxane yielded 422 mg (85%) of a clear oil which could not be crystallized but gave a single ninhydrin positive spot on TLC (R_f: 0.08 (B). ^{1}H NMR (DMSO-D_6) δ: 8.61 (1H, d, C-term. Ala amide NH), 8.38 (2H, d, Lys and Ala NH), 8.18 (3H, m, NH_3^+), 7.98-8.01 (1H, m, Lys εNH), 7.43 (2H, m, benzo H_3 and H_5), 6.98 (1H, m, benzo H_6), 4.36 (1H, qn, N-term. Ala αCH), 4.23 (2H, m, Lys and Ala αCH), 3.84 (1H, qn, N-term. Ala αCH), 3.59 (3H, s, -OCH_3), 3.51-4.11 (20H, m, crown CH_2), 3.21 (2H, m, Lys εCH_2), 1.33 (3H, d, C-term. Ala CH_3), 1.30-1.60 (6H, m, Lys β, δ and γCH2), 1.25 (3H, d, Ala CH_3), 1.22 (3H, d, N-term. Ala CH_3).

N$^{\alpha}$-*tert*-Butoxycarbonyl-L-alanyl-(N$^{\varepsilon}$-benzyloxycarbonyl)-L-lysyl-L-alanyl-L-alanyl-L-alanyl-(N$^{\varepsilon}$-(benzo-18-crown-6)-4-carbonyl))-L-lysyl-L-alanine methyl ester 25. To a solution of peptide **21** (290 mg, 0.56 mmol) in DMF was added BOP (248 mg, 0.56 mmol), the free amino peptide **24** (417 mg, 0.56 mmol) and 150 μL of triethylamine. The resulting mixture was stirred for 18h at room temperature then loaded on a Sephadex G-10-120 column. The desired product **25** was obtained after two elutions with acetic acid and gave one spot on TLC ninhydrin negative at first then positive after HCl exposure. Yield: 270 mg (40%) of a white hygroscopic powder; TLC, R_f: 0.69 (B); $[\alpha]_D$ = -34° (c = 0.5, AcOH), $[\alpha]_{546}$ = -38° (c = 0.5, AcOH); ^{1}H NMR (DMSO-D_6) δ: 8.31 (2H, m, amide NH and crown Lys εNH), 7.34-7.43 (7H, m, benzo H_5 and H_3 and benzyl arom. H), 7.21 (1H, t, (Z)-Lys εNH), 6.94-7.01 (2H, m, benzo H_6 and BOC NH), 4.98 (2H, s, benzyl CH_2), 4.23 (6H, m, 6 αCH), 3.95 (1H, qn, N-term. Ala αCH), 3.59 (3H, s, OCH_3), 3.51-4.11 (20H, m, crown CH_2), 3.21 (2H, m, crown Lys εCH_2), 2.95 (2H, m, (Z)-Lys εCH_2), 1.36 (9H, s, BOC CH_3), 1.31-1.71 (12H, m, 2 Lys β, δ and γ CH_2),1.13-1.27 (15H, m, 5 Ala CH_3).

N$^{\alpha}$-*tert*-Butoxycarbonyl-L-alanyl-L-alanyl-L-alanyl-L-lysyl-L-alanyl-(N$^{\varepsilon}$-(benzo-18-crown-6)-4-carbonyl))-L-lysyl-L-alanine methyl ester 1. The peptide **16** (40 mg, 0.03 mmol) was dissolved in mixture of methanol/ acetic acid (1:1) and 50 mg of 100% Pd on charcoal were added. The solution was hydrogenated at 50 psi pressure for 1.5h, the filtered on celite. The methanol was evaporated and the acetic acid lyophilized to yield 37 mg (98%) of **1** as a hygroscopic white powder which gave a single peak in analytical HPLC. Overall yield from starting amino acids: 20%. TLC, R_f: 0.09 (A); FAB-MS ($C_{50}H_{83}N_9O_{17}$): 1082 ($M+H^+$) and 1120 ($M+K^+$); ^{1}H NMR (DMSO-D_6) δ: 8.29 (2H, m, 2 amide NH), 7.80-8.08 (5H, m, 4 amide NH and crown Lys ε NH), 7.40-7.44 (2H, m, benzo H_3 and H_5), 6.95-7.01 (2H, m, benzo H_6 and BOC NH), 4.23 (6H, m, 6 αCH), 3.96 (1H, m, N-term. Ala αCH), 3.58 (3H, s, -OCH_3), 3.52-4.11 (20H, m, crown CH_2), 3.22 (4H, m, 2 Lys εCH_2), 1.88 (3H, s, CH_3COO^-),

1.36 (9H, s, BOC CH_3), 1.30-1.60 (12H, m, 2 Lys β, δ and γCH_2), 1.13-1.31 (15H, m, 5 Ala CH_3). The NH_3^+ is screened.

N$^{\alpha}$-*tert*-Butoxycarbonyl-L-alanyl-L-alanyl-L-lysyl-L-alanyl-L-alanyl-(N$^{\varepsilon}$-(benzo-18-crown-6)-4-carbonyl))-L-lysyl-L-alanine methyl ester 2. Peptide **20** (123 mg, 0.1 mmol) was hydrogenolyzed as for **16** at 50 psi of H_2 for 3h, then the solution was filtrered on celite. After evaporation of the methanol, the mixture was loaded on a Sephadex G-10-120 column. The desired peptide **2** was eluted with acetic acid to yield 66 mg (60%) of a white hygroscopic powder after lyophilization. Analytical reverse phase HPLC showed a single peak. TLC, R_f: 0.09 (A); overall yield: 39%, FAB-MS ($C_{50}H_{83}N_9O_{17}$): 1082 ($M+H^+$) and 1104 ($M+Na^+$); ^1H NMR (DMSO-D_6) δ: 8.34 (2H, m, 2 amide NH), 7.85-8.07 (3H, m, 3 amide NH), 7.65-7.71 (2H, m, amide NH and crown Lys ε NH), 7.40-7.44 (2H, m, benzo H_3 and H_5), 7.00 (2H, m, benzo H_6 and BOC NH), 4.23 (6H, m, 6 αCH), 3.95 (1H, m, N-term. Ala αCH), 3.59 (3H, s, -OCH_3), 3.51-4.11 (20H, m, crown CH_2), 3.20 (4H, m, 2 Lys εCH_2), 1.90 (3H, s, CH_3COO^-), 1.36 (9H, s, BOC CH_3), 1.30-1.60 (12H, m, 2 Lys β, δ and γCH_2), 1.13-1.32 (15H, m, 5 Ala CH_3). The NH_3^+ is screened.

N$^{\alpha}$-*tert*-Butoxycarbonyl-L-alanyl-L-lysyl-L-alanyl-L-alanyl-L-alanyl-(N$^{\varepsilon}$-(benzo-18-crown-6)-4-carbonyl))-L-lysyl-L-alanine methyl ester 3. The hydrogenolysis of peptide **25** (225 mg, 0.18 mmol) was performed as described above for **20**. After filtration on celite and evaporation, the mixture was loaded on a Sephadex G-10-120 column and chromatographed with acetic acid to yield 112 mg (56%) of a white hygroscopic powder after lyophilisation. Overall yield: 11%; TLC, R_f: 0.10 (A); FAB-MS ($C_{50}H_{83}N_9O_{17}$): 1082 ($M+H^+$) and 1104 ($M+Na^+$); ^1H NMR (DMSO-D_6) δ: 8.35 (2H, m, 2 amide NH), 8.10 (3H, m, 3 amide NH), 7.85 (2H, m, amide NH and crown Lys ε NH), 7.42-7.46 (2H, m, benzo H_3 and H_5), 7.00 (2H, m, benzo H_6 and BOC NH), 4.23 (6H, m, 6 αCH), 3.94 (1H, m, N-term. Ala αCH), 3.59 (3H, s, -OCH_3), 3.52-4.11 (20H, m, crown CH_2), 3.20 (4H, m, 2 Lys εCH_2), 1.86 (3H, s, CH_3COO^-), 1.36 (9H, s, BOC CH_3), 1.30-1.60 (12H, m, 2 Lys β, δ and γCH_2), 1.13-1.28 (15H, m, 5 Ala CH_3). The NH_3^+ is screened.

Conformational studies

Circular dichroism studies were performed in 2,2,2-trifluoroethanol (TFE) and acetonitrile (Spectro Grade) using solutions of peptides **1**, **2**, **3**, and **16**, **20**, and **25** at a concentration of 3.3-3.5 x 10^{-5}M. The solutions were made from peptides 1.75 X 10^{-3} M stock solutions in TFE and in acetonitrile containing 4% of TFE. In every cases, a 5 mm pathlenght quartz cell was used. The experiments with K^+ were performed by adding solid $KClO_4$ to the stock peptide solutions. After vigourous shaking, the excess of salt was filtered and then the CD curves were recorded. The assays with the

neutralized peptide **1-3** were done using a 100 fold equivalent of quinuclidine. All the data are reported in molar ellipticity ([θ]) in mdeg cm^2dmol^{-1}.

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