Synthesis and Solution Conformations of Cyclo(Pro–Leu–Gly)$_2$ and Cyclo(Pro–Leu–Gly)$_4$

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Two cyclic peptides containing the C-terminal tripeptide sequence of oxytocin, cyclo(Pro–Leu–Gly)$_2$ and cyclo(Pro–Leu–Gly)$_4$, have been synthesized from the corresponding linear hexapeptide active ester under high dilution conditions. Cyclo(Pro–Leu–Gly)$_4$ serves as an example of the synthesis of cyclic peptides from linear peptides containing an even number of amino acid residues through dimerization. These two compounds are characterized by TLC, HPLC, amino acid analysis, NMR, and mass spectra. Cyclo(Pro–Leu–Gly)$_2$ has also been synthesized from the linear tripeptide precursor independently, and its properties have been shown to be identical to that from linear hexapeptide. Circular dichroism indicated that cyclo(Pro–Leu–Gly)$_2$ contains β-turns as expected; in contrast, cyclo(Pro–Leu–Gly)$_4$ exhibited a random structure pattern. The NMR spectra demonstrated that cyclo(Pro–Leu–Gly)$_2$ is fixed in only one conformer in DMSO, while in a $CDCl_3$ solution, several conformations with a rapid interconversion are present. Unlike the cyclohexapeptide, cyclo(Pro–Leu–Gly)$_4$ showed more complicated NMR spectra in the two solvents; two or more conformers are considered to be present.

Studies on tocinamide and deaminotocinamide[1,2] emphasized the importance of the C-terminal linear part of oxytocin, Pro–Leu–Gly–NH_2. This can be supported by investigating the biological activity of Pro–Leu–Gly–NH_2 or its derivatives directly.[3,4] On the other hand, since many peptides with biological activities have cyclic structures,[5] and cyclic peptides are also nice models to investigate the reverse turns in proteins, they have attracted a great deal of attention. Recently, some new application of cyclic peptides on organic nanotubes was also reported.[6] At present, all widely available selective agonists of neurohypophyseal hormones are cyclic;[7] therefore, investigating the properties of cyclic peptides containing the C-terminal tripeptide sequence of oxytocin may be helpful in understanding the structure–activity relationship in these peptides.

Cyclic peptides are generally synthesized from the corresponding linear peptides by intramolecular cyclization under high-dilution conditions; this strategy was successfully applied to the synthesis of gramicidin S by Schwyzer et al.[8] In a later study on a related linear pentapeptide active ester, instead of the expected monomer, cyclopentapeptide, a dimer, cyclodecapeptide, through intermolecular cyclization was obtained.[9] Schwyzer pointed out that this phenomenon might be expected to occur especially effectively with peptides containing an odd number of amino acid residues.[9,10] Afterwards, other researchers demonstrated that usually both monomers and dimers could be obtained in different ratios, depending on the activation methods of the linear peptides and concentration of reactants[11,12] when using linear pentapeptides as models. To investigate the formation of the cyclotripeptide, cyclohexapeptide, cyclononapeptide, and cyclododecapeptide with repeated C-terminal tripeptide sequence of oxytocin, two routes both from the linear tripeptide precursor and linear hexapeptide precursor were tried. The conformations of the resulting cyclic peptides in solutions are discussed based on CD and NMR spectra.

Results and Discussion

The linear tripeptide Boc–Pro–Leu–Gly–OMe (**1**) was prepared via coupling Boc–Pro–OH with the dipeptide H–Leu–Gly–OMe·HCl, which was obtained by deprotecting Boc–Leu–Gly–OMe with 4 M HCl in dioxane (1 M = 1 mol dm^{-3}). Part of the fully protected tripeptide was saponified with NaOH, followed by acidification to give Boc–Pro–Leu–Gly–OH (**2**); another part of the tripeptide was treated with HCl to give the free N-terminal derivative. The linear hexapeptide Boc–(Pro–Leu–Gly)$_2$–OMe (**6**) with repeating tripeptide sequence was prepared by the conventional peptide bond-formation reaction.

As shown in Figs. 1a and 1b, the fully protected tripeptide **1** and hexapeptide **6** were saponified, converted into the corresponding active esters with HOSu. Deprotection was by trifluroacetic acid (TFA) to give the free N-terminal linear tripeptide and hexapeptide derivatives, respectively. They were cyclized under high dilution conditions, and the crude products were then purified by chromatography on Sephadex LH-20 or silica-gel columns. In the cyclization reaction from linear tripeptide active ester, only the product of dimerization was obtained, and no cyclotripeptide, cyclononapeptide or cyclododecapeptide could be isolated. Interestingly, in

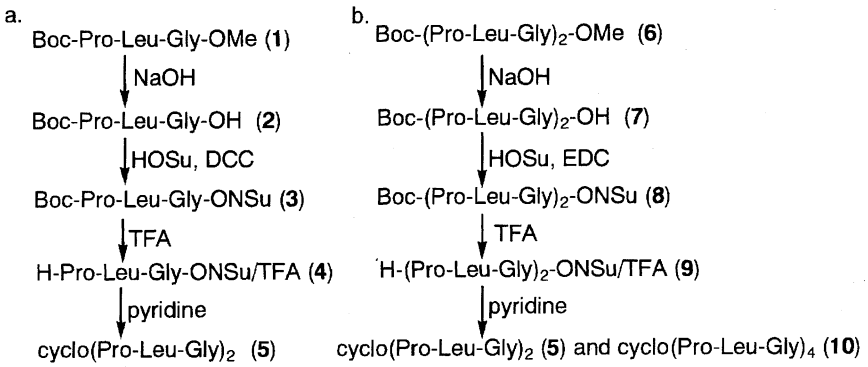

Fig. 1. a. Synthetic scheme of cyclo(Pro–Leu–Gly)$_2$ (**5**) from linear tripeptide precursor. b. Synthetic scheme of cyclo(Pro–Leu–Gly)$_2$ (**5**) and cyclo(Pro–Leu–Gly)$_4$ (**10**) from linear hexapeptide precursor.

the cyclization reaction from linear hexapeptide, in addition to the monomer cyclo(Pro–Leu–Gly)$_2$ (**5**), a dimer cyclo(Pro–Leu–Gly)$_4$ (**10**) has also been isolated, although in relatively low yield (4.3%). This can serve as an example of the synthesis of cyclic peptides from linear peptide precursors containing an even number of amino acids. The molecular weights of the two cyclic peptides have been confirmed by the mass spectra. Cyclo(Pro–Leu–Gly)$_2$ (**5**) from linear tripeptide precursor is identical to that from a linear hexapeptide precursor in all physical properties and spectral patterns.

In the CD spectra shown in Fig. 2a, cyclo(Pro–Leu–Gly)$_2$ (**5**) from linear tripeptide precursor **1** and that from hexapeptide precursor **6** have the same pattern, both show minima at 204 and 221 nm, falling into one type of the CD spectra of a series of cyclohexapeptides (X–Pro–Y)$_2$ reported before.[13] This indicates cyclo(Pro–Leu–Gly)$_2$ has β-turn structures; in contrast, the dimer cyclo(Pro–Leu–Gly)$_4$ shows a minimum at 199 nm, a random structure pattern.

To compare the conformations before and after cyclization, the CD spectra of two linear peptides, Boc–Pro–Leu–Gly–OH and Boc–(Pro–Leu–Gly)$_2$–OH, and cyclic peptides were measured (Fig. 2b). Boc–Pro–Leu–Gly–OH has a minimum at 198 nm and Boc–(Pro–Leu–Gly)$_2$–OH has a minimum at 200 nm, and both show the typical pattern of random coil, different from the β-turn structures

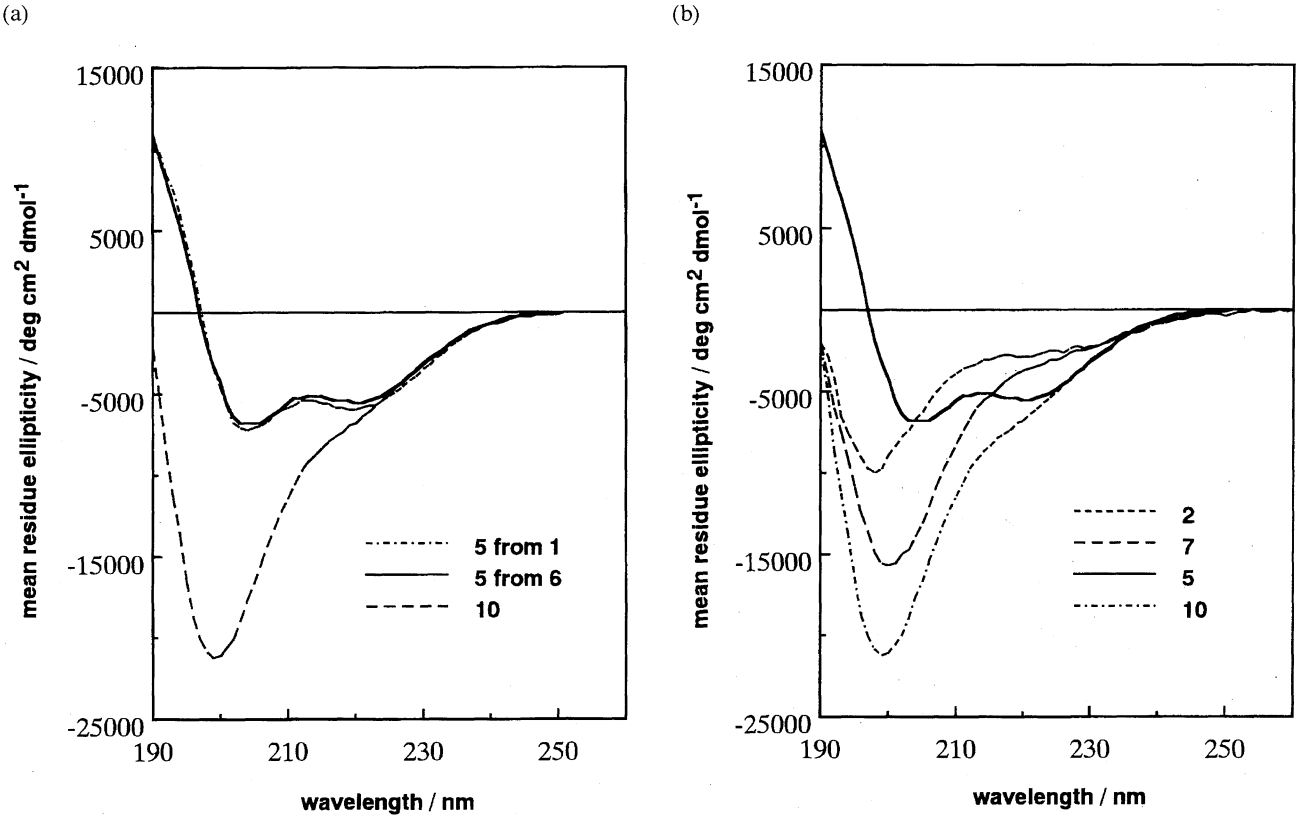

Fig. 2. CD spectra of linear and cyclic peptides in MeOH. Peptide concentrations were at 2 mM. a. cyclohexapeptide **5** from linear tripeptide precursor **1**, from linear hexapeptide precursor **6**, and cyclododecapeptide **10** from linear hexapeptide precursor. b. linear tripeptide precursor (**2**), linear hexapeptide precursor (**7**), cyclohexapeptide (**5**), and cyclododecapeptide (**10**).

taken by cyclo(Pro–Leu–Gly)$_2$. The shape of the CD curve of cyclo(Pro–Leu–Gly)$_4$ is closely similar to that of Boc–(Pro–Leu–Gly)$_2$–OH, but increases in the magnitude of the mean residue ellipticity.

In the conformation of cyclohexapeptide, Pro can only occupy the $i+1$ and $i+2$ positions in a β-turn with the four residues, numbered from i to $i+3$, as shown in Fig. 3. Previous studies[13] on cyclohexapeptides containing prolines generalized that Pro occupy the $i+1$ positions in most cases, but can occur in the $i+2$ positions when (a) D residues precede the L-Pro and L residues follow it or (b) L-Val follows L-Pro. In our case, both form A and form B shown in Fig. 4 are possible since Gly can be considered as either D or L residues. Torchia et al.[14] also pointed out that when the Pro C_α–C=O bond is *cis'* and Pro is preceded by a Gly or D residue, it is possible to construct a model with a peptide backbone, like form B, in which the residues following the Pro are internally hydrogen bonded.

To determine whether form A or form B occurs in solution, we turned to the NMR spectra. Figure 5 gives the spectra of the amide protons resonances of the cyclohexapeptide and cyclododecapeptide in $CDCl_3$ and DMSO-d_6, and the results of temperature dependence experiments for the amide resonances of cyclo(Pro–Leu–Gly)$_2$ are summarized in Fig. 6. In DMSO-d_6 the temperature coefficient of amide NH of Leu is much higher than that of Gly NH (Fig. 6a and Table 1), indicating that the amide NH of Gly are shielded from the solvent by the intramolecular hydrogen bonds.[15] Thus, in DMSO cyclo(Pro–Leu–Gly)$_2$ occurs in form A. This result is consistent with the predominant conformations of cyclo-(Pro–Ser–Gly)$_2$[16] and cyclo(Pro–Phe–Gly)$_2$[13] in the same solvent, and is also supported by the characteristic double-minima at 204 and 221 nm for type I β-turn in CD spectrum measured in MeOH,[17] and our data fit quite well with theirs. In this conformation, all peptide bonds are in *trans'*. As for the one-*cis'* structure, which led to the asymmetric

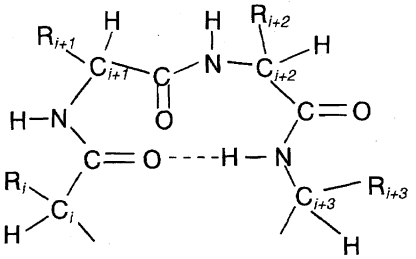

Fig. 3. A β-turn with 1←4 hydrogen bond.

Table 1. Temperature Coefficients $\Delta\delta/\Delta T$ of Amide Resonances for Cyclo(Pro–Leu–Gly)$_2$ and Cyclo-(Pro–Leu–Gly)$_4$ ($\times 10^{-3}$ ppm deg^{-1})

		Cyclo(PLG)$_2$	Cyclo(PLG)$_4$			
$CDCl_3$	Gly NH	3.3	2.8	2.4		
	Leu NH	3.7	1.4	2.3		
DMSO-d_6	Gly NH	0.37	4.0	2.7	2.5	0.18
	Leu NH	6.8	5.6	5.5	4.6	0.57

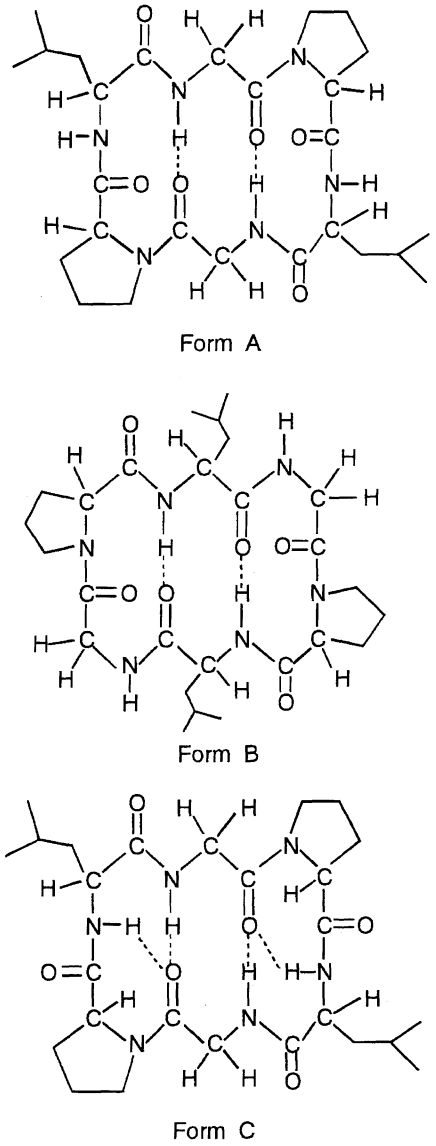

Fig. 4. Conformations of cyclo(Pro–Leu–Gly)$_2$ in solution.

amide resonances, it was almost undetectable in our NMR spectrum.

In contrast to this, when measured in the $CDCl_3$ solution, which has not been studied before, the temperature coefficients of Leu NH and Gly NH are comparable (Fig. 6b and Table 1); these values can't be unambiguously assigned to either hydrogen-bonded or nonhydrogen-bonded residues, but are equivalent to the average of temperature coefficients in the case of hydrogen bonded and nonhydrogen bonded. Along with the possibility of two forms, A and B, as mentioned above, both conformations are supposed to be present in the $CDCl_3$ solution, with rapid interconversion on the NMR time scale. In addition, form C (see Fig. 4) with the presence of β-turns and γ-turns at the same time is also possible to account for the deviation from the usual temperature coefficients for Leu and Gly NH, since the typical CD pattern could not be detected in $CHCl_3$ solution. In fact, cooling the solution of cyclo(Pro–Leu–Gly)$_2$ in $CDCl_3$

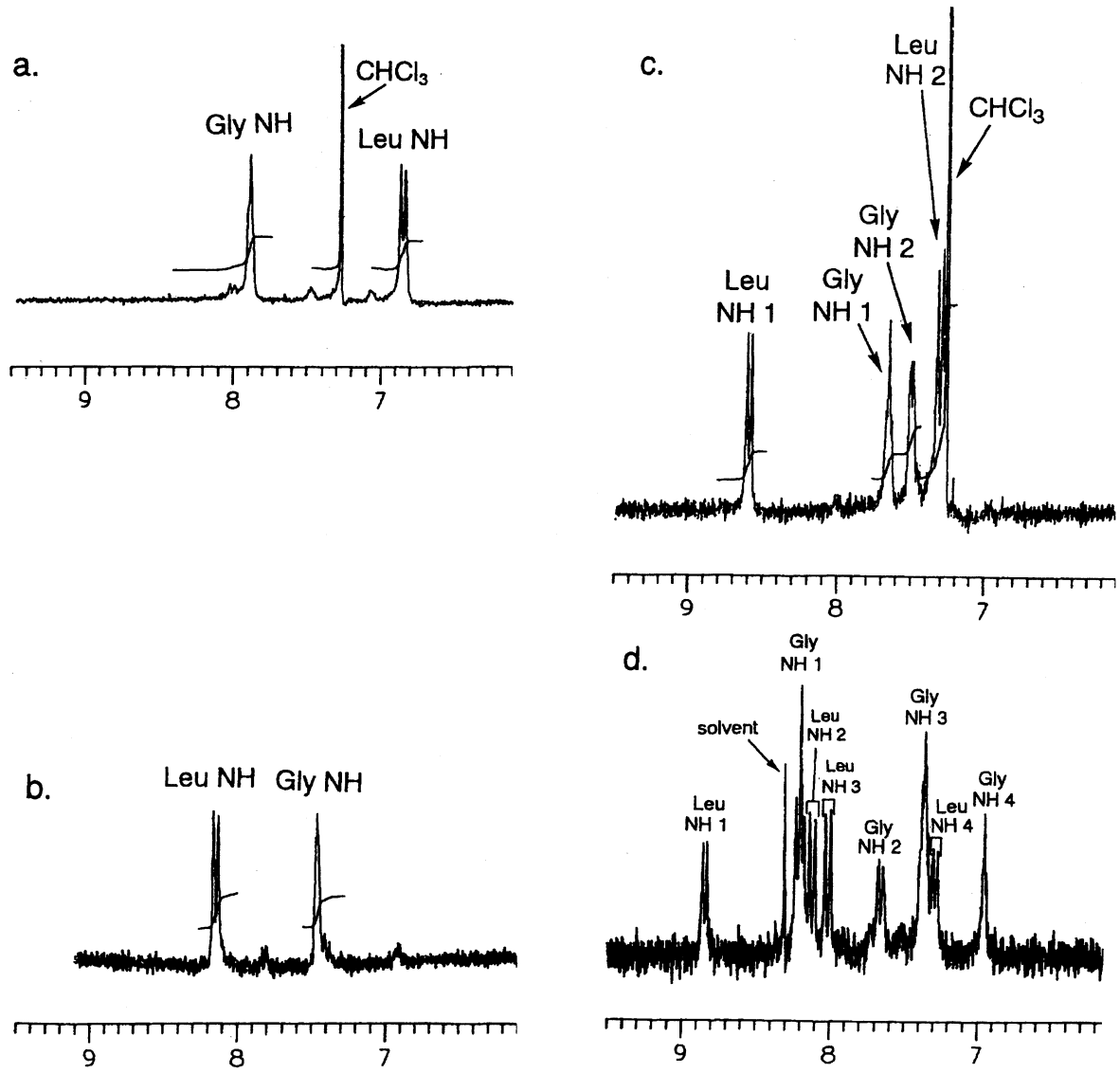

Fig. 5. The 270-MHz ^{1}H NMR spectrum of the amide NH resonances of cyclic peptides. a. cyclo(Pro–Leu–Gly)$_2$ in $CDCl_3$, peptide concentration 11.4 mM, the temperature 22 °C. b. cyclo(Pro–Leu–Gly)$_2$ in DMSO-d_6, peptide concentration 6.2 mM, the temperature 21 °C. c. cyclo(Pro–Leu–Gly)$_4$ in $CDCl_3$, peptide concentration 3.7 mM, temperature 22 °C. d. cyclo(Pro–Leu–Gly)$_4$ in DMSO-d_6, peptide concentration 4.0 mM, temperature 30 °C. The singlet at 8.31 ppm was due to a little of $CHCl_3$ in DMSO.

caused a broadening of the signals, evidence for the existence of interconverting conformers.

The difference between conformations in the two kinds of solvents discussed above can be elucidated in the following way. Usually, there are many possible conformers for a given peptide; that is also the case in solvents like CH_2Cl_2 or $CHCl_3$, in which the interaction between the solvent and peptide molecules is very weak. However, in a strong proton-donor or proton-acceptor solvent like DMSO, the previous conformers tend to convert to more stable or more favorable structures in potential energy under the specific solvent effect.

Although much has been done on the conformations of cyclohexapeptides, relatively little has been done on cyclododecapeptides, except for the analogs of valinomycin and their complexes.[18—21] However, due to the specificity of repeating tetrapeptide sequence of valinomycin, on which the C_3 or S_6 symmetry is based, much similarity between our repeating tripeptide sequence and them is not expected.

In the NMR spectrum of cyclo(Pro–Leu–Gly)$_4$ in $CDCl_3$ (Fig. 5c) there are two sets of proton resonances for each kind of amide proton. Temperature-dependence experiments (Table 1) suggested that the amide protons of all residues seem to be hydrogen bonded if only one conformer is present. The higher temperature coefficients than the usual H bonded case prompted us to construct a model in which all amide protons were involved in 1←3 H bonds, as in the γ-turns, in which the nonlinear H bonds might contribute to the slightly higher temperature coefficients; however, the model proved that it forms a loose "bracelet" structure, requiring that every kind of residue should be equivalent, contradictory to the conclusion from NMR spectrum, therefore, the loose bracelet structure must be excluded. Further attempts lead to the assumption of conformation D or E (Fig. 7). Form D should be

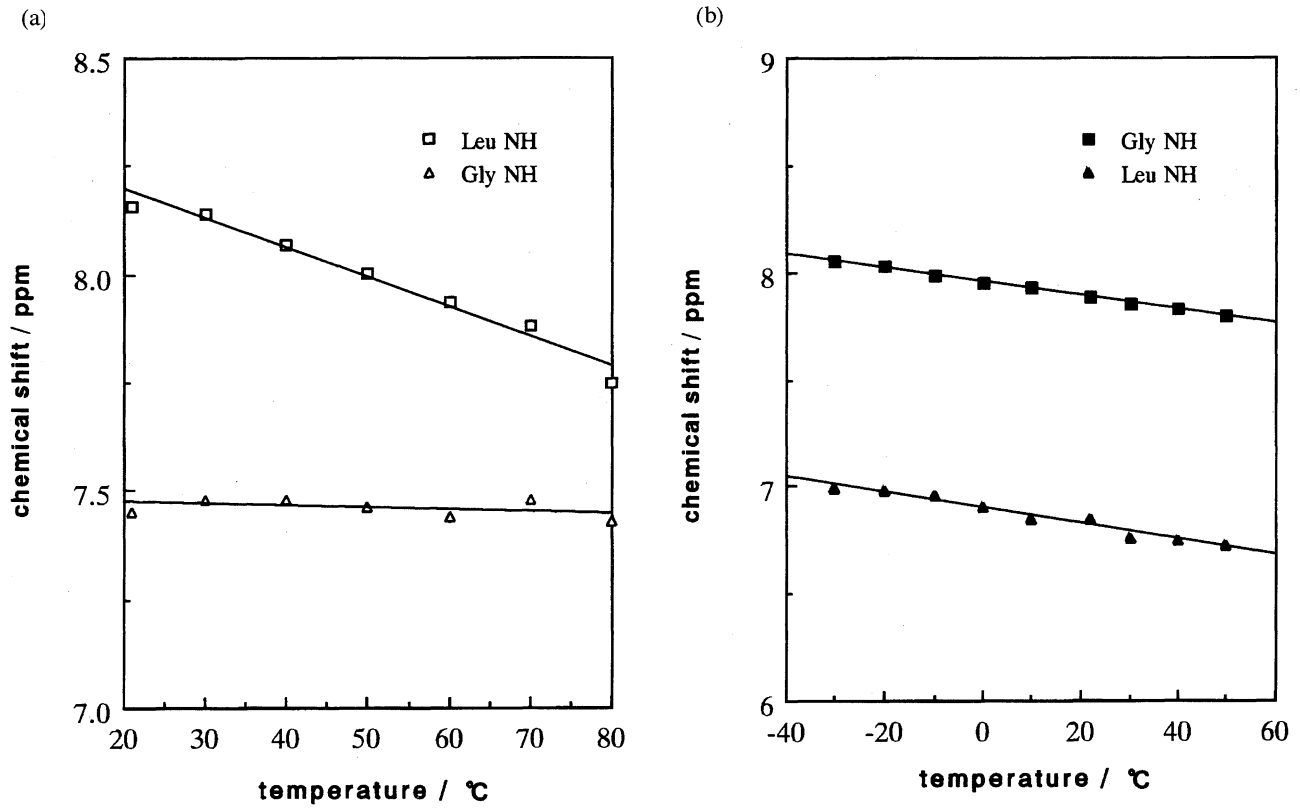

Fig. 6. The temperature dependence of chemical shifts for the amide protons of cyclo(Pro–Leu–Gly)$_2$, concentration 6.2 mM. (a) in DMSO-d_6, (b) in $CDCl_3$.

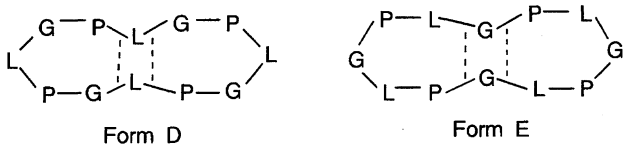

Fig. 7. The possible conformations for cyclo(Pro–Leu–Gly)$_4$ (assumption). The letters represent the location of the amino acid α-carbon atoms and dashed lines represent the H bonds.

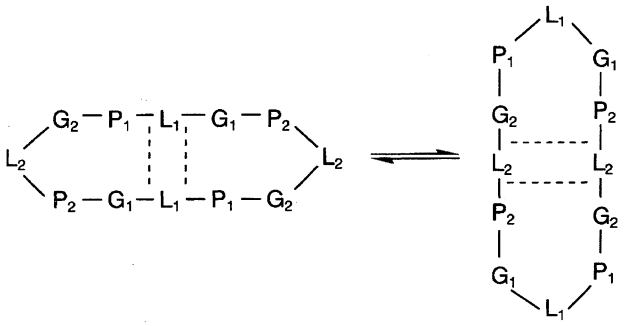

Fig. 8. The frame-shift structures of form D in Fig. 7 and interconversion between them.

more preferable, since the bulky side chains of Leu residues extend outside, favorable in potential energy, while form E is sterically crowded in the molecular model. Furthermore, form D is consistent with the observation in NMR spectrum that the difference in the chemical shifts between two sets of Gly is smaller than the difference between the Leu residues. When the temperature was raised, the two sets of Gly signals even tended to merge into one set (data not shown). This result can indicate that an equilibrium of interconversion of two frame-shift structures exists as depicted in Fig. 8.

On the other hand, in DMSO solution, the NMR spectrum of the cyclododecapeptide (Fig. 5d) becomes even more complicated, none of the amide NH is equivalent, and there are four sets of amide proton resonances. With regard to the conformations of cyclo(Pro–Leu–Gly)$_4$ in DMSO, several conclusions can be deduced from the NMR studies and CD spectra. First, the magnetic environments of all residues are different, and the molecule should have no symmetry if only one conformer is present. Secondly, as depicted in the CD spectra, cyclo(Pro–Leu–Gly)$_4$ did not show the β-turn pattern as cyclo(Pro–Leu–Gly)$_2$; thus, the 1←4 H bonds must be excluded in the cyclododecapeptide molecules. Moreover, the temperature coefficients (Table 1) show that both types of amide protons, those exposed to solvent and those shielded from solvent, are present. Finally, the characteristics of cyclization do not have much effect on the conformation. In other words, the conformational change from the linear hexapeptide to the cyclododecapeptide (Fig. 2b) is not obvious, except in magnitude. Most probably, in DMSO cyclo(Pro–Leu–Gly)$_4$ takes a random structure or exists as a mixture of several conformers with high energy barriers preventing a free interchange between them.

In conclusion, circular dichroism indicated that cyclo(Pro–Leu–Gly)$_2$ contains the β-turns as expected, in contrast, cyclo(Pro–Leu–Gly)$_4$ exhibited a random structure pat-

tern. The NMR spectra demonstrated that in DMSO cyclo-(Pro–Leu–Gly)$_2$ only exists in the conformation in which Pro occupy the $i+1$ positions of β-turns (form A), consistent with the results of other investigators, while in $CDCl_3$ solution, both the conformations of which Pro occupy either $i+1$ or $i+2$ positions (form B) are present, along with form C, and a rapid interconversion between them exists. Unlike the cyclohexapeptide composed of the same tripeptide sequence, cyclo(Pro–Leu–Gly)$_4$ showed more complicated NMR spectra in the two solvents, two or more conformers are considered to be present in solution and high energy barriers prevent the interchange between one another.

Experimental

Abbreviations Used: AAA, amino acid analysis; Boc, *t*-butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; HOBt, 1-hydroxybenzotriazole; HOSu, *N*-hydroxysuccinimide; –ONSu, hydroxysuccinimide ester.

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. Amino acids were of L-configuration.

The melting points were not corrected. Amino acid analyses were performed on a Wakopak WS-PTC system and the hydrolysis of peptides was carried out in 6 M HCl at 110 °C for 24 h, then at 150 °C for 1 h. Thin-layer chromatography (TLC) 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, column was Wakosil 5C4 200 ϕ4.6×250 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 content of solution B from 0 to 100% in 30 min, and back to 0% during the next 5 min, flow rate 1 ml min^{-1}. Mass spectra were recorded on a MALDI-TOF mass spectrometer of Voyager.

^{1}H NMR Spectroscopy. The proton magnetic resonance spectra were recorded on a JEOL JNM-GX270 FT NMR spectrometer. The chemical shifts were all downfield from tetramethylsilane, which was used as an internal reference. Temperature-dependence studies were performed with the temperature controlled to ±1 °C.

CD Spectroscopy. Circular dichroism measurements were made on a JASCO J-720 spectropolarimeter, the concentrations of all peptide compounds were 2 mM in MeOH. The spectra were scanned at room temperature (25 °C) in a capped, quartz optical cell with a 0.1 mm path length. Spectra were obtained at a wavelength of 260—190 nm. Ten scans were taken for each peptide at a scan rate of 20 nm min^{-1}. Mean residue ellipticities were expressed in deg cm^{2}dmol^{-1} and calculated as follows:

$$m_\theta = 100\theta/(nCl)$$

where θ is the measured ellipticity in degrees, l is the path length in centimeters, C is the molar concentration, and n is the number of residues.

Peptide Synthesis. The linear precursors of the cyclic peptides were prepared by a stepwise synthesis of tripeptide fragments, which were coupled to form the open-chain hexapeptide. All 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. For further details concerning specific peptide derivatives, they are described below.

Boc–Pro–Leu–Gly–OH (2). The blocked tripeptide Boc–Pro–Leu–Gly–OMe was saponified with NaOH to get the C-terminal free derivative. 1.598 g (4 mmol) Boc–Pro–Leu–Gly–OMe was dissolved in 12 ml methanol and cooled to 0 °C, to the solution 5.6 ml ca. 1.1 M NaOH aqueous solution (6 mmol) was added and stirred at 0 °C for 1 h, then at room temperature for 1 h. The solvent was removed by evaporation under reduced pressure and the residue was distributed between 20 ml water and 10 ml ether; the aqueous solution was separated and adjusted to ca. pH 2 with $KHSO_4$; the white solid precipitated was extracted with ethyl acetate three times, each with 20 ml; the EtOAc solutions were combined, washed with saturated NaCl solution, dried over anhydrous sodium sulfate and concentrated. Upon the addition of hexane, the product precipitated as white crystals, yield 1.286 g, 83.3%, showing the same properties as that prepared by Zaoral et al. through different method,[22] $[\alpha]_D^{25}$ −81.5° (*c* 1.01, EtOH). Anal. Found: C, 54.56; H, 8.16; N, 10.69%. Calcd for $C_{18}H_{31}N_3O_6\cdot0.5H_2O$: C, 54.81; H, 8.18; N, 10.65%.

Boc–Pro–Leu–Gly–ONSu (3). 386 mg (1 mmol) Boc–Pro–Leu–Gly–OH (2) and 172 mg (1.5 mmol) HOSu were dissolved in 3 ml CH_3CN, cooled to 0 °C, 15 min later, 206 mg (1 mmol) DCC was added, and the reaction mixture was allowed to warm up to room temperature and stirred for 26 h. The solvent was evaporated and the residue was taken up in 10 ml ethyl acetate. The insoluble substances were removed by filtration and the filtrate was washed with 5 ml water; evaporation of the solvent to dryness in vacuo afforded a white microcrystalline solid 440 mg, 91.2%.

H–Pro–Leu–Gly–ONSu·TFA (4). The above-described product Boc–Pro–Leu–Gly–ONSu (**3**) was treated with 1 ml trifluroacetic acid, and stirred at 0 °C for 30 min. The remaining TFA was removed by a stream of nitrogen gas, the residue was taken up in several 2-ml portions of anhydrous ether, and the supernatants were decanted; a chromatographic homogenous product was obtained in an amorphous solid, and dried over potassium hydroxide pellets, yield 452 mg, 99.8%.

Cyclo(Pro–Leu–Gly)$_2$ (5) (from Tripeptide). The cyclohexapeptide was obtained both from the corresponding linear tripeptide active ester by cyclodimerization and from the linear hexapeptide active ester, as shown below. 452 mg H–Pro–Leu–Gly–ONSu·TFA (**4**) was dissolved in 1 ml DMF, the solution was dropped into 150 ml pyridine precooled to 0 °C, 1 ml DMF was used for rinsing and also dropped into pyridine solution; then, the reaction mixture was stirred at room temperature for 3 d. The solvent was evaporated in vacuo, and the residue was taken up in 10 ml water, extracted with ethyl acetate for three times, each with 10 ml, the organic solutions were combined, and concentrated to about 1 ml of volume, from which on storage for several days crystals precipitated; yield 19 mg, mp 255—258 °C. The mother liquor was concentrated to dryness and dissolved in 1 ml methanol, and chromatographied on a column of Sephadex LH-20; fractions containing the product were combined, and concentrated to afford another portion of 51 mg, total yield 70 mg, 26.2% from Boc–Pro–Leu–Gly–OH (**2**). It was homogenous in several TLC solvent systems and showed negative ninhydrin test, R_f 0.56 in $CHCl_3$/MeOH 5 : 1 (v/v), HPLC retention time 9.09 min. Molecular weight by mass spectrometry gave base peak at *m/z* 535.4 (M+H$^+$) and *m/z* 557.4 (M+Na$^+$), theoretical value *m/z* 534.66 for $C_{26}H_{42}N_6O_6$, AAA Pro 1.03(1), Leu 0.99(1), Gly 0.97(1).

H–Pro–Leu–Gly–OMe·HCl. Boc–Pro–Leu–Gly–OMe was dissolved in 4 M HCl/dioxane(10 equiv) and kept at 37—41 °C for one hour, the solvent was evaporated under reduced pressure and the residue was triturated with several portions of anhydrous ether. After drying over phosphorus pentaoxide and potassium hydroxide, TLC homogenous product was obtained in amorphous solid, yield 98.0%.

Boc–(Pro–Leu–Gly)$_2$–OMe (6). 994 mg (2.96 mmol) H–Pro–Leu–Gly–OMe·HCl was dissolved in 13.5 ml DMF, cooled to 0 °C, 1.141g (2.96 mmol) Boc–Pro–Leu–Gly–OH (**2**) was added, followed by 0.33 ml (2.96 mmol) *N*-methylmorpholine and 544 mg (3.55 mmol) HOBt·H_2O, 15 min later, 610 mg (2.96 mmol) DCC was added, and the reaction mixture was stirred at 0 °C for 1 h and then at room temperature for 3 d. Several drops of acetic acid were added and the DCU was removed by filtration. After DMF was evaporated in vacuo, the residue was taken up in 140 ml EtOAc, insoluble substances were removed by filtration, the resulting organic solution was washed successively with 70 ml 0.5 M $NaHCO_3$ aq, 70 ml 5% $KHSO_4$ aq, and 70 ml saturated NaCl solution for three times, and dried over Na_2SO_4. Evaporation of EtOAc afforded 1.35 g (68.4%) hexapeptide, showing only faint traces of minor components on thin-layer chromatographic analysis.

Boc–(Pro–Leu–Gly)$_2$–OH (7). 1.065 g (1.6 mmol) Boc–(Pro–Leu–Gly)$_2$–OMe (**6**) was dissolved in 5 ml methanol, cooled to 0 °C, and to it 2.4 ml 1.018 M aqueous NaOH solution (2.4 mmol) was added dropwise while stirring. After 1 h at 0 °C, the reaction mixture was allowed to warm up to room temperature and stirred for 1 h. The solvent was evaporated under reduced pressure, and the residue was distributed between 50 ml water and 20 ml ether; the aqueous layer was separated and adjusted to pH 2 with 2M $KHSO_4$ aq, and a white solid precipitated. EtOAc was used to extract the product twice, each with 50 ml, and the combined organic solution was washed with saturated NaCl solution 50 ml. After drying over Na_2SO_4 and concentration of the solution to a volume of about 1 ml, 3 ml ether, and 9 ml hexane were added, and allowed to stand overnight. 0.817 g (78.4%) product precipitated and was collected. It was further purified by chromatography on silica gel using chloroform/methanol/acetic acid = 95 : 6 : 1 as eluent, 646.4 mg chromatographic homogenous product was obtained. Mp 124—128 °C, $[\alpha]_D^{25}$ −106.6° (*c* 0.68, EtOH). Anal. Found: C, 53.79; H, 8.02; N, 11.94%. Calcd for $C_{31}H_{52}N_6O_9 \cdot 2H_2O$: C, 54.05; H, 8.19; N, 12.20%.

Boc–(Pro–Leu–Gly)$_2$–ONSu (8). To a clear solution of 300 mg (0.46 mmol) Boc–(Pro–Leu–Gly)$_2$–OH (**7**) in 2 ml CH_3CN at 0 °C, were added 58 mg (0.506 mmol, 1.1 equiv) HOSu and 97 mg (0.506 mmol, 1.1 equiv) EDC. After 5.5 h, another portion of 58 mg HOSu and 97 mg EDC were added. 5 h later, the solvent was evaporated and the residue was taken up in 20 ml EtOAc, washed with 5 ml water and 10 ml saturated NaCl solution, and dried over anhydrous Na_2SO_4. Evaporation of the solvent to dryness afforded oil 345 mg with purity >90%.

H–(Pro–Leu–Gly)$_2$–ONSu·TFA (9). The above-described compound (**8**) (345 mg) was dissolved in 2 ml TFA and stirred at 0 °C for 30 min; then, the TFA was removed by a stream of nitrogen gas and the residue was treated with three 3-ml portions of anhydrous ether, and the supernatants were decanted. The residue was evaporated to dryness in vacuo; an oil was obtained, dried over potassium hydroxide pellets, yield 316 mg, 99.9%.

Cyclo(Pro–Leu–Gly)$_2$ (5) and Cyclo(Pro–Leu–Gly)$_4$ (10) (from Hexapeptide). A solution of 316 mg H–(Pro–Leu–Gly)$_2$–ONSu·TFA (**9**) in 1 ml DMF was added dropwise into 150 ml pyridine which had been precooled to 0 °C. 1 ml DMF was used for rinsing, and also dropped into the pyridine solution. The reaction mixture was allowed to warm up to room temperature and stirred for 3.5 d; the solvent was evaporated under reduced pressure and then in vacuo; the residue was distributed between 10 ml EtOAc and 10 ml water; the aqueous solution was separated, and further extracted successively with EtOAc and $CHCl_3$ three times, each with 5 ml. The EtOAc and $CHCl_3$ solutions were dried over anhydrous Na_2SO_4 and combined. After concentration to about 2 ml of volume and standing overnight, some crystals precipitated, they were collected by filtration and washed with a little of EtOAc, yielded 96.6 mg, the mother liquor was concentrated and passed through a column of $\phi 2.2 \times 30.7$ cm on silica gel, using a mixture of chloroform and methanol 5 : 1 (v/v) as eluent, the component with R_f 0.56 was collected and provided a second crop of 10.4 mg after concentration to dryness and recrystallization from ethyl acetate. The total yield was 107 mg, 43.5% from Boc–(Pro–Leu–Gly)$_2$–OH (**7**), mp 255—258 °C, $[\alpha]_D^{25}$ −50.4° (*c* 0.98, EtOH). HPLC retention time 9.09 min, ^{1}H NMR ($CDCl_3$, δ/ppm) Gly NH (7.89, t, 2H), Leu NH (6.85, d, 2H), Pro α-CH (4.52, t, 2H), Leu α-CH (4.40, m, 2H), Gly α-CH_2 (4.29, 3.85, dd, 4H), Pro δ-CH_2 (3.70—3.55, t, 4H), Leu γ-CH (2.28, m, 2H), Pro β,γ-CH_2 (2.04, m, 8H), Leu β-CH_2 (1.55, q, 4H), Leu CH_3 (0.95, m, 12H), molecular weight by mass spectrometry gave base peak at *m/z* 535.5 ($M+H^+$) and *m/z* 557.4 ($M+Na^+$), theoretical value *m/z* 534.66 for $C_{26}H_{42}N_6O_6$. Amino acid analysis Pro 1.04(1), Leu 1.00(1), Gly 0.96(1). Anal. Found: C, 55.09; H, 7.99; N, 14.93%. Calcd for $C_{26}H_{42}N_6O_6 \cdot 2H_2O$: C, 54.72; H, 8.12; N, 14.73%. Also collected was the component of R_f 0.67, which was characterized as cyclo(Pro–Leu–Gly)$_4$, yield 10.5 mg, 4.3% from Boc–(Pro–Leu–Gly)$_2$–OH (**7**), mp >300 °C, $[\alpha]_D^{25}$ −165.6° (*c* 0.98, EtOH). HPLC retention time 12.39 min. ^{1}H NMR ($CDCl_3$, δ/ppm) Leu NH-1 (8.61, d, 2H), Gly NH-1 (7.69, t, 2H), Gly NH-2 (7.52, t, 2H), Leu NH-2 (7.32, d, 2H), Gly α-CH_2-1 (4.94, 4.36, m, 4H), Pro α-CH-1 (4.58, t, 2H), Leu α-CH (4.47, 3.95, m, 4H), Pro α-CH-2 (4.11, t, 2H), Gly α-CH_2-2 (4.05, 3.66, m, 4H), Pro δ-CH_2 (3.51, m, 8H), Leu γ-CH (2.50, m, 4H), Pro β,γ-CH_2 (2.17—1.79, m, 16H), Leu β-CH_2 (1.60, q, 8H), Leu CH_3 (0.89, m, 24H). Molecular weight by mass spectrometry gave *m/z* 1069.8 ($M+H^+$) and *m/z* 1091.7 ($M+Na^+$), theoretical value *m/z* 1069.3 for $C_{52}H_{84}N_{12}O_{12}$. Amino acid analysis Pro 1.12(1), Leu 0.99(1), Gly 0.89(1). Anal. Found: C, 54.61; H, 7.99; N, 14.58%. Calcd for $C_{52}H_{84}N_{12}O_{12} \cdot 4H_2O$: C, 54.72; H, 8.12; N, 14.73%.

The cyclo(Pro–Leu–Gly)$_2$ from linear tripeptide active ester by cyclodimerization and that from linear hexapeptide active ester were indistinguishable from each other by the following criteria:(1) same melting point, (2) amino acid analysis, (3) same R_f value in TLC, (4) identical CD spectra, (5) identical NMR spectra, (6) identical MS spectra, and (7) same retention time in HPLC. Both of them showed negative ninhydrin test.

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