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Solution State Conformation of an Immunosuppressive Cyclic Dodecapeptide, Cycloleonurinin [1]

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Abstract: The cyclic dodecapeptide, cycloleonurinin, *cyclo*(-Gly-Pro-Thr-Gln-Tyr-Pro-Pro-Tyr-Tyr-Thr-Pro-Ala-), isolated from the fruits of *Leonurus heterophyllus*, showed potent immunosuppressive effect on human peripheral blood lymphocytes. The solution state conformation of cycloleonurinin was examined by high field NMR methods, distance geometry calculation and restrained energy minimization from NMR data. Calculation using 277 different initial structures led to a uniquely determined backbone conformation with a root mean square deviation value of 0.80 Å. The backbone structure of cycloleonurinin consists of two β-turns, a β VI turn at Pro^6-Pro^7, and a β I turn at Pro^{11}-Ala^{12}. In addition to two transannular 4 → 1 backbone hydrogen bonds, which constructed two β-turns, two intramolecular hydrogen bonds between Tyr^9-NH and Pro^7-CO, and between Thr^{10}-NH and Tyr^8-CO, constructing γ-turns, and those between Thr^3-NH and Tyr^8-CO, and between Ala^{12}-NH and Thr^{10}-OH, were observed. © 1997 Elsevier Science Ltd.

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] the structures and conformations of four new cyclic peptides, cycloleonuripeptides A - D, from the fruits of *L. heterophyllus*, showing cell growth inhibitory or cyclooxygenase inhibitory activities, were reported. Kinoshita et al. reported the structure of cyclic dodecapeptide, cycloleonurinin, from the same plant in 1991.[4]

Many naturally occurring cyclic peptides have unique structures and biological activities, and we have focused our attention on various cyclic peptides from higher plants,[5] having various biological activities. We found that cycloleonurinin showed potent immunosuppressive effect on human peripheral blood lymphocytes, *i.e.* the antiproliferative effects on concanavalin A-stimulated human peripheral blood lymphocytes, which is comparable with that of a well-known immunosuppressive agent, cyclosporin A.

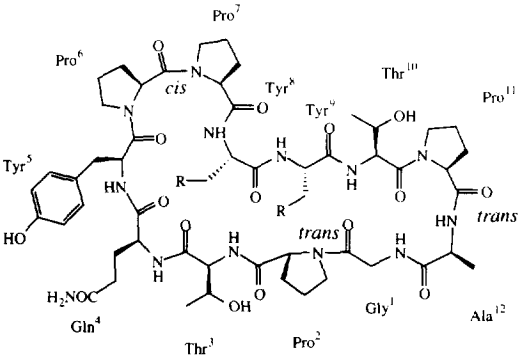

Fig. 1. Structure of cycloleonurinin, R=Ph-OH

Cycloleonurinin was a proline-rich cyclic peptide, containing four proline residues with Pro-Pro sequence. 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, a stable single conformation of cycloleonurinin was observed in DMSO-d_6 solution.

A knowledge of three dimensional structure of cycloleonurinin must be of great important to understand its immunological activity and to elucidate correlation between conformation and pharmacodynamic action. The solution conformational analysis of the immunosuppressive cyclic dodecapeptide, cycloleonurinin, was carried out by NMR data such as interatomic distances from a phase sensitive ROESY experiment, temperature effects and D_2O exchange rate on NH protons, and torsion angles calculated from $^3J_{NH\text{-}C\alpha H}$ coupling constants. For further conformational elucidation, a distance geometry (DG)-molecular dynamics (MD) procedure using distance constraints from a phase sensitive ROESY experiment was examined. We describe here the immunosuppressive activity and conformational preference of cycloleonurinin in DMSO-d_6 solution examined by DG calculation and restrained energy minimization from NMR data.

RESULTS AND DISCUSSION

Immunosuppressive activity of cycloleonurinin

Cycloleonurinin showed the inhibitory effects on mitogen (concanavalin A) - induced response of human peripheral-blood lymphocytes (IC_{50}: 28 ng/ml). A final concentration of 5 μg/ml of concanavalin A was used for the assay, this being the optimum lectin concentration for mitogenic stimulation of human lymphocytes as determined at our laboratory.[6] The activity was shown dose-dependently. No change of lymphocyte viability before or after agent treatment could be detected by a trypane blue dye exclusion test. Neither did cell lysis appear responsible for the reduction in thymidine uptake since the cells were still intact after 72 hr exposure. Thus possibly, cycloleonurinin may not be lymphocytotoxic but rather only inhibitory toward DNA synthesis. The IC_{50} in this system of a well-known immunosuppressive agent, cyclosporin A, was shown to be 3 ng/ml, which is comparable to that of cycloleonurinin.

Solution state conformation of cycloleonurinin

^1H and ^{13}C signal assignments. The assignments of ^1H and ^{13}C signals of cycloleonurinin, being essential for conformational elucidation, were made by the combination of DQF-COSY, TOSCY, HMQC for direct $^1J_{H\text{-}C}$ connectivities and HMBC for long range $^2J_{H\text{-}C}$ and $^3J_{H\text{-}C}$ ones, all of which were detected in FG-inverse detected mode at 600 MHz in DMSO-d_6 (320K). Though a part of assignment is reported by Kinoshita et al.,[4] a close inspection of the NMR spectra at 600 MHz led to complete ^1H and ^{13}C assignments of individual amino acid, as shown in Tables 1 and 2. Each of the four proline amide bonds is promising to produce *cis/trans* conformers that interconvert at a rate slow enough to give the separate signals. However, the NMR spectra in DMSO-d_6 at 320K gave well-resolved sharp signals and the presence of minor conformers was not observed. One of the proline amide bonds (the geometry between Pro^6 and Pro^7) in DMSO-d_6 was shown to be *cis* by the ^{13}C chemical shifts (δ 30.13 and 21.27) of β and γ positions in Pro^7 residue,[7] and the occurrence of a doublet signal of Hα in Pro^7.[8]

NOE enhancements. NOE derived distance information between protons provide the most important information for solution conformation of peptides. Conformational preference of cycloleonurinin was examined by using the NMR data such as interatomic distances from a phase sensitive ROESY experiment, temperature effects on NH protons, and torsion angles calculated from $^3J_{NH\text{-}C\alpha H}$ coupling constants.

Table 1. ^{1}H NMR signal assignments and NOE relationship of cycloleonurinin in DMSO-d_6 at 320K

assignment		δ_H [int. mult. J(Hz)]	NOE relationship
Gly^1			
	α	3.67 (1H, br d, 15.8)	Pro^2: Hδ(m); Gly^1: Hα(s)
		4.50 (1H, dd, 9.0, 15.8)	Gly^1: NH(m)
	NH	7.40 (1H, d, 9.0)	Ala^{12}: Hα(s), Hβ(w)
Pro^2			
	α	4.95 (1H, br s)	Pro^2: Hβ(w); Tyr^9: Hα(s); Thr^3: NH(m)
	β	1.80 (1H, m)	
		2.11 (1H, m)	
	γ	1.90 (2H, m)	
	δ	3.52 (1H, m)	Pro^2: Hδ(s)
		3.58 (1H, m)	
Thr^3			
	α	4.54 (1H, dd, 4.4, 8.4)	Thr^3: NH(m), Hβ(s); Gln^4: NH(s)
	β	3.95 (1H, m)	Thr^3: Hγ(s), OH(s); Gln^4: NH(w)
	γ	0.98 (3H, d, 6.2)	Thr^3: OH(w); Tyr^8: Hα (m)
	OH	4.96 (1H, br s)	
	NH	8.03 (1H, d, 8.1)	Tyr^3: Hα(s), Hβ(m)
Gln^4			
	α	4.08 (1H, ddd, 4.0, 7.0, 9.5)	Gln^4: NH(m), Hβ(m), Hγ(m); Tyr^8: Hδ(m); Tyr^5: Hδ(m)
	β	1.58 (1H, m)	
		1.85 (1H, m)	
	γ	2.05 (2H, m)	Gln^4: NH$_2$(w)
	NH_2	6.62 and 7.06 (each 1H, br s)	
	NH	8.09 (1H, d, 7.0)	Gln^4: Hβ(m), Hγ(w)
Tyr^5			
	α	4.59 (1H, dt, 3.3, 9.5)	Tyr^5: Hβ(m), NH(w), Hδ(m), Hε(w); Pro^6: Hδ(s)
	β	2.46 (1H, dd, 11.0, 13.9)	Tyr^5: Hβ(s), Hδ(m)
		2.70 (1H, dd, 2.6, 13.9)	Tyr^5: Hδ(m); Pro^6: Hδ(m)
	δ	7.00 (2H, d, 8.4)	
	ε	6.58 (2H, d, 8.4)	Pro^6: Hδ(w)
	NH	7.28 (1H, d, 8.8)	Tyr^5: Hβ(w)
Pro^6			
	α	4.42 (1H, m)	Pro^7: Hα(s)
	β	1.46 (1H, m)	
		1.90 (1H, m)	
	γ	1.95 (2H, m)	
	δ	3.42 (1H, m)	Pro^7: Hδ(s)
		3.62 (1H, m)	
Pro^7			
	α	3.96 (1H, d, 7.3)	Tyr^8: Hδ(m)
	β	1.85 (1H, m)	
		2.44 (1H, m)	
	γ	1.60 (1H, m)	
		1.80 (1H, m)	
	δ	2.97 (1H, br t, 10.3)	
		3.28 (1H, m)	
Tyr^8			
	α	4.48 (1H, ddd, 5.1, 7.0, 12.5)	Tyr^8: NH(s), Hδ(s), Hβ(m); Tyr^9: NH(w)
	β	2.78 (1H, dd, 5.1, 13.6)	Tyr^8: Hβ(s), Hδ(m)
		3.26 (1H, m)	Tyr^8: Hδ(m)
	δ	6.87 (2H, d, 8.1)	
	ε	6.59 (2H, d, 8.1)	
	NH	8.51 (1H, d, 7.0)	
	OH	8.99 (1H, br s)	
Tyr^9			
	α	4.71 (1H, ddd, 5.1, 8.1, 8.8)	Tyr^9: NH(m), Hβ(m), Hδ(m); Thr^{10}: NH(s)
	β	2.62 (1H, dd, 8.1, 13.9)	Tyr^9: Hβ(s), Hδ(m)
		2.82 (1H, dd, 5.1, 13.9)	Tyr^9: Hδ(m)
	δ	6.99 (2H, d, 8.4)	
	ε	6.61 (2H, d, 8.4)	
	NH	7.67 (1H, d, 8.8)	Tyr^9: Hβ(w)
	OH	9.06 (1H, br s)	
Thr^{10}			
	α	4.90 (1H, dd, 3.3, 9.2)	Thr^{10}: NH(w), Hβ(s), Hγ(w), OH(m); Pro^{11}: Hδ(s)
	β	4.38 (1H, m)	Thr^{10}: Hγ(s), OH(w); Pro^{11}: Hδ(s); Ala^{12}: NH(w)
	γ	1.02 (3H, d, 5.9)	Thr^{10}: OH(w)
	OH	5.30 (1H, d, 9.9)	Pro^{11}: Hδ(m), Ala^{12}: NH(m)
	NH	7.45 (1H, d, 9.2)	Thr^{10}: OH(w), Hβ(w)
Pro^{11}			
	α	4.24 (1H, t, 8.4)	Ala^{12}: NH(m)
	β	1.75 (1H, m)	
		2.27 (1H, m)	
	γ	1.90 (2H, m)	
	δ	3.67 (1H, m)	Pro^{11}: Hδ(s)
		3.87 (1H, m)	
Ala^{12}			
	α	4.27 (1H, dq, 9.2, 7.3)	Ala^{12}: NH(m), Hβ(s)
	β	1.19 (3H, d, 7.3)	Ala^{12}: NH(m)
	NH	7.39 (1H, d, 9.2)	

In the NOE column, s, m and w in parenthesis indicate strong, medium and weak NOE enhancements, respectively.

Table 2. ^{13}C assignments of cycloleonurinin in DMSO-d_6.

		δ_C			δ_C			δ_C
Gly^1	α	40.72	Tyr^5	α	51.57	Tyr^9	α	54.13
	C=O	168.09		β	35.80		β	38.26
				γ	127.14[b]		γ	128.09[b]
Pro^2	α	59.21		δ	130.06[c]		δ	130.18[c]
	β	29.89		ε	114.68[d]		ε	114.88[d]
	γ	24.45		ζ	155.32[e]		ζ	155.85[e]
	δ	46.37		C=O	169.35		C=O	170.08
	C=O	170.50[a]	Pro^6	α	58.04	Thr^{10}	α	56.13
				β	27.60		β	67.32
Thr^3	α	55.60		γ	24.13		γ	18.82
	β	66.74		δ	46.50		C=O	170.55[a]
	γ	17.47		C=O	169.71			
	C=O	169.02				Pro^{11}	α	61.65
			Pro^7	α	60.81		β	28.95
Gln^4	α	53.65		β	30.13		γ	25.19
	β	26.50		γ	21.27		δ	47.43
	γ	31.60		δ	45.66		C=O	170.24
	δ	173.70		C=O	171.89			
	C=O	170.62	Tyr^8	α	57.88	Ala^{12}	α	47.77
				β	32.60		β	17.83
				γ	127.27[b]		C=O	171.45
				δ	130.18[c]			
				ε	114.75[d]			
				ζ	155.72[e]			
				C=O	171.81			

a-e) Assignment with the same superscripts may be interchanged.

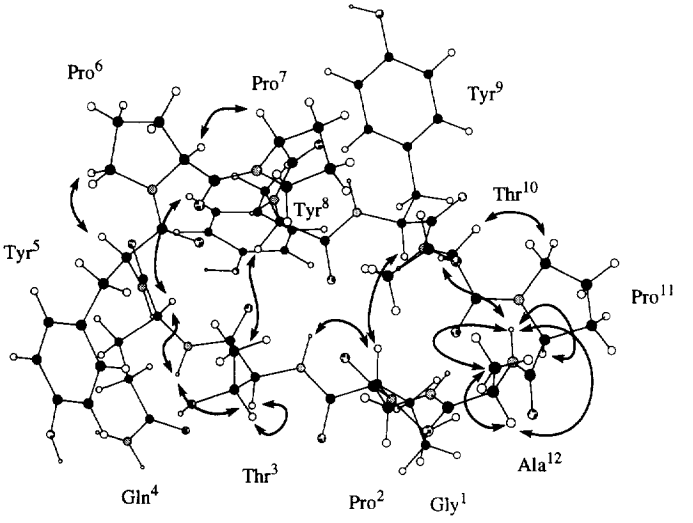

Fig. 2. Selected NOE correlations of cycloleonurinin observed by a phase sensitive ROESY spectrum.

The NOE relationship measured by its phase sensitive ROESY spectrum in DMSO-d_6 is shown in Fig. 2 and listed in Table 1. The strong NOE enhancement between Pro^6-Hα and Pro^7-Hα is related to the *cis* Pro^6-Pro^7 bond and also indicates that it takes type VI β-turn from Tyr^5 to Tyr^8. The NOE cross peaks between Ala^{12}-NH and Ala^{12}-Hα, between Ala^{12}-NH and Ala^{12}-Hβ, between Ala^{12}-NH and Pro^{11}-Hα, and between Ala^{12}-NH and Thr^{10}-OH, suggested that β-turn is formed from Thr^{10} to Gly^1 like that (type I) of dichotomin A, which has been reported in our previous paper.[9] If cycloleonurinin take β turn at this sequence, Gly^1-NH may take part in a transannular 4 → 1 hydrogen bond between Gly^1-NH and Thr^{10}-CO, which was indicated

by the NMR and simulation studies as shown below. In addition, three characteristic bond-through interactions indicating two protons are close to each other and they may limit considerably backbone conformation, were observed between Pro^2-Hα and Tyr^9-Hα, between Thr^3-Hβ and Tyr^8-Hα and between Gln^4-Hα and Tyr^8-Hδ.

Hydrogen bonding of the amide protons. In such a solvent as DMSO-d_6, the temperature effect on NH chemical shifts is often used to identify the external or internal NH orientations.[10] The temperature coefficients (dδ/dT) of cycloleonurinin given in Table 3 clearly show that the following five amide protons; Gly^1, Thr^3, Tyr^8, Tyr^9, and Ala^{12}, are strongly shielded from the solvent, which are characteristic of a proton forming a strong hydrogen bond. It was reported that values $<-3\times10^{-3}$ ppm/K usually suggest solvent-shielded and presumably hydrogen-bonded NH groups, whereas values $>-4\times10^{-3}$ ppm/K are for exposed groups.[11] The intermediate value for Tyr^5 and Thr^{10} do not lead to definitive conclusions, at this stage. In contrast, the strong temperature dependence of the signal of the amide proton in Gln^4 indicates that this amide group is exposed to the solvent and may not be involved in intramolecular hydrogen bonding.

On the other hand, another method that hydrogen bonds are evaluated is rates of hydrogen-deuterium (H-D) exchange. The experiment in DMSO-d_6-D_2O mixtures showed that the exchange half-life time ($t_{1/2}$) for Gly^1-NH, Thr^3-NH, Tyr^5-NH, Tyr^8-NH, Tyr^9-NH, Thr^{10}-NH, and Ala^{12}-NH were 17.0, 24.3, 20.8, 29.0, 28.0, 27.5, and 17.0 min., respectively. These results were almost corresponding to those of the temperature effect. Two amide protons of Tyr^5 and Thr^{10} were considered to be involved in intramolecular hydrogen bonds or be shielded from the solvent.

Table 3. Temperature coefficients ($-d\delta/dT \times 10^{-3}$ ppm/K) of NH chemical shifts in ten intervals over the range 300 - 330 K in DMSO-d_6 and rate of deuterium exchange (min.) of NH protons of cycloleonurinin.

	Gly^1	Thr^3	Gln^4	Tyr^5	Tyr^8	Tyr^9	Thr^{10}	Ala^{12}
Temp. coefficients	1.6	1.8	4.8	3.7	1.8	-0.9	3.9	1.6
D_2O exchange rate	17.0	24.3	4.5	20.8	29.0	28.0	27.5	17.0

Proton vicinal coupling of NH/Hα. Three-bond couplings gave very useful information for the determination of the backbone conformation because they can directly be converted into dihedral angles *via* Karplus-type equations proposed by Bystrov et al.[12] The dihedral angles, ϕ, calculated from vicinal NH-CαH coupling constants are shown in Table 4. Uniformity of this calculation resultant and the values obtained from distance geometry simulation is shown later.

Side chain conformations. The side chain conformations of three tyrosine residues: Tyr^5, Tyr^8 and Tyr^9 were elucidated by the HMBC correlations[13] between Hβ protons of each Tyr and amide carbonyl carbons of the corresponding Tyr, and vicinal proton coupling constant[14] between Hα and Hβ. The coupling constants (2.6 and 11.0 Hz) of Hα-Hβ in Tyr^5 and those (5.1 and 12.5 Hz) in Tyr^8 indicated that the rotamers in Tyr^5 and Tyr^8 were *gauche*$^-$ or *trans*. Furthermore, the vicinal coupling constants (5.1 and 8.1 Hz) between Hα and Hβ in Tyr^9 suggested that the aromatic ring in Tyr^9 rotated freely.

These rotamers were also supported by the cross peaks in HMBC spectrum.[13] Strong correlation between Hβ (*pro R*; δ 2.46) and amide carbonyl carbon (δ 169.35) more than that of Hβ (*pro S*: δ 2.70) in Tyr^5 indicated that the rotamer take *trans* orientation. The lack of HMBC correlation between Hβ (*pro R*, δ 3.26) and amide carbonyl carbon (δ 171.81) in Tyr^8 indicated that the rotamer take *gauche*$^-$ orientation, which causes the ring current effect to one of Hδ protons in Pro^7. In addition, it was suggested that the side chain in Tyr^9 had

allocation of *trans* or *gauche*$^-$ because there was not variation by intensity of two HMBC correlations between Hβ protons and the amide carbonyl carbon in Tyr^9.

Table 4. Backbone dihedral angles (°) in cycloleonurinin, calculated from vicinal NH-CαH coupling constants (Hz) and the mean structure obtained by DG calculations.

residues	Hz	φ angle(°)[a]	cycloleonurinin[b]		
			φ	ψ	ω
Gly^1	9.0	51, **149**	176.3	72.6	174.4
Pro^2			-64.3	118.9	-177.2
Thr^3	8.1	75, 45, **-152**, -88	-157.5	-175.1	-179.8
Gln^4	7.0	86, 34, -159, **-81**	-82.7	-29.5	-174.9
Tyr^5	8.8	-147, **-93**	-92.6	152.6	-176.2
Pro^6			-52.9	141.0	-1.1
Pro^7			-82.6	16.5	173.6
Tyr^8	7.0	**86**, 34, -159, -81	69.3	-70.7	-173.2
Tyr^9	8.8	-147, **-93**	-101.3	62.1	171.7
Thr^{10}	9.2	-144, **-96**	-106.6	133.5	-178.5
Pro^{11}			-60.8	-30.7	173.6
Ala^{12}	9.2	-144, **-96**	-77.4	1.9	180.0

The calculated φ angles shown by bold letters are close to those calculated by DG calculations
[a] Calculated by using the Karplus-Bystrov equation: $^3JHN\alpha=9.4\cos^2|60-\phi|-1.1\cos|60-\phi|+0.4$ for non-Gly residues and and $\Sigma^3JHN\alpha2=6.0\cos^2\phi-1.5\cos\phi+12.5\sin^2\phi$ for Gly residue.
[b] dihedral angles calculated by the mean structure of group **A** of cycloleonurinin obtained by DG calculations

Distance geometry calculation

The conformational properties of cycloleonurinin have been extensively studied by distance geometry (DG) calculation. Interatomic distances were calculated from the integrated volumes of the ROESY cross peaks and classified into three ranges, 1.9 - 2.5, 1.9 - 3.5 and 1.9 - 5.0 Å, corresponding to strong, medium and weak NOEs, respectively. Because of the lack of stereospecific assignments in some methylene protons, the upper distances of these methylene and methyl protons were further relaxed by means of the pseudoatoms corrections. In addition, amide torsion angle constraints were taken into consideration. No hydrogen bonding constraints were used.

Initial structures satisfying the experimental restraints were embedded by DG calculations. Structural calculations were carried out using simulated annealing (SA) protocol with the program SYBYL,[15] and the produced conformers were then subjected to restrained energy minimization with the AMBER all-atom force field.[16] In SA simulation, each system was equilibrated for 5000 fs in a thermal bath at 800K, and thereafter successively for 2700 fs, the temperature was decreased 54 times until a final temperature of 100K was reached. Each frozen conformation was finally minimized.

The converged group was selected as those whose pairwise backbone RMSDs are less than 1.00Å and resulted in four structural families (groups **A** - **D**). 17 structures among 277 structures generated by the DG method were defined as the converged group **A**, whose mean RMSD of the restraint violations was 0.01 Å. Fig. 3 shows a superposition of the backbone heavy atoms of these 17 refined structures. The mean structure generated, followed by energy minimization, is shown in Fig. 4. The overall atomic RMSDs between the individual structures and the mean coordinate positions are 0.80

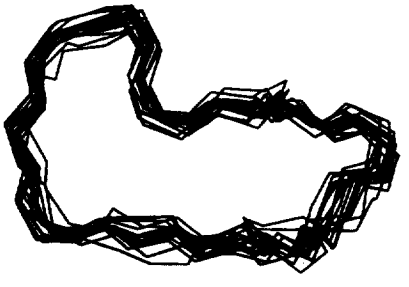

Fig. 3. Superimposed 17 backbone structures of group **A** (RMSD 0.80 Å)

(0.22) Å for the backbone atoms.

It is obvious from the dihedral angles of the mean backbone conformation listed in Table 4 that it adopts a type I β turn from Thr^{10} to Gly^{1} and a type VI β turn from Tyr^{5} to Tyr^{8} with a peptide bond between Pro^{6} and Pro^{7} in the *cis* configuration. In addition, the presence of γ-turn at Pro^{7}-Tyr^{8}-Tyr^{9} sequence was suggested. These turns were also suggested by two 4 → 1 hydrogen bonds between Gly^{1}-NH and Thr^{10}-CO (N-H...O 1.90Å, 156.5°), and between Tyr^{8}-NH and Tyr^{5}-CO (N-H...O 1.87Å, 142.2°), and one 3 → 1 hydrogen bond between Tyr^{9}-NH and Pro^{7}-CO (N-H...O 1.89Å, 140.4°). Furthermore intramolecular hydrogen bonds and short contacts listed in Table 6 were indicated. The low temperature coefficients, as shown in Table 3, well corresponded to these hydrogen bonds. The backbone dihedral angles (ϕ), calculated from $^{3}J_{NH\text{-}C\alpha}$ coupling constants, shown in Table 4 are approximately the same as this conformer (Bold letters in Table 4 corresponded closely to this conformer).

18 DG conformers were selected as group **B** among 277 structures generated. As can be seen from the mean structure of group **B** in Fig. 4, the backbone conformation is very similar to that of group **A**, though a total energy of **B** was a little higher than that of **A**. A major different point each other is the direction of amide bond in Tyr^{5}. The amide proton of **B** is being outside from the backbone ring, whereas that of **A** interior. This direction of the amide proton in **B** is not consistent with the NMR data concerning with the hydrogen bonds (Table 3), and the torsion angle (**B**: ϕ 73.6°) in Tyr^{5} also not with those (ϕ -147°, -93°) obtained from the coupling constant (Table 4). 12 and 18 conformers were selected as groups **C** and **D**, respectively. Total energy, especially, group **D** of them is higher than that of **A**. In addition, the correlation with the NMR data concerning to hydrogen bonds was not found at all in **C** and **D**.

The assessment of a low energy group **A**, which considered ^{1}H NMR information (NOE effects, hydrogen bonds, and torsion angles calculated from vicinal coupling constants), led to a proposal of the solution conformation for cycloleonurinin.

Table 5. Results of distance geometry calculation for cycloleonurinin.

Structural parameters	group **A**	group **B**	group **C**	group **D**
No. of converged conformer[a)]	17	18	12	18
Energy (kcal/mol)[b)]	50.244	53.116	52.308	68.674
RMS NOE[b)]	0.01	0.02	0.02	0.03
RMSD for backbone heavy atoms of mean structures (Å)	0.80 (0.22)	0.68 (0.21)	0.94 (0.25)	0.61 (0.24)

a) the number of the produced conformers whose pairwise RMSD for heavy atoms is less than 1.00.
b) the value of each mean structure.

Table 6. Intramolecular hydrogen bonds and short contacts (Å) of the mean structure of group **A** obtained by DG calculations.

From	To	Distance (Å)		Angle (°)
		O...N	O...HN	O...HN
Gly^{1}-NH	Thr^{10}-CO	2.86	1.90	156.5
Ala^{12}-NH	Thr^{10}-OH	3.55	2.70	142.0
Thr^{10}-NH	Gly^{1}-CO	4.30	3.42	146.5
Thr^{3}-NH	Tyr^{8}-CO	2.87	1.86	173.8
Tyr^{8}-NH	Tyr^{5}-CO	2.75	1.87	142.2
Tyr^{9}-NH	Pro^{7}-CO	2.75	1.89	140.4
Thr^{10}-NH	Tyr^{8}-CO	3.49	2.65	140.0

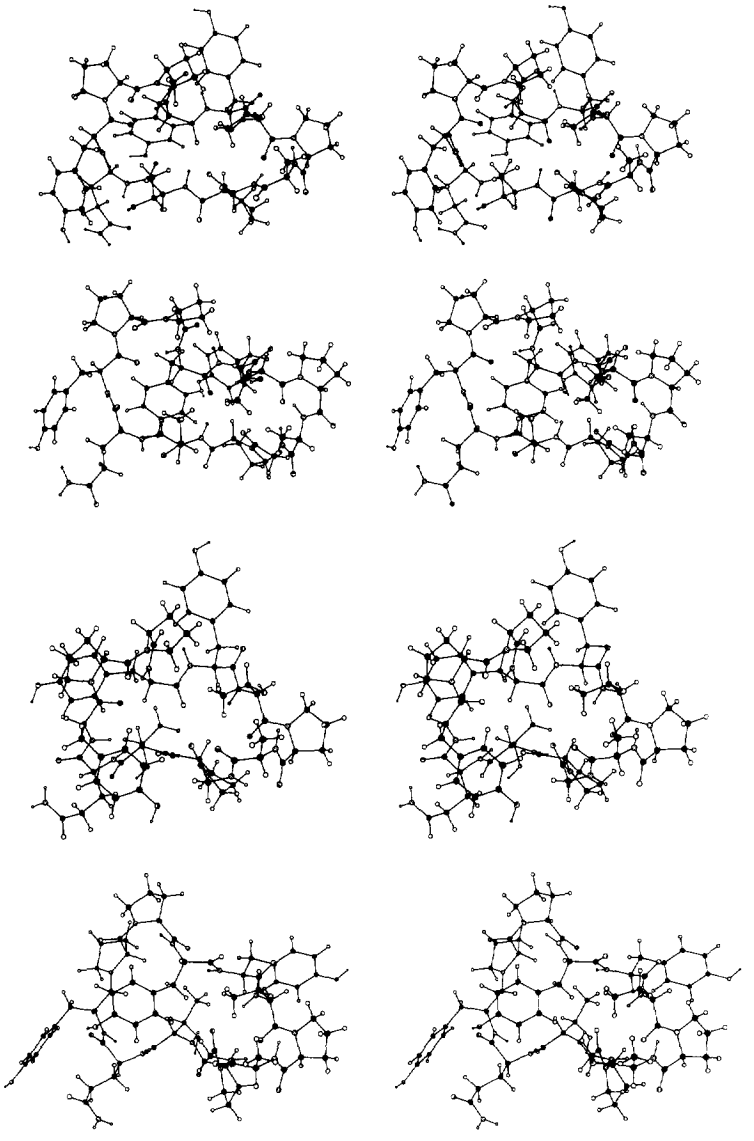

Fig. 4. From top to bottom, stereoscopic views of the mean structure of each group (**A** - **D**) by DG calculation, group **A** was proposed conformation of cycloleonurinin in DMSO-d_6.

CONCLUSION

In the present studies, conformational preference for cycloleonurinin was analyzed by extensive NMR methods and DG calculation. Cycloleonurinin showed a potent immunosuppressive activity comparable with that of cyclosporin A.

Cyclosporin A, cyclolinopeptide A (CLA) from linseed oil[17]: *cyclo* (-Pro-Pro-Phe-Phe-Leu-Ile-Ile-Leu-Val-), and antamanide (ANT) from the mushroom *Amanita phalloides*[18]: *cyclo* (-Pro-Pro-Ala-Phe-Phe-Pro-

Pro-Phe-Phe-Val-) are well known as immunosuppressive cyclic peptides. Cyclolinopeptide A, antamanide and the present cycloleonurinin were demonstrated to possess amino acid sequences of a definite similarity in their structures: -Val-Pro-Pro-Phe-Phe- in CLA, -Val-Pro-Pro-Ala-Phe-Phe- in ANT, and -Pro-Pro-Tyr-Tyr- in cycloleonurinin. The comparison of these cyclic peptides suggests that the immunosuppressive activity of these cyclic peptides requires the presence of two adjacent prolines and two adjacent aromatic amino acid residues. In these specific peptides, the amide bonds located between two Pro residues, are of *cis* configuration. Furthermore, the conformation of cyclolinopeptide A, which has already been analyzed in $CDCl_3$,[19] and that of cycloleonurinin in DMSO-d_6, are also similar to each other in the region of the above amino acid sequences: the type VI β-turn formed by the two adjacent Pro residues and γ-turn formed at the aromatic residue next to the proline residue. From our present study, such sequential and conformational features are considered to be necessary for their biological activities. Detailed conformational analysis in the same solvent may have to be conducted to obtain a definite conclusion.

EXPERIMENTAL

Material

After the fruits of *Leonurus heterophyllus* (10 kg) were defatted with n-hexane two times, they were extracted with hot 70% methanol two times to give a methanol extract (ca. 500 g) which was treated with *n*-butanol and water. The *n*-butanol soluble fraction (ca. 250 g) was subjected to Diaion HP-20 column chromatography using a water - methanol gradient system (1:0 - 0:1). The fractions (47 g) eluted by 80% methanol were further subjected to silica gel column chromatography using a methylene chloride - methanol gradient system (1:0 - 0:1). The fraction eluted by 20% methanol was subjected to ODS HPLC with 50 % MeOH solvent system to give cycloleonurinin (0.01%) as colorless powder, whose spectroscopic data were completely identical with those reported in the literature.[4]

Biological Assay

Venous blood (10 ml) was taken from a healthy volunteer, heparinized, and then centrifuged at 1300 × g fir 15 min at room temperature. Mononuclear lymphocytes were separated by a Ficoll-Hypaque density gradient. The cells were suspended in RPMI 1640 medium containing 100,000 IU/l of penicillin, 100 μg/l of streptomycin, and 10% of fetal calf-serum to a cell density of 1×10^6 cells/ml. Two hundred microliters of this suspension were placed into each well of a microtiter plate with 96 flatbottom wells. Concanavalin A was added to each well to a final concentration of 5.0 μg/ml. Subsequently, 4 μl of an ethanol solution containing the test compounds were added to a final concentration of 1-10000 ng/ml. Four microliters of ethanol were added to a control well. The plate was incubated for 4 days in 5 % CO_2/95% air at 37 °C. The cells were pulsed with 0.5 μCi/well of [^3H]-thymidine for the last 16 h of incubation and then collected on glass fiber filter paper using a multiharvester device and dried. The radioactivity retained on the filter was further processed for liquid scintillation counting. The mean of the counts of a triplicate for each sample was determined. Agent concentration that would give 50 % inhibition of lymphocyte mitosis (IC_{50}) was determined from the dose response curve. The viability of lymphocytes was assessed by a dye exclusion test using trypane blue as a dye as described elsewhere.

NMR

^1H and ^{13}C-NMR spectra were recorded on JEOL α600 and Bruker AM500 spectrometers. 10 mg of cycloleonurinin in a 5mm tube (0.5ml DMSO-d_6, degassed) was used for the homonuclear and heteronuclear measurements. The spectra were recorded at 320K. A phase sensitive ROESY experiment was made with a mixing time of 200 msec. Temperature effects on NH chemical shifts were measured to assess the solvent accessibilities to the amide protons at 10°C intervals, over the range of 300 - 330 K, by using a linear regression analysis. ^1H and ^{13}C assignments are shown in Tables 1 and 2.

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. DG, SA and molecular mechanics

calculations were performed with the AMBER all-atom force field.[16] The dielectric constant (ε) was assumed to be proportional to the interatomic distances (r) as ε=r. Solvent molecules were not included in the calculations. The NOE relationships shown in Table 1 (total 57 constraints) were taken into account in the calculations of the constrained minimizations and dynamics, with an extra harmonic term of the form $E=1/2k(d-d^{low})^2$ for $d<d^{low}$, $1/2k(d^{high}-d)^2$ for $d^{high}<d$ and E=0.0 for $d^{low}\leq d\leq d^{high}$ added to the force field [k=200 kcal/(mole)(°)^2]. Torsion constraints (the amide bond between Pro^6 and Pro^7 was only *cis* geometry), with an extra harmonic form of the form $E=1/2k(\omega-\omega^0)^2$ [k=0.01 kcal/(mole)(Å^2)] were also added to the force field. 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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