Conformational Preference of Cycloleonuripeptides A, B, and C, Three Proline-Rich Cyclic Nonapeptides from *Leonurus heterophyllus*[1)]

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Three-dimensional structures in dimethyl sulfoxide (DMSO)-d_6 of three proline-rich cyclic nonapeptides, cycloleonuripeptides A: *cyclo* (–Gly–Pro–Pro–Pro–Tyr–Pro–Pro–Met–Ile–), B: *cyclo* (–Gly–Pro–Pro–Pro–Tyr–Pro–Pro–Met(O)–Ile–), and C: *cyclo* (–Gly–Pro–Pro–Pro–Tyr–Pro–Pro–Met(O)–Ile–), which have been isolated from the fruits of *Leonurus heterophyllus*, were determined by distance geometry calculation and restrained energy minimization from NMR data. Calculation using 272 different initial structures led to a uniquely determined backbone conformation with a root mean square deviation value of 0.57 Å. The backbone structures of cycloleonuripeptides A, B, and C consist of two β-turns, a β VI turn at Pro[3]–Pro[4], and a β I turn at Pro[7]–Met[8]. In addition to a transannular 4→1 backbone hydrogen bond between Tyr[5]–NH and Pro[2]–CO, two intramolecular hydrogen bonds between Gly[1]–NH and Pro[6]–CO, and between Ile[9]–NH and Pro[6]–CO, which constructed a β-bulge conformation, were observed.

Key words cycloleonuripeptide; conformation; distance geometry calculation; ^{1}H-NMR; β-bulge; *Leonurus heterophyllus*

The fruits of *Leonurus heterophyllus* (Labiatae) have been used as a Chinese drug to invigorate blood circulation, regulate menstrual disturbance and dispel edema.[2)] In our previous paper,[3)] three new cyclic nonapeptides, named cycloleonuripeptides A (**1**), B (**2**), and C (**3**), **2** and **3** being isomers of each other and showing cell growth inhibitory activity, were reported. Cycloleonuripeptides A, B, and C were proline-rich cyclic peptides, containing five proline residues with Pro–Pro and Pro–Pro–Pro sequences. The presence of Pro residues in the primary sequence, in general, could lead to a number of possible stable conformations due to the *cis-trans* isomerization of a Pro amide bond. In spite of this, each stable single conformation of **1**—**3** was observed in dimethyl sulfoxide-d_6 (DMSO-d_6) solution. It is conceivable that the inherent constraints in **1**—**3** containing five prolines would reduce the conformational space available to the peptide, allowing one to ascertain the accessible conformations independently from the environment.

The solution conformational analysis of unique proline rich cyclic nonapeptides, cycloleonuripeptides A, B, and C, was carried out by NMR data such as interatomic distances from a phase sensitive rotating-frame Overhauser enhancement (ROE) spectroscopy (ROESY) experiment, temperature effects on NH protons, and torsion angles calculated from $^3J_{NH-C\alpha H}$ coupling constants. For further conformational elucidation, a distance geometry (DG)-molecular dynamics (MD) procedure using distance constraints from the phase sensitive ROESY experiments was examined. We describe here the conformational preference of **1**—**3** in DMSO-d_6 solution examined by DG calculation and restrained energy minimization from NMR data.

Results and Discussion

NMR Study Complete ^{1}H and ^{13}C assignments, essential for conformational elucidation, were reported in our previous paper,[3)] determined by the use of various NMR measurements such as ^{1}H–^{1}H correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC)[4)] for direct $^1J_{H-C}$ connectivities and heteronuclear multiple-bond correlation spectroscopy (HMBC)[5)] for long range $^2J_{H-C}$ and $^3J_{H-C}$ ones. In spite of five Pro residues (Pro–Pro and Pro–Pro–Pro sequences), a single stable conformer in DMSO-d_6 on the NMR time scale was observed. In the amide bonds of the five Pro residues, only the geometry of one, between Pro[3] and Pro[4], was assignable to *cis* by the ^{13}C chemical shifts of β and γ positions in Pro[4] [**1**: 29.39 (β), 21.58 (γ); **2**: 30.59 (β), 21.60 (γ); **3**: 30.59 (β), 21.60 (γ)],[6)] and by a strong ROE effect[7)] between Pro[3]-Hα and Pro[4]-Hα in a phase sensitive ROESY spectrum.[8)]

The relationship of ROE enhancements by a phase sensitive ROESY spectrum gave us information about the relationship of interatomic distances, as shown in Fig. 1. The strong ROEs between Pro[3]-Hα and Pro[4]-Hα indicated the presence of a type VI β-turn between Pro[3] and Pro[4] residues. Another β-turn is considered to be sited from Pro[6] to Gly[1], which has not been determined yet. In addition to a sequential ROE correlation, two characteristic ROE correlations, between Pro[7]-Hα and Gly[1]-NH, and between Pro[2]-Hδ and Ile[9]-Hδ, were observed, indicating that they are close to each other.

Temperature dependence studies of the amide proton chemical shifts indicated the existence of several intramolecular hydrogen bonds.[9)] The temperature effect on the NH proton chemical shift was measured in ten intervals over the range 300—330 K in DMSO-d_6, using a linear regression analysis. The results are shown in Table 1. Of the four amide protons due to Gly[1], Tyr[5], Met[8], and Ile[9], the ones due to Gly[1], Tyr[5], and Ile[9] did not change significantly when the temperature was increased, indicating that they were shielded for the solvent or involved in intramolecular hydrogen bondings. If cycloleonuripeptides A—C take VI β-turn between Pro[3] and Pro[4], Tyr[5]-NH may take part in a transannular 4→1 hydrogen

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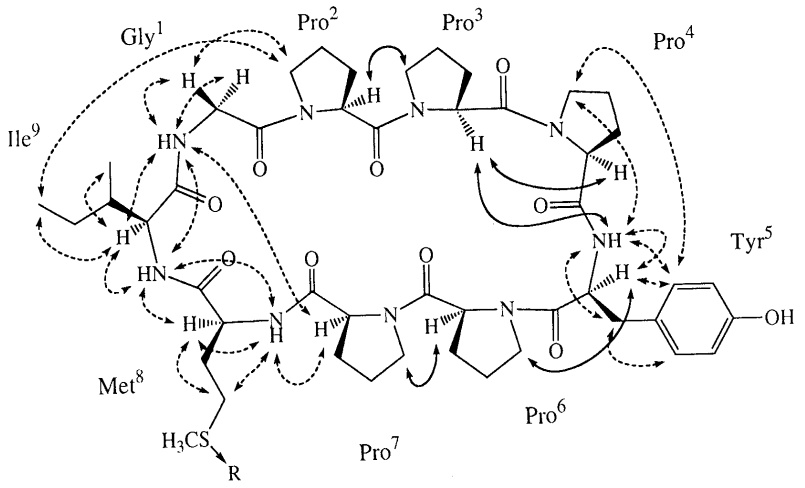

Fig. 1. Structures of Cycloleonuripeptides A (**1**), B (**2**) and C (**3**)

The arrows show a strong ROE relationship and dashed arrows show medium and weak ROE relationships in phase sensitive ROESY spectra. The residue 8 of **1** is Met, and **2** and **3** are isomers with R=O.

Table 1. Temperature Coefficients, $-d\delta/dT$ (10^{-3} ppm/K), of NH Protons of Cycloleonuripeptides A—C (**1**—**3**) in Ten Intervals over the Range 300—330 K in DMSO-d_6

Compounds	Gly[1]	Tyr[5]	Met[8]	Ile[9]
Cycloleonuripeptide A (**1**)	0.8	1.8	7.6	0.7
Cycloleonuripeptide B (**2**)	1.0	1.7	5.1	0.3
Cycloleonuripeptide C (**3**)	1.0	1.7	4.7	0.3

bond between Tyr[5]-NH and Pro[2]-CO. However, another amide proton in Met[8] shifted significantly upfield as temperature increased, demonstrating that it was not involved in hydrogen bonding. Judging from the similar relationship of ROE correlations and temperature dependence values among **1**, **2**, and **3**, cycloleonuripeptides A—C were regarded to have similar solution conformations in DMSO-d_6, that is, the sulfoxide groups in **2** and **3** were considered not to affect the solution conformation. The populations of side chain rotamers of Tyr[5] in **1**, **2**, and **3** were regarded to be either *gauche*$^-$ or *trans* conformation by the ^3J proton coupling constant between Hα and Hβ in Tyr[5] (**1**: $J_{\alpha\beta}=2.4$, 11.3 Hz; **2**: $J_{\alpha\beta}=2.7$, 11.2 Hz; **3**: $J_{\alpha\beta}=3.0$, 10.8 Hz). However, the stereospecific assignment of the Hβ proton in the side chain of Tyr[5] was not definite because of the overlapping of each Hβ methylene proton.

DG Calculation For analysis of the conformational preference of cycloleonuripeptide A, it is useful to use a computational method, which does not depend on the starting structures. The initial 272 structures satisfying the experimental restraints were embedded by DG calculations. Structural calculations were carried out using simulated annealing (SA) protocol with the program SYBYL,[10] and the produced conformers were then subjected to restrained energy minimization with the AMBER all-atom force field.[11] In SA simulation, each system was equilibrated for 5000 fs in a thermal bath at 800 K, and thereafter successively for 2700 fs, the temperature was decreased 54 times until a final temperature of 100 K was reached. Each frozen conformation was finally minimized.

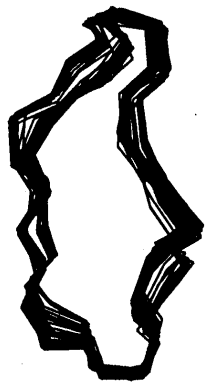

Fig. 2. Superimposed Backbone Structures of the 40 Lowest Energy Conformers in Conformer A (Backbone RMSD=0.57 Å)

Interatomic distances were calculated from the integrated volumes of the ROESY cross peaks and classified into three ranges, 1.8—2.5, 1.8—3.5 and 1.8—5.0 Å, corresponding to strong, medium and weak ROEs, respectively. Because of the lack of stereospecific assignments in Proδ methylene protons, the distance constraints were relaxed by means of the pseudoatom corrections (+1.0 Å). No hydrogen bonding or torsion angle constraints were used.

The conformational determination of cycloleonuripeptide A by DG and restrained SA resulted in two structural families, conformers A and B.

Conformer A: 79 structures among 272 structures generated by the DG method were defined as conformer A, whose mean root mean square deviation (RMSD) of the restraint violations was 0.005 Å. Figure 2 shows a superposition of the backbone heavy atoms of the 40 lowest energy refined structures. The mean structure generated, followed by energy minimization, is shown in Fig. 3, which exhibited a twisting left turn. The overall atomic RMSDs between the individual structures and the mean coordinate positions are 0.57 Å for the backbone atoms.

The mean backbone conformation adopts a type VI β-turn between Pro[2] and Tyr[5], with a peptide bond between Pro[3] and Pro[4] in the *cis* configuration, and a type I β-turn

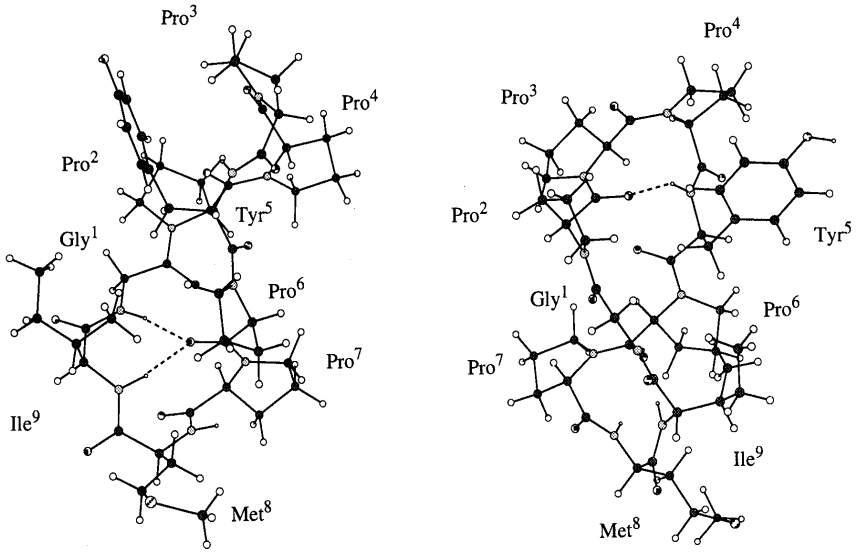

Fig. 3. Mean Structure of Stable Conformer A of Cycloleonuripeptide A

Three hydrogen bonds were dipicted by dotted lines. The right-hand structure was viewed by rotating conformer A 90° along the y axis of the left structure.

Table 2. Backbone Dihedrals (ϕ) in Cycloleonuripeptides A—C (**1**—**3**) Calculated from Vicinal NH–CαH Coupling Constants (Hz)[a]

Compounds	Residues	Hz	ϕ angle[a]
1	Tyr^5	7.1	85.2, 34.8, −158.0, **−82.0**
	Met^8	6.3	91.5, 28.5, −162.6, **−77.4**
	Ile^9	10.2	**−135.4**, −104.6
2	Tyr^5	6.9	86.9, 33.1, −159.2, **−80.8**
	Met^8	6.0	93.6, 26.4, −164.3, **−75.7**
	Ile^9	10.4	**−132.9**, −107.1
3	Tyr^5	7.0	86.0, 34.0, −158.6, **−81.4**
	Met^8	5.9	94.3, 25.7, −164.9, **−75.1**
	Ile^9	10.3	**−134.2**, −105.8

a) Calculated using the Karplus–Bystrov equation: $^3J_{HN\alpha}=9.4\cos^2|60-\phi|-1.1\cos|60-\phi|+0.4$. Calculated dihedral angles shown in bold letters correspond to those in conformer A calculated in Table 3.

Table 3. Backbone Dihedral Angles in the Mean Structures of Conformers A and B of Cycloleonuripeptide A Calculated from DG Calculations

Residues	Conformer A			Conformer B		
	ϕ	ψ	ω	ϕ	ψ	ω
Gly^1	−175.0	−160.6	−180.0	46.1	18.0	180.0
Pro^2	−85.3	163.6	170.7	−161.3	117.0	170.6
Pro^3	−41.4	133.3	7.5	−65.6	146.1	0.3
Pro^4	−78.5	4.8	−179.0	−98.2	36.7	−173.0
Tyr^5	−94.9	150.8	−177.1	−153.5	74.6	−163.2
Pro^6	−76.6	148.1	170.6	−92.7	97.9	155.8
Pro^7	−33.7	−39.7	175.8	−77.9	−161.5	−176.0
Met^8	−47.3	−35.9	180.0	−31.7	−58.7	180.0
Ile^9	−122.8	−37.3	−180.0	−75.4	8.4	179.9

between Pro^6 and Ile^9 residues. The dihedral angles of the mean structures are listed in Table 3. Conformer A shows three intramolecular hydrogen bonds. Two of them are involved in 4→1 hydrogen bonds between Tyr^5-NH and Pro^2-CO (N–H···O 1.893 Å, 146.7°), and between Ile^9-NH and Pro^6-CO (N–H···O 1.866 Å, 165.6°) at two β-turn structures (type VI and type I, respectively). The temperature coefficients of Tyr^5- and Ile^9-NH, as shown in Table 1, corresponded to the above hydrogen bonds. An additional hydrogen bond is suggested to exist between Gly^1-NH and Pro^6-CO (N–H···O 1.866 Å, 168.3°), which is consistent with the low temperature gradient of the Gly^1-NH in Table 1.

The backbone conformation of conformer A contains a β bulge unit[12] formed by two consecutive β-type hydrogen bonds, including two residues. An α-helical conformation in Ile^9 ($\phi=-123°$, $\psi=-37°$) and a normal β conformation ($\phi=-175°$, $\psi=-161°$) in Gly^1 formed the classical β-bulge unit.[12]

The backbone dihedral angles (ϕ), calculated from $^3J_{NH-C\alpha}$ coupling constants by the use of a Karplus type equation proposed by Bystrov *et al.*,[13] are approximately the same as those of conformer A (bold letters in Table 2 corresponded closely to conformer A). The side chain conformation of Tyr^5 in conformer A was *gauche*$^-$, as deduced from $^3J_{H\alpha-H\beta}$ coupling constants.

Conformer B: 20 DG structures were selected among 272 structures generated. The average RMSD of the restraint violations was 0.005 Å. Figures 4 and 5 show the mean structure and a superposition of the backbone heavy atoms. Pairwise composition of the backbone atoms in these 20 structures gave an average RMSD of 0.83 Å. The total energy of conformer B (101.1 kcal/mol) was a little higher than that of conformer A (85.3 kcal/mol).

The mean structure takes two β-turn conformations; one is constructed between the Pro^3 and Pro^4 residues similarly to conformer A, and the other between Met^8 and Ile^9 residues. The latter formed a type I β-turn. The amide proton of Ile^9 in conformer B did not participate in hydrogen bonding and is not consistent with the temperature coefficient value of Ile^9-NH (0.7 ppb/K). In addition, the ϕ angle in conformer B deviated from the calculated values more than those of conformer A.

The assessment of a low energy conformer A, obtained by DG and SA calculations which considered ^{1}H-NMR information (ROE effects, hydrogen bonds, and torsion

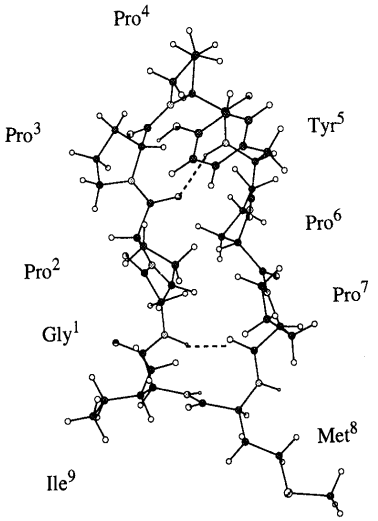

Fig. 4. Mean Structure of Stable Conformer B
Two hydrogen bonds are dipicted by dotted lines.

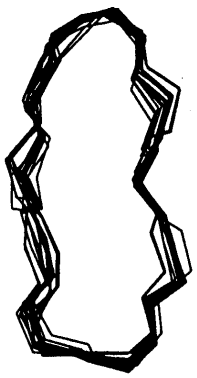

Fig. 5. Superimposed Backbone Structures of the 20 Lowest Energy Conformers in Conformer B (Backbone RMSD = 0.83 Å)

angles calculated from vicinal coupling constants), led to a proposal of the solution conformation for cycloleonuripeptides A—C. Interestingly, the whole structure of conformer A exhibits twist in the left turn. Conformer A is completely correlated with the distance constraints from the ROE correlations. In addition, the distances between Tyr^5-NH and Pro^2-CO, between Ile^9-NH and Pro^6-CO, and between Gly^1-NH and Pro^6-CO in conformer A, involving intramolecular hydrogen bondings, correspond to the temperature dependence on NH chemical shifts. The conformational preference of the proline-rich cyclic nonapeptides, cycloleonuripeptides A—C, involves two β-turn structures (type VI β-turn between Pro^3 and Pro^4 at two corners, and type I β-turns between Pro^7 and Met^8 at two corners) with a β-bulge motif.

Attempts to obtain a single crystal for **1**—**3**, as well as details from NMR studies, and to examine various biological assays are currently being done in our laboratories.

Experimental

Isolation After the fruits (10 kg) of *Leonurus heterophyllus* (Labiatae) were defatted with *n*-hexane two times, they were extracted with hot 70% methanol twice to give a methanol extract (*ca.* 500 g) which was treated with *n*-butanol and water. From the *n*-butanol soluble fraction (*ca.* 250 g), cycloleonuripeptides A (**1**), B (**2**), and C (**3**) were isolated according to the method described previously.[3)]

NMR ^{1}H- and ^{13}C-NMR spectra were recorded on Varian Unity 400 spectrometers. Each 10 mg of cycloleonuripeptide A—C in a 5 mm tube (0.5 ml DMSO-d_6, degassed) was used for the homonuclear and heteronuclear measurements. The spectra were recorded at 300 K. A phase sensitive ROESY experiment was made with a mixing time of 200 msec. The temperature effect on NH chemical shifts was measured to assess the solvent accessibilities to the amide protons at 10 intervals, over the range of 300—330 K, using a linear regression analysis.

Computational Methods Computer modeling and all calculations were carried out using the molecular-modeling software package SYBYL ver. 6.22 (Tripos, Inc., St. Louis, MO) on an IRIS 4D computer. Molecular mechanics and SA calculations were performed with the AMBER all-atom force field.[11)] The dielectric constant (ε) was assumed to be proportional to the interatomic distances (r) as $\varepsilon = r$. Solvent molecules were not included in the calculations. The ROE relationships shown in Fig. 1 (total 32 constraints) were taken into account in the calculations of the constrained minimizations and dynamics, with an extra harmonic term of the form $E = \Sigma K(r - r_{max})^2$ for $r > r_{max}$ and $E = 0.0$ for $r < r_{max}$ added to the force field ($K = 200$). The conformers after minimization were divided into two groups (conformers A, B). Each energy minimization was carried out until the derivatives became less than 0.01 kcal·mol^{-2}·Å^{-1} using the MAXMIN program.

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