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Conformational Studies of Cyclo(Pro–Leu–Aib)$_2$ by ^{1}H Nuclear Magnetic Resonance Spectroscopy and Circular Dichroism[1]

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The synthesis and conformational analysis of cyclo(Pro–Leu–Aib)$_2$ by means of NMR technique are reported. The cyclohexapeptide was synthesized in the classical high-dilution solution method. Its yield in the cyclization reaction from linear hexapeptide active ester was lower than that of the analogous cyclo(Pro–Leu–Gly)$_2$ previously reported. Two sets of amide proton signals for Leu and Aib in NMR spectra and temperature coefficients indicated an asymmetric conformation which contains both *cis* and *trans* Aib–Pro bonds. Here Pro amino acids occupy the positions $i+2$ of β-turns. The CD spectrum gave two negative bands at 202 and 230 nm, which is considered to be the sum of type II′ (when Aib–Pro is *trans*) and type VI (when Aib–Pro is *cis*) β-turns.

Incorporation of unusual amino acids into peptide chains is an active field in the design of artificial peptides and proteins. Generally, unusual amino acids are introduced to increase resistance to protease, to improve stability, to impose steric hindrance, or to generate various functionalities. For reviews concerning this subject, see Roberts and Vellaccio[2] or Ishida et al.[3] α-Aminoisobutyric acid (Aib), the prototype of $C^{\alpha,\alpha}$-disubstituted amino acids, is considered to bring severe steric hindrance to the peptide chains and to reduce the flexibilities due to the two methyl groups attached to the α-carbon atom. Apparently the sterically allowed regions of conformational angles ϕ and ψ^4 must be restricted only to those regions which are allowed for both L- and D-alanines. A large number of peptides containing Aib have been investigated to examine the influence of this structurally unique amino acid on the folding of peptide backbones or formation of secondary structures; most were linear peptides.[5]

Cyclic peptides are excellent models for reverse turns, and it is well established that cyclohexapeptides contain two β-turns. In a study of β-turns with cyclo(Pro–Leu–Gly)$_2$,[1] we observed the two β-turns structure involving two 4→1 intramolecular hydrogen bonds as expected. When the glycine is replaced by an unusual amino acid Aib, one can investigate the influence of the two methyl groups of Aib on the formation of β-turns. Furthermore, the successful preparation of cyclo(Pro–Leu–Aib)$_2$ can probably guarantee the synthesis of both cyclo(Pro–Leu–Ala)$_2$ and cyclo(Pro–Leu–D-Ala)$_2$, while the former is a rare example of cyclohexapeptides consisting of all L-configuration residues.[6]

Results and Discussion

Although Aib is also an achiral amino acid which can be free of racemization, it was not chosen as the C-terminal residue because of the difficulty which might be encountered in the cyclization step resulting from the steric hindrance. Instead, proline was selected in the synthesis of cyclo(Pro–Leu–Aib)$_2$ because its chiral integrity remains unaffected under most of the usual conditions of peptide synthesis.[7] For example, proline was chosen as the C-terminal residue of the open chain precursor in the synthesis of gramicidin S.

The synthetic routes are shown in Fig. 1. Cyclo(Pro–Leu–Aib)$_2$ was synthesized from both linear tri- and

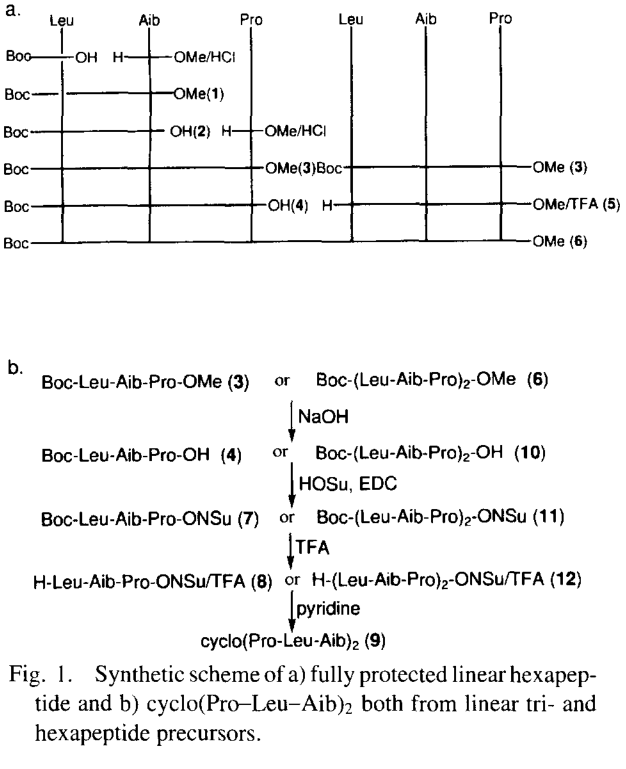

Fig. 1. Synthetic scheme of a) fully protected linear hexapeptide and b) cyclo(Pro–Leu–Aib)$_2$ both from linear tri- and hexapeptide precursors.

linear hexapeptide active esters through cyclization in pyridine under high-dilution conditions. The linear precursors were prepared in the conventional liquid phase method. Coupling of Boc–Leu–OH with H–Aib–Pro–OMe•TFA in DCC/HOBt method gave the fully protected tripeptide Boc–Leu–Aib–Pro–OMe (**3**) only in extremely low yield, probably due to the formation of diketopiperazine.[5] However, it was prepared smoothly in satisfactory yield from dipeptide Boc–Leu–Aib–OH (**2**) and H–Pro–OMe•HCl instead. A portion of Boc–Leu–Aib–Pro–OMe (**3**) was saponified in aqueous NaOH solution, followed by acidification, to give the free acid derivative **4**. Another portion of the tripeptide was treated with trifluroacetic acid to remove Boc group. Subsequent coupling of the two tripeptide components afforded hexapeptide Boc–Leu–Aib–Pro–Leu–Aib–Pro–OMe (**6**).

The protected linear tripeptide **2** and linear hexapeptide **6** were saponified respectively to set the C-terminal free, and converted to active esters with HOSu. After removal of the Boc groups by TFA, cyclization reactions proceeded in pyridine solution. Cyclo(Leu–Aib–Pro)$_2$ (**9**) was isolated after column chromatography on silica-gel in 17 and 19.5% yield from linear tripeptide **4** and hexapeptide **10**, respectively, lower than 26 and 43.5%, from linear tripeptide and hexapeptide, respectively, for cyclo(Pro–Leu–Gly)$_2$.[1]

The ^{1}H NMR spectra of cyclo(Pro–Leu–Aib)$_2$ are shown

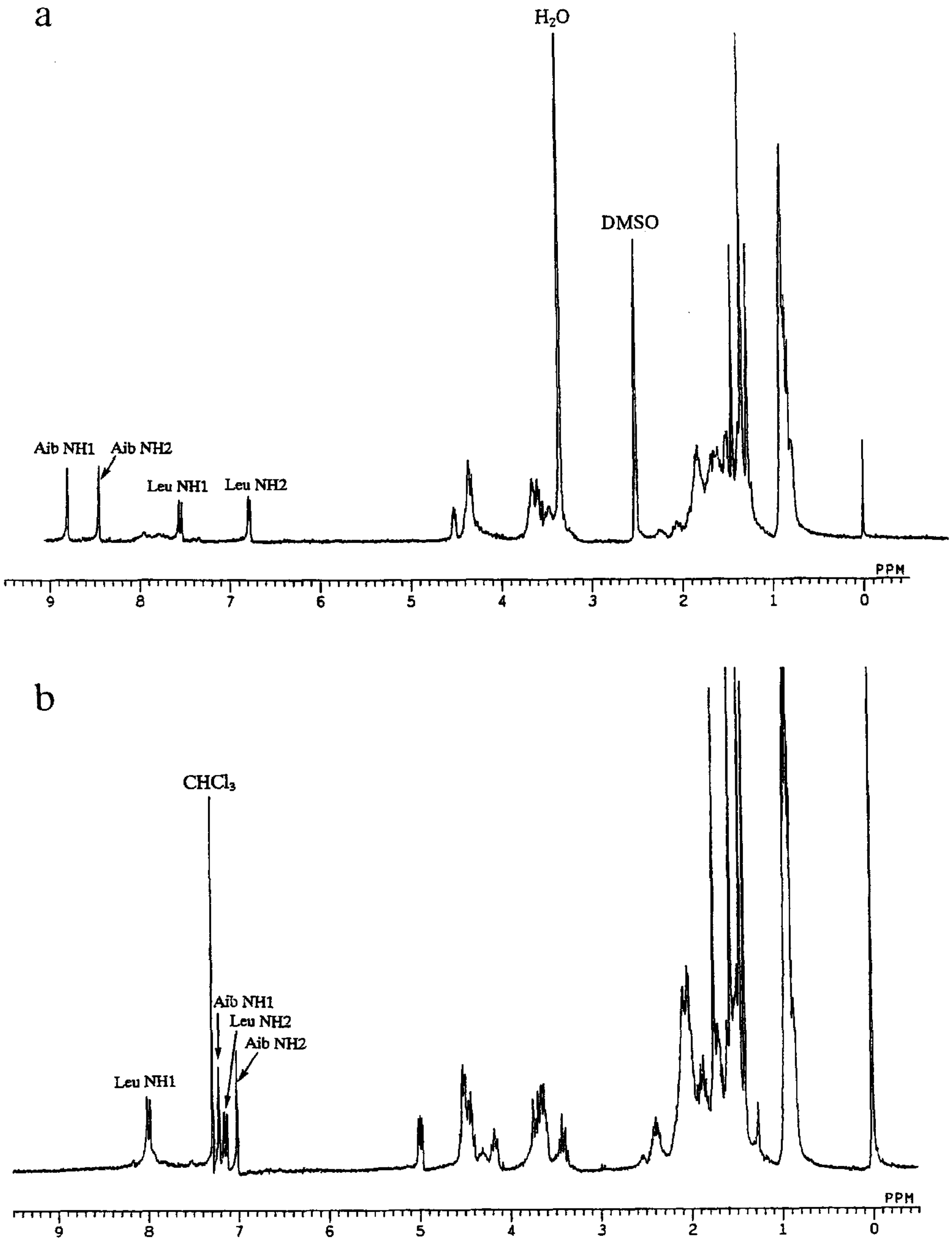

Fig. 2. 270 MHz ^{1}H NMR spectra of cyclo(Pro–Leu–Aib)$_2$ at 21 °C in a) DMSO-d_6, 17.5 mg ml^{-1} and b) CDCl$_3$, 19.0 mg ml^{-1}. Two sets of amide protons were numbered in series from left sides, respectively.

in Fig. 2. Unlike cyclo(Pro–Leu–Gly)$_2$, the analogous cyclo(Pro–Leu–Aib)$_2$ gave two sets of amide proton NH signals for Leu and Aib residues both in DMSO-d_6 and $CDCl_3$. Because of the absence of α-H in Aib, the two singlets should come from Aib residues, and the two doublets were split by a α-carbon hydrogen; they accordingly could be assigned to the Leu. When two drops of CD_3OD were added into the DMSO solution of cyclo(Pro–Leu–Aib)$_2$, the H–D exchange rate was almost the same for NH of both Leu and Aib residues. Thus the isotope exchange experiment could not distinguish these two kinds of amide protons.

In peptides with Aib incorporated, the sterically allowed regions of conformational angles ϕ and ψ must apparently be restricted only to those regions which are allowed for both L- and D-alanine. A number of peptides containing Aib have been investigated to examine the influence of this structurally unique amino acid on the folding of peptide backbone or formation of secondary structures. Most of the studies were focused on linear peptides,[5,8,9] while cyclic peptides were rare except for cyclodipeptides (diketopiperazines), the only one cyclohexapeptide containing Aib—cyclo(Gly-Aib-Gly)$_2$ [10] was reported as a byproduct during the synthesis of copolymers of Gly and Aib by conventional peptide procedures. It was well established that Aib is preferable for α-helix with $\phi = -55°$, $\psi = -45°$ and 3_{10}-helix with $\phi = -60°$, $\psi = -30°$.[11–16] γ-Turns (C_7 conformations) have been shown to be energetically less favorable even though the steric contacts are generally acceptable. β-Turns (also called C_{10}-conformations) were also found in linear Aib-containing peptides,[17] for example, Z–Aib–Pro–Aib–Ala–OMe and Z–Aib–Pro–Aib–Pro–OMe.

The temperature coefficients of the amide protons of cyclo(Pro–Leu–Aib)$_2$ are summarized in Table 1. For the purpose of comparison, the data for cyclo(Pro–Leu–Gly)$_2$ are also listed. It can be seen that change of chemical shifts for amide protons NH of Aib residues are more dependent on temperature than that of Leu residues. For instance, 4.29 and 6.66 ppm/°C of Aib vs. 0.69 and 0.94 ppm/°C of Leu in DMSO. Moreover, it was observed that replacement of the strongly H-bonding solvent DMSO by $CDCl_3$ leads to a considerable shift to higher field of NMR signals of exposed NH-groups.[18,19] When the solvent was changed from DMSO to $CDCl_3$, the chemical shifts of Aib amide proton resonance moved upfield as high as 1.59 and 1.44 ppm. In contrast, those of Leu moved downfield by only 0.45 and 0.37 ppm. All these results suggest that the backbone contains two Aib–Pro turns connected by extended Leu residues and the amide protons of Leu are more shielded from solvent than those of Aib and less sensitive to temperature. This argues that Pro amino acids occupy positions $i+2$ of β-turns numbered from i to $i+3$, which is contrary to the conformation of cyclo(Pro–Leu–Gly)$_2$ in DMSO in which Pro amino acids occupy positions $i+1$.[1] Further, there is difference between the amide protons of these two extended Leu residues, especially in $CDCl_3$ solution.

The crystal structures of Boc–Aib–Pro–OBzl were analyzed and the conformations of pyrrolidine ring of Pro residue were preferred for type III β-turn and the unfolded backbone of the dipeptide sequence.[20] Furthermore, theoretical calculations have led to the conclusion that β-turns with corner residues of L-X-Pro are energetically unfavorable due to nonbonded contacts between the C^{δ} atom of the pyrrolidine ring and the C^{β} group of the X residue.[21] This conclusion has been reinforced by a survey of the Pro-containing β-turn structures observed in proteins.[22] Of the 459 β-turns examined from 29 proteins whose crystal structures are known, 58 β-turns occur with Pro at position $i+1$ and 12 with Pro at position $i+2$. Similar results from statistical analysis of β-turns in proteins from a greater data set of 3899 β-turns using 205 protein chains were reported more recently.[23] The difficulty in the formation of β-turns with Pro at the position $i+2$ might account for the lower yield of cyclo(Pro–Leu–Aib)$_2$ in the cyclization reaction from linear active ester than that with the analogous sequence Pro–Leu–Gly. In this case, the steric effect of the two methyl groups of the α-carbon atom on Aib residue forces the Pro into the less favored $i+2$ position in the case of cyclo(Pro–Leu–Aib)$_2$.

Furthermore, since in Fig. 2 there are two sets of amide protons for each kind of amino acid residue, the conformation must be asymmetric. That is, one of the Aib–Pro peptide bonds should be cis, as shown in Fig. 3. The measured coupling constants of two doublets for Leu are 9.28 and 5.37. According to correlation[24] $J_{HN-CH} = 7.9\cos 2\theta - 1.55\cos\theta + 1.35\sin 2\theta$, they should correspond to 171° and 132°, and these could be confirmed by manual model-building. Of course, the possibility of an equilibrium of two-*cis* structure

Table 1. Amide Proton NMR Signals of Cyclic Peptides
The data are shown in chemical shifts δ in ppm at 21 °C, the values in parentheses are temperature coefficients $\Delta\delta/\Delta T$ in $\times 10^{-3}$ ppm/°C.

Cyclic peptides	Residues	DMSO-d_6	$CDCl_3$
Cyclo(Pro–Leu–Gly)$_2$	Gly NH	7.48 (0.37)	7.88 (3.3)
	Leu NH	8.18 (6.8)	6.84 (3.7)
Cyclo(Pro–Leu–Aib)$_2$	Aib NH	8.81 (4.29)	7.22 (12.2)
		8.46 (6.66)	7.02 (1.94)
	Leu NH	7.56 (0.69)	8.01 (3.83)
		6.80 (0.94)	7.17 (0.95)

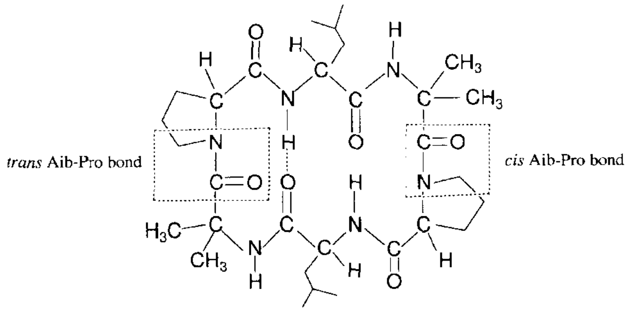

Fig. 3. Proposed model of cyclo(Pro–Leu–Aib)$_2$ in solution. The hydrogen bond in the type II′ β-turn with *trans* Aib–Pro bond on the left side is indicated by dashed line.

and all-*trans* structure can not be ruled out, since it was well established in cyclohexapeptides that *cis* and *trans* peptide bond equilibrium exists.[25]

When Pro occupies position $i+2$ and the Aib–Pro peptide unit is *trans*, it falls into type II′ β-turn; when the Aib–Pro bond is *cis*, it forms a type VI β-turn. The definitions used for the ϕ, ψ angles of the type VI were taken from Richardson.[26] This type of β-turns was also found in proteins. In the survey of 3899 β-turns from 205 protein chains as mentioned above, 55 were identified as type VI.[23]

In the crystal structure of cyclo(Gly–Aib–Gly)$_2$,[10] Aib amino acids also occupy positions 2 of β-turns, but it has a center of symmetry. All peptide units are in *trans* conformations. Two glycines (1 and 4) have *trans* conformations of the C^{α}–C' bonds (ψ_1 = 178.55°), whereas the other two glycines are near *cis* conformations ($\psi_3 = -2.69°$). The observed values of ϕ and ψ for the Aib residues ±(55.3, 35.2) agree broadly with the predicted values for the low-energy form of an Aib residue ±(50, 42), and are midway between expected values for a α-helix and a 3_{10}-helix. It contains two β-turns stabilized by two $1 \leftarrow 4$ hydrogen bonds.

In the circular dichroism of cyclo(Pro–Leu–Aib)$_2$ together with cyclo(Pro–Leu–Gly)$_2$ shown in Fig. 4, the spectrum of cyclo(Pro–Leu–Aib)$_2$ displays two negative bands at 202 and 230 nm with molar ellipticities per residue of about -5000, a pattern neither similar to the α-helix-like curve of type I β-turns, nor similar to classical type II cyclohexapeptides.[4,27,28] Since what the CD spectrum shows is the overall molecular picture of conformations in solution, the curve of cyclo(Pro–Leu–Aib)$_2$ should be a sum of both type II′ and type VI β-turns, as discussed above.

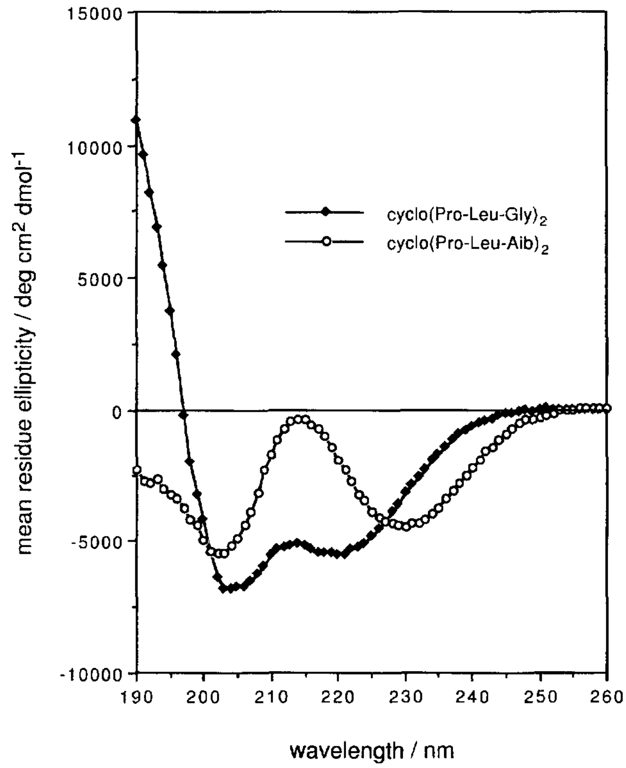

Fig. 4. CD spectra of cyclo(Pro–Leu–Aib)$_2$ and cyclo(Pro–Leu–Gly)$_2$ in MeOH. Peptide concentrations were 2 mM.

Conclusion

In the proton NMR spectra of cyclo(Pro–Leu–Aib)$_2$ in DMSO-d_6 and $CDCl_3$ solutions, there were two sets of amide proton signals for both Leu and Aib, indicating that cyclo(Pro–Leu–Aib)$_2$ takes an asymmetric conformation. Temperature coefficients proved that Pro occupy the positions $i+2$ of β-turns, different from the conformation of cyclo(Pro–Leu–Gly)$_2$ in DMSO solution in which prolines occupy the positions $i+1$ of β-turns. Cyclo(Pro–Leu–Aib)$_2$ contains both *trans* (type II′ β-turn) and *cis* (type VI β-turn) Aib–Pro bonds. Its CD spectrum gave two negative bands at 202 and 230 nm, and should be the sum of these two types of β-turns.

Experimental

Abbreviations: Aib, α-aminoisobutyric acid; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; EA, ethyl acetate; EDC, 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide hydrochloride; HPLC, high-performance liquid chromatography; HOBt, 1-hydroxybenzotriazole; HOSu, *N*-hydroxysuccinimide; –ONSu, hydroxysuccinimide ester; LP, light petroleum ether (30—60 °C); NMM, *N*-methylmorpholine; TLC, thin-layer chromatography.

Materials and Methods. Dimethyl-d_6 sulfoxide (99.9 atom% D) and chloroform-*d* (99.96% atom% D) were purchased from Isotec Inc., USA. Analytical grade solvents were used routinely without further purification. Optical active amino acids were of L-configuration. Melting points were uncorrected. Amino acid analyses were performed on a Wakopak WS-PTC system and the hydrolysis of peptides was carried out in 6 M HCl (1 M = 1 mol dm^{-3}) at 110 °C for 24 h, then at 150 °C for 1 h. Thin-layer chromatography was run on silica gel (Merck 60 GF_{254}). HPLC was conducted on a JASCO chromatography recorder with a UV-970 Intelligent UV/vis detector at 220 nm and 807 IT Intelligent Integrator. The column was Wakosil 5C4 200 $\phi 4.6 \times 250$ mm (D), gradient eluents were solution A (H_2O/MeCN/TFA 95 : 5 : 0.05) and solution B (H_2O/MeCN/TFA 5 : 95 : 0.04), with the amount of solution B increasing from 0 to 100% in 30 min, and returning to 0% in the next 5 min. Flow rate was 1 ml min^{-1}. Mass spectra were recorded on a MALDI-TOF mass spectrometer of Voyager. The proton magnetic resonance spectra were recorded on a JEOL JNM-GX270 FT NMR spectrometer. Tetramethylsilane was used as internal standard. Temperature-dependence studies were performed with the temperature controlled to ±1 °C. Circular dichroism measurements were made on a JASCO J-720 spectropolarimeter, the concentrations of peptides were 2 mM in MeOH. Spectra were scanned at room temperature in a cylindrical quartz cell with a 0.1 mm path length. The spectra were obtained over the range of 260—190 nm. Ten scans were taken for each peptide at a scan rate of 20 nm min^{-1}. Molar ellipticities per residue were expressed in deg cm^2 dmol^{-1}.

Peptide Synthesis. The linear tripeptide fragment was prepared by a stepwise strategy, and it was coupled to form the open-chain hexapeptide. Some of the intermediate peptides were not thoroughly purified to give analytical samples, but their purities were checked by TLC in a variety of solvent systems to give one spot or a major component of the expected products before the next steps were undertaken. Further details concerning specific peptide derivatives are described below.

Boc–Leu–Aib–OMe (1). 9.47 g of Boc–Leu–OH•H_2O (38.0 mmol) and 5.84 g of H–Aib–OMe•HCl (38.0 mmol) were dissolved in 115 ml of DMF and cooled to 0 °C. 4.2 ml of NMM (38 mmol) was added for neutralization, followed by 5.13 g of HOBt (38.0 mmol). 10 min later, DCC (7.84 g, 38.0 mmol) was added for activation. The reaction mixture was stirred at room temperature for 48 h. DCU was filtered off and the solvent was evaporated completely in vacuo. The residue was taken up in 120 ml of EtOAc. Insoluble substances were filtered off and the organic solution was washed successively with 0.5 M $NaHCO_3$ aq (40 ml×3), 5% $KHSO_4$ aq (40 ml×3), and saturated NaCl solution (40 ml×3). After drying over anhydrous sodium sulfate overnight, evaporation of the solvent afforded the protected dipeptide. It was washed with petroleum ether (20 ml×3). Yield 9.34 g, 74.4%, mp 154—156 °C. From the mother liquid of petroleum ether solution, a second crop of 506 mg was obtained, total yield 78.4%.

Boc–Leu–Aib–OH (2). 9.34 g of Boc–Leu–Aib–OMe (28.3 mmol) was dissolved in 70 ml of MeOH and cooled to 0 °C. To the solution 56.0 ml of 1.01 M NaOH solution (56.6 mmol) was added. The mixture was allowed to warm up to room temperature. After 3 h, the solvent was evaporated and the residue was taken up in 30 ml of water, then extracted with 10 ml of ether three times to remove byproducts. The aqueous solution was acidified to pH 2 with 2 M $KHSO_4$ (ca. 30 ml). Precipitated white solid was extracted with EA (50 ml×3). After drying with Na_2SO_4, the solvent was evaporated and the resulting crystalline solid was washed with 20 ml LP, yield 8.50 g, 95.0%, $[\alpha]_D^{27}$ −31.4° (*c* 1.00, EtOH), mp 160—162 °C (Ref.[29] 128—130 °C, Ref.[30] 142—145 °C).

Boc–Leu–Aib–Pro–OMe (3). 7.94 g of Boc–Leu–Aib–OH (25.1 mmol) and 3.39 g of HOBt (25.1 mmol) were activated with 5.18 g of DCC (25.1 mmol) in 31 ml of DMF at 40 °C for 20 min. A solution of 4.99 g of H–Pro–OMe•HCl (30.1 mmol) and 3.3 ml of NMM (30.1 mmol) dissolved in 32 ml of DMF was added; the mixture was stirred at 40 °C for 1 h, then at room temperature for 24 h, finally at 0 °C for 1 h. The DCU was filtered off and the solvent was evaporated in vacuo. The residue was taken up in 150 ml of EA; this mixture was washed successively with 40 ml of water, 0.5 M $NaHCO_3$ aq (50 ml×3), 5% $KHSO_4$ aq (50 ml×3) and saturated NaCl solution (50 ml×3); and dried over Na_2SO_4. After removal of the solvent, the crystals were washed with 20 ml of LP to give 8.45 g of product. Yield 78.8%, $[\alpha]_D^{27}$ −97.1° (*c* 1.13, EtOH), mp 175—176 °C (Ref.[30] 167 °C). Anal. Found: C, 58.96; H, 8.74; N, 9.89%. Calcd for $C_{21}H_{37}N_3O_6$: C, 59.00; H, 8.72; N, 9.83%.

Boc–Leu–Aib–Pro–OH (4). 3.85 g of Boc–Leu–Aib–Pro–OMe (9.00 mmol) was dissolved in 27 ml of MeOH and cooled to 0 °C, to the solution 9.3 ml of 1.95 M NaOH solution was added. Thirty min later, the reaction mixture was stirred at room temperature for 13 h. The solvent was evaporated and the residue was taken up in 15 ml of water. The aqueous solution was extracted with 10 ml of ether three times, and then acidified to pH 2 with 2 M $KHSO_4$. The product was extracted with EA (40 ml×3). After drying over sodium sulfate, evaporation of the solvent afforded 3.05 g of crystals, 82.0%, $[\alpha]_D^{27}$ −83.3° (*c* 1.23, EtOH), mp 110—119 °C. Recrystallization from ethanol raised the melting point to 115—123 °C. Anal. Found: C, 57.35; H, 8.95; N, 9.25%. Calcd for $C_{20}H_{35}N_3O_6 \cdot 0.5H_2O$: C, 56.85; H, 8.59; N, 9.95%.

Boc–Leu–Aib–Pro–ONSu (7). 827 mg of Boc–Leu–Aib–Pro–OH (2.00 mmol) was dissolved in 13 ml of DMF and cooled to 0 °C. To it 254 mg of HOSu (2.20 mmol) and 421 mg of EDC (2.20 mmol) were added. The reaction mixture was stirred at 0 °C for 2 h and then at room temperature for 26 h. DMF was evaporated in vacuo, and the residue was distributed between 10 ml of water and 30 ml of EA. The organic solution was separated and washed with saturated NaCl solution (10 ml×2); then dried over anhydrous sodium sulfate. Evaporation of the solvent afforded active ester in quantitative yield; this was used in the next step without purification.

H–Leu–Aib–Pro–ONSu•TFA (8). To the above Boc–Leu–Aib–Pro–ONSu (7), 5 ml of TFA was added and the mixture was kept at 0 °C for 60 min. The excess TFA was evaporated and the residue was taken up in 10 ml of ether three times; the supernatants were discarded and the residue was evaporated to dryness. White amorphous solid (910 mg) was obtained in 86.8% yield. The freshly prepared N-terminal free active ester was used for the next cyclization without further purification.

Cyclo(Leu–Aib–Pro)$_2$ (9) (from linear tripeptide). A solution of 900 mg of H–Leu–Aib–Pro–ONSu•TFA (1.72 mmol) in 2 ml of DMF was dropped into 330 ml of pyridine. 1 ml of DMF was used for rinsing and also dropped into the pyridine solution. After 3 h at 0 °C, the reaction mixture was stirred at room temperature for 3 d. The solvent was removed by evaporation and the residue was taken up in 30 ml of EA. Followed by washing with 10 ml of water for three times. The aqueous layers were combined and re-extracted with chloroform (15 ml×3). After drying with anhydrous sodium sulfate and removal of the solvent by evaporation, 475 mg of crude product was obtained. Purification of the cyclic peptide was achieved by chromatography on a silica-gel column of $\phi 2.7 \times 71$ cm, using chloroform/methanol 5 : 1 (v/v) as eluent. The fractions were collected and concentrated, and 86 mg cyclo(Leu–Aib–Pro)$_2$ (yield 17.0%) was obtained. R_f 0.69 (chloroform/methanol 5 : 1).

H–Leu–Aib–Pro–OMe•TFA (5). To a solution of 1.28 g of Boc–Leu–Aib–Pro–OMe (3.00 mmol) in 6 ml of dichloromethane at 0 °C, 3 ml of TFA was added. The solution was stirred at 0 °C for 10 min, and at room temperature for 3 h. The solvent was evaporated and the residue was taken up in 10 ml of ether for 3 times; the supernatants were discarded and the residue was evaporated to dryness. After drying over potassium hydroxide, 1.25 g TLC homogenous product was obtained, yield 94.7%.

Boc–Leu–Aib–Pro–Leu–Aib–Pro–OMe (6). 1.09 g of Boc–Leu–Aib–Pro–OH (2.64 mmol) was activated with 0.60 g of EDC (3.13 mmol) in 10 ml of DMF in the presence of 384 mg of HOBt (2.84 mmol) for 15 min at 0 °C. To it a solution of 1.25 g of H–Leu–Aib–Pro–OMe•TFA (2.84 mmol) and 0.3 ml of NMM in 10 ml of DMF was added. The reaction mixture was stirred at 0 °C for 1 h, then at room temperature for 30 h. DMF was evaporated in vacuo and the residue was taken up in 45 ml chloroform. The solution was washed successively with water (10 ml×2), 0.5 M $NaHCO_3$ aq (30 ml×2), 5% $KHSO_4$ aq (30 ml×2) and saturated NaCl aq (30 ml×3); it was then dried with anhydrous sodium sulfate. Evaporation of the solvent afforded 1.73 g of crystals, 90.9%, mp 226—230 °C. Recrystallization from EA raised the melting point to 235—236 °C. $[\alpha]_D^{28}$ −50.0° (*c* 1.22, EtOH). Anal. Found: C, 59.70; H, 8.64; N, 11.70%. Calcd for $C_{36}H_{62}N_6O_9$: C, 59.81; H, 8.64; N, 11.63%.

Boc–Leu–Aib–Pro–Leu–Aib–Pro–OH (10). 1.67 g of Boc–(Leu–Aib–Pro)$_2$–OMe (2.31 mmol) was dissolved in 20 ml of MeOH and cooled to 0 °C. To it 4.5 ml of 1.02 M NaOH aqueous solution was added. After completion of the reaction (checked by TLC), the solvent was evaporated and the residue was treated with 30 ml of water. The solution was extracted with ether (20 ml) to remove byproducts, and acidified to pH 2 with 2 M $KHSO_4$ aq. The product was extracted with 20 ml of chloroform and 20 ml of EA three times. After drying and removal of the solvent, 1.30 g hexapeptide was obtained, 79.5%, mp 216—221 °C. Recrystallization from EA gave the analytical sample, $[\alpha]_D^{28}$ −44.1° (*c* 1.29, EtOH), mp 225.5—227 °C. Anal. Found: C, 58.77; H, 8.50; N, 11.64%. Calcd for $C_{35}H_{60}N_6O_9\cdot 0.5H_2O$: C, 58.56; H, 8.56; N, 11.71%.

Boc–Leu–Aib–Pro–Leu–Aib–Pro–ONSu (11). 1.30 g of Boc–(Leu–Aib–Pro)$_2$–OH (1.84 mmol) was dissolved in 10 ml of DMF and cooled to 0 °C. To it 422 mg of HOSu (3.67 mmol) and 527 mg of EDC (2.75 mmol) were added. After 60 min at 0 °C, the solution was stirred at room temperature for 24 h. DMF was removed in vacuo and the residue was taken up in 20 ml of chloroform. The organic solution was washed with water and saturated NaCl solution 10 ml×2. The resulting aqueous solution was re-extracted with EA 15 ml×2. Removal of the organic solvents after drying with Na_2SO_4 gave 1.46 g active ester, 98.6%, which was used in the next step without further purification.

H–Leu–Aib–Pro–Leu–Aib–Pro–ONSu·TFA (12). To a solution of 1.43 g Boc–(Leu–Aib–Pro)$_2$–ONSu (1.77 mmol) in 2 ml dichloromethane at 0 °C, 4 ml TFA was added. The solution was stirred for 15 min and further at room temperature for 4 h. Solvent was evaporated and the residue was rubbed with several changes of ether; the supernatants were discarded and the residue was evaporated to dryness. White amorphous solid was obtained quantitatively.

Cyclo(Leu–Aib–Pro)$_2$ (9) (from linear hexapeptide). A solution of 1.39 g H–(Leu–Aib–Pro)$_2$–ONSu·TFA (1.70 mmol) in 2 ml DMF was dropped into 650 ml pyridine at 0 °C. 2 h later, the solution was stirred at room temperature for 3 d. Solvent was removed by evaporation and the residue was distributed between 10 ml water and 20 ml chloroform. The aqueous layer was separated and further extracted with 20 ml chloroform, followed by EA 20 ml×3. The organic solutions were dried with anhydrous sodium sulfate and evaporated under reduced pressure. The crude product was passed through silica gel on a column of $\phi 4.7\times 65$ cm, using chloroform/methanol 5 : 1 (v/v) as eluent. Fractions containing the cyclic peptide were collected and concentrated. Crystals appeared upon addition of LP, yielded 196 mg cyclo(Leu–Aib–Pro)$_2$, 19.5%, $[\alpha]_D^{28}$ −81.9° (*c* 0.598, EtOH), mp 152—157 °C. Retention time $t_R = 11.54$ min. MS gave the parent ion of $[M+H^+]$ 591.5, theoretic value 590.76 for $C_{30}H_{50}N_6O_6$. Anal. Found: C, 58.73; H, 8.43; N, 13.98%. Calcd for $C_{30}H_{50}N_6O_6\cdot H_2O$: C, 59.19; H, 8.61; N, 13.80%.

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