Pergamon

Tetrahedron, Vol. 52, No. 8, pp. 2795-2802, 1996

0040-4020/96 $15.00 + 0.00

0040-4020(95)01094-7

Configurational and Conformational Analyses of a Cyclic Octapeptide, Lyciumin A, from *Lycium chinense* MILL.[1)]

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Abstract : Configurational and conformational analysis of a unique cyclic octapeptide, lyciumin A, showing an inhibitory activity on angiotensin-converting enzyme, was made by the spectroscopic and computational chemical methods. The homo- and heteronuclear 2D NMR analysis at 600 MHz in pyridine-d_5 enable us to determine the complete stereostructure of lyciumin A, which agrees with the structure obtained by the Monte Carlo (MC) and restrained molecular dynamics (MD) calculation using AMBER* force field. A major solution form of lyciumin A in pyridine-d_5, analyzed by NH-CαH coupling constants, temperature dependence on NH protons, and NOE constrained MD calculations, was shown to have a type II β-turn-like conformation between the Val and Gly residues constituting the cyclic backbone.

Many naturally occurring cyclic peptides have unique structures and biological activities, and we have focused our attention on various cyclic peptides from higher plants,[2)] having various biological activities. Despite their importance, only a few studies on cyclic peptides from higher plants have been reported.[3)] Nohara et al. reported the structures of unique monocyclic octapeptides, lyciumins, showing an inhibitory activity on angiotensin-converting enzyme, from the roots of *Lycium chinense* MILL. in 1989.[4)]

Lyciumins have a novel C-N linkage between the tryptophan N1 and the glycine Cα. Schmidt et al. conducted a total synthesis of lyciumins A and B in 1992.[5)] However, the configuration of the C-N linkage at the glycine Cα is not known yet. We are interested in the conformation of lyciumins, especially of the cyclic pentapeptide backbone incorporating a tryptophan. In this paper, we describe the elucidation of the complete stereostructure and conformational analysis of lyciumin A by extensive

analysis of the 2D NMR spectra in pyridine-d5 at 600 MHz and computational calculations including the Monte Carlo (MC) and the molecular dynamics (MD) conformational search methods.

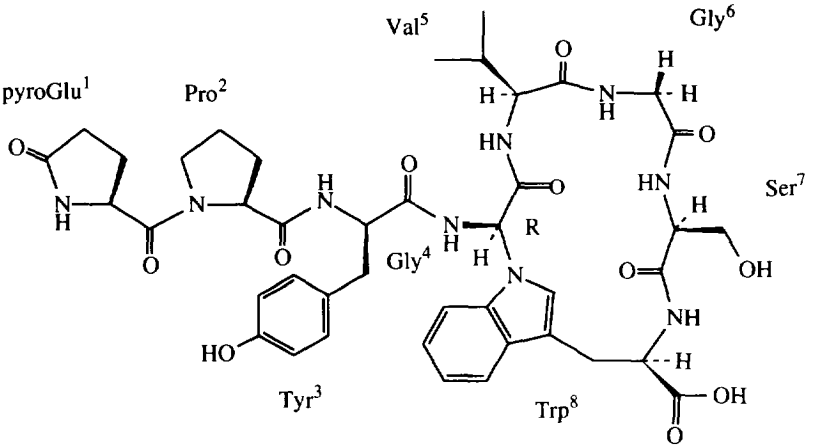

Fig. 1. Structure of lyciumin A (**1**); pyroGlu residue was provisonally numbered as first amino acid.

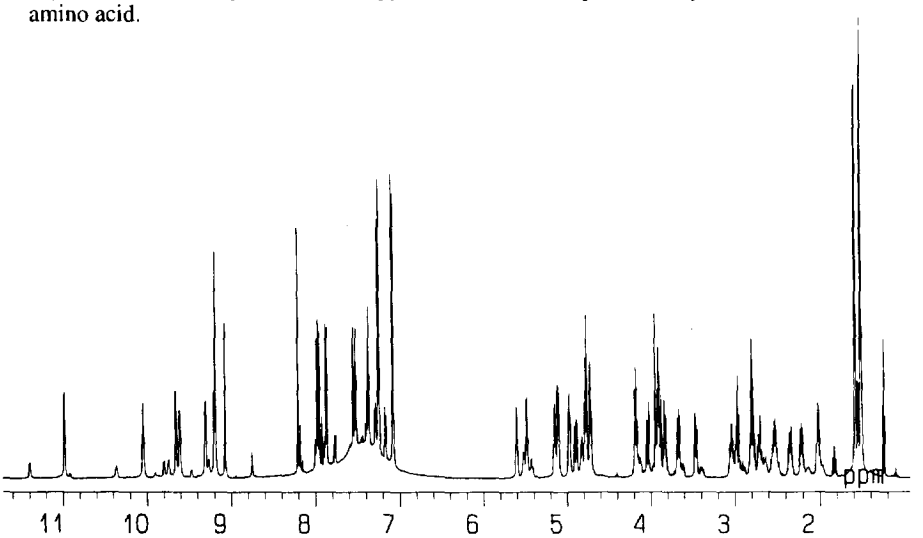

Fig.2. ^{1}H NMR spectrum of lyciumin A in pyridine-d5 at 600 MHz.

Complete assignments of ^{1}H and ^{13}C NMR signals in pyridine-d5

To determine the configuration at Gly^{4}-Cα of lyciumin A and the dynamic structure of the compound, complete assignments of the ^{1}H and ^{13}C signals of lyciumin A were made in various NMR measurements such as ^{1}H-^{1}H COSY, HOHAHA,[6] HMQC[7] for direct $^{1}J_{H\text{-}C}$ connectivities, and HMBC[8] for long range $^{2}J_{H\text{-}C}$ and $^{3}J_{H\text{-}C}$ ones. The NMR data of lyciumin A in DMSO-d6

have already been reported.[4] However, in DMSO-d6, about 20% of lyciumin A takes the form of a minor conformer, due to *cis-trans* isomerization of the proline amide bond, which complicates the NMR analysis. The ratio of *cis-* and *trans* isomers varies depending on the solvent. We used pyridine-d_5 as the solvent because in pyridine-d_5, lyciumin A gave well-resolved sharp signals and the minor conformer content of only about 5 %, as shown in Fig. 2. The complete assignments of the ^{1}H-NMR signals of the major conformer are shown in Table 1. In the major conformer, the chemical shifts of the β and γ carbons of the Pro residue are δ 28.71 and 25.28, respectively, which gives an evidence of a trans proline peptide bond between pyroGlu[1] and Pro[2].[9] The double doublet signal of Hα in Pro[2] is another evidence of the trans proline peptide bond.[10]

Table 1. ^{1}H NMR signal assignments and ROE relationship of lyciumin A (**1**) in pyridine-d5

assignment		δ_H (int. mult, J(Hz))	ROE relationship
pyroGlu[1]			
	α	4.97 (1H, dd, 6.0, 6.6)	pyroGlu[1]: Hβ(m), NH(m); Pro[2]: Hδ(s)
	β	2.70 (1H, m)	
		2.80 (1H, m)	
	γ	2.80 (1H, m)	
		2.97 (1H, t, 10.2)	
	NH	9.18 (1H, s)	
Pro[2]			
	α	5.10 (1H, dd, 4.5, 8.0)	Pro[2]: Hβ(m), Hγ(w); Tyr[3]: NH(s)
	β	2.21 (1H, m)	
		2.53 (1H, m)	
	γ	2.02 (1H, m)	
		2.34 (1H, m)	
	δ	3.83 (1H, m)	
		3.90 (1H, m)	
Tyr[3]			
	α	5.15 (1H, ddd, 6.6, 7.5, 5.5)	Tyr[3]: Hβ(m), Hδ(m), Hε(w)
	β	3.46 (1H, dd, 6.6, 13.8)	Tyr[3]: Hδ(m), Hε(w)
		3.67 (1H, dd, 7.5, 13.8)	
	δ	7.25 (2H, d, 8.4)	
	ε	7.08 (2H, d, 8.4)	
	NH	9.65 (1H, d, 5.5)	Tyr[3]: Hα(s), Hβ(m)
Gly[4]			
	α	7.86 (1H, d, 8.4)	Gly[4]: NH(m); Val[5]: NH(m); Trp[8]: H2(w), H7(s)
	NH	10.98 (1H, d, 8.4)	Val[5]: NH(m); Trp[8]: H2(m); Tyr[3]: Hα(m)
Val[5]			
	α	4.77 (1H, m)	Val[5]: Hβ(m), NH(m)
	β	3.04 (1H, m)	
	γ	1.51 (3H, d, 6.6)	
		1.58 (3H, d, 7.2)	
	NH	9.61 (1H, d, 6.9)	Val[5]: Hβ(m), Hγ(m), Hγ(w)
Gly[6]			
	α	4.18 (1H, dd, 5.7, 15.6)	Gly[6]: NH(s); Ser[7]: NH(w)
		4.89 (1H, dd, 5.7, 15.6)	Gly[6]: NH(w); Ser[7]: NH(m)
	NH	10.05 (1H, t, 5.7)	Val[5]: Hα(s); Ser[7]: NH(w)
Ser[7]			
	α	5.48 (1H, ddd, 3.6, 5.0, 6.6)	Ser[7]: NH(m), Hβ(m); Trp[8]: NH(s)
	β	4.72 (1H, dd, 5.0, 11.0)	
		4.79 (1H, dd, 3.6, 11.0)	
	NH	9.19 (1H, d, 6.6)	Trp[8]: H2(m); Ser[7]: Hβ(m)
Trp[8]			
	α	5.60 (1H, td, 3.6, 7.8)	Trp[8]: H2(s)
	β	3.92 (1H, m)	Trp[8]: Hα(m), H2(w)
		4.04 (1H, dd, 3.6, 15.6)	Trp[8]: Hα(s), H2(m)
	NH	9.30 (1H, d, 7.8)	Trp8: H2(m), Hα(m), Hβ(w)
	2	8.20 (1H, s)	
	4	7.97 (1H, d, 7.0)	Trp[8]: Hβ(m)
	5	7.37 (1H, t, 7.0)	
	6	7.52 (1H, t, 7.0)	
	7	7.95 (1H, d, 7.0)	

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

ROE relationship of lyciumin A in pyridine-d5

To elucidate the configuration of the C-N linkage at Gly^4-Cα and the conformational characteristics of lyciumin A, a phase sensitive ROESY spectrum[11] of lyciumin A was measured at 600 MHz (Table 1), with special reference to the ROE relationships around Gly^4 and Trp^8 residues. Strong ROE enhancements between Gly^4-Hα and Trp^8-H7, and between Trp^8-Hα and Trp^8-H2, midium ones between Trp^8-H4 and both of Trp^8-Hβ were observed and also the presence of a weak ROE between Trp^8-H2 and Gly^4-Hα indicate that the indole ring of Trp^8 does not rotate freely. Furthermore, a weak ROE between Trp^8-NH and Trp^8-H2, and medium ROEs among Gly^4-NH, Gly^4-Hα, Val^5-NH and Trp^8-H2 shown in Table 1, suggest that the configuration at Gly^4-Cα is *R*. The *R* configuration of Gly^4-Cα was proved by a computer-simulated experiment using the Monte Carlo (MC) and ROE restrained molecular dynamics (MD) calculation (See MC and MD section).

In addition to the above enhancements, strong ROEs were observed between Gly^6-NH and Val^5-Hα, and between Gly^6-NH and one of Gly^6-Hα together with a weak ROE between Gly^6-NH and Ser^7-NH, suggesting a turn structure between Val^5 and Gly^6. Since no ROE was observed between Val^5-NH and Gly^6-NH, the β-turn between Val^5 and Gly^6 was considered to be of type II. The geometries of the amide bonds including a *trans* Pro^2 amide bond were all defined as *trans* by the ROEs between Hαi and NHi+1.

MC and restrained MD calculation

To confirm that lyciumin A takes *R* configuration at Gly^4-Cα, as revealed by the NMR study, and to analyze the conformational features of **1**, a series of computational methods were used. Exhaustive conformational searches of **1** was conducted by use of systematic pseudo Monte Carlo (MC) search[12] for two possible isomers of **1** (*R* and *S* isomers at Gly^4-Cα), followed by molecular dynamics simulation. Each conformation generated by each MC and MD calculation was minimized by the use of molecular mechanics calculation of AMBER* all-atom force field with GB/SA solvation method[13] implemented in MacroModel/Batchmin[14] (Ver. 4.5).

Firstly, the starting stereostructures of **1** for MD simulation were produced by the MC search. To discriminate between the conformational energy of the cyclic rings and that of the side chains, the MC calculation was made with a hypothetical model compound (**2**) whose side chain was CH=O, in stead of pyroGlu-Pro-Tyr. The pseudosystematic MC procedure of Still and Goodman[12] was performed, in a pseudosystematic way, by changing the torsion angles of 11 non-amide bonds in the 17-membered ring in the rane of 0 - 180°. A total of 10,000 MC steps were performed by using different starting geometries to confirm the reproducibility of calculation results. After the MC conformational search, each of the resulting conformations was subjected to the energy-minimization calculation by the GB/SA method to reduce the gradient rms to less than 0.001 kcal/Å mol. Of them, those conformers whose energy level was less than 25 kJ/mol above the global minimum-energy conformation were chosen. To eliminate possible duplicate conformations, a comparison was performed on the heavy atoms only. The ROE relationships around Trp^8 (Trp^8-H7 - Gly^4-Hα: strong, Trp^8-H4 - Trp^8-Hβ: medium) suggest that the indole ring is not allowed to rotate. Therefore,

of the possible conformations, those having the distance above 2.5 Å between Trp^8-H7 - Gly^4-Hα and the distance above 3.5 Å between Trp^8-H4 - Trp^8-Hβ were discarded. Accordingly, 3 conformers for the *R* isomer and 5 conformers for the *S* isomer, whose energy levels were within a range of 1 kcal/mol from the each global minimum were obtained. All these structures possess similar 3-dimentional arrangements of the backbone ring skeleton (mean RMSD: 0.639 Å for the *R* conformers; 0.833 Å for the *S* conformers).

Comparison of the dihedral angles in each global minimum of the above conformers with those estimated from the vicinal NH-CαH coupling constants by the Karplus-type equation proposed by Bystrov et al.,[15] is shown in Table 2. Thus, the φ angles of *R* isomer agree fairly well with those calculated from the coupling constants, however, those of the *S* isomer did nor.

Table 2. Backbone dihedral angles (φ) in global minima of lyciumin A (**1**) calculated by MD/MM simulations and compound **2** calculated by MC simulations, and those calculated by vicinal NH-CαH coupling constants (Hz).

residues	Hz	φ angle(°)[a]	*R* isomer (φ)[b]		*S* isomer (φ)[b]	
			1	**2**	**1**	**2**
Val^5	6.9	-159, **-81**, 33, 87	-89	-73	-68	-55
Gly^6	5.7	-63, -12, **132**, 183	142	137	170	157
Ser^7	6.6	**-161**, -79, 31, 89	-158	-158	-160	-159
Trp^8	7.8	-155, **-85**, 39, 81	-104	-104	-175	-171

The calculated φ angles shown by bold letters are close to those calculated for *R* isomer
[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 $\Sigma^3JHN\alpha 2=6.0\cos^2\phi-1.5\cos\phi+12.5\sin^2\phi$ for Gly residue.
[b] dihedral angles calculated from each stable conformer of **1** and **2**

In the next step, MD simulations were performed from each of the above lowest energy backbones of the two possible stereoisomers of **1** with a side chain, PyroGlu-Pro-Tyr. A preliminary survey using ROE relationships (Table 1) into the conformation of **1** was made to understand the conformational properties. The distance constraints derived from the volume of the cross peaks in ROESY spectrum were classified into three ranges, 1.8 - 2.5 Å, 1.8 - 3.5 Å and 1.8 - 5.0 Å, corresponding to strong, medium and weak ROEs, respectively (Table 1). No hydrogen bonding and dihedral angle restraints were taken into consideration. Each system was equilibrated for a duration of 200 ps with a thermal bath at 500K. SHAKE[16] was used for the bonds to hydrogen constrained. The 2000 sampled conformers were finally minimized by the molecular mechanics calculation of AMBER* force field with GB/SA solvation treatment. Comparison of non hydrogen-atom structures was performed to eliminate possible duplicate conformations again and the maximum atomic deviation allowed for identical conformation was set to 0.25 Å. In each MD calculation, 5 conformers for the *R* isomer (having *R* configuration at Gly^4-Hα) within a range of 1 kcal/mol appeared 31 times, whereas 5 conformers of *S* isomer 60 times (mean RMSD: 0.266 Å for the heavy atoms of *R* conformers; 0.803 Å for those of *S* conformers). The global minimum structure of *R* isomer was shown in Fig. 3. The total energy of the lowest energy conformer of *R* isomer was -662.9 kJ/mol, which was considerably lower than that of *S* isomer (-571.7 kJ/mol). In addition, the conformer of *R* isomer completely satisfied the distance constraints derived from the characteristic ROE relationship, whereas, in that of *S* isomer, some ROE violations from the used distance constraints were relatively large. For example, Gly^4-NH - Val^5-NH: 0.271, Gly^6-NH - Gly^6-Hα:

0.321, Ser^7-NH - Trp^8-H2: 0.110, Trp^8-Hα - Trp^8-H2: 1.073, and Val^5-NH - Trp^8-H2: 0.030 Å. From the foregoing evidences, the configuration of the C-N linkage at Gly^4-Cα was concluded to be *R* configuration, which agree with the solution structure of **1** derived from the NMR data.

The global minimum structure of *R* isomer of lyciumin A obtained from the combination of MC and MD conformational search with AMBER* force field gives the best agreement with that expected from the ROE relationship. The global minimum structure (*R* isomer) of **1** contains a β-turn structure between Val^5 and Gly^6 residues, suggested by the ROE enhancements around the two residues. Hydrogen bond between Ser^7-NH and Gly^4-CO was not detected and the distance between Ser^7-NH and Trp^8-H2 (3.557 Å) explains the presence of ROE enhancement between Ser^7-NH and Trp^8-H2. The absence of ROEs between the peptide backbone and Tyr^3-Hβ and the coupling constants of 6.6 and 7.5 Hz between Tyr^3-Hα and Tyr^3-Hβ suggest that the aromatic side chain of Tyr^3 has some rotational mobility in solution.

Temperature effect on amide protons

For the conformational determination of peptides in solution by NMR, it is essential to determine whether NH protons are exposed to the solvent or shielded from the solvent sterically or through hydrogen bonding. The temperature dependencies[17] of amide protons in pyridine-d_5 and DMSO-d_6 indicated no strong intramolecular hydrogen bonds as shown in Table 3. The conformation of *R* isomer does not include strong hydrogen bonds. The amide proton of Ser^7 gave a relatively small temperature coefficient in DMSO-d_6, suggesting that it is shielded from the solvent sterically, because Ser^7-NH is situated inside the 17-membered backbone ring.

Table 3 Temperature coefficients ($-d\delta/dT \times 10^3$ ppm/K) of NH chemical shifts of lyciumin A in DMSO-d_6 and pyridine-d_5.[a]

	PyroGlu[1]	Tyr^3	Gly^4	Val^5	Gly^6	Ser^7	Trp^8
DMSO-d_6	5.8	4.8	4.4	5.3	4.3	2.9	4.8
pyridine-d_5	15.1	12.0	16.4	10.8	9.5	9.0	11.0

[a] Measurement made at 5 °C intervals over the range 300 - 330 K.

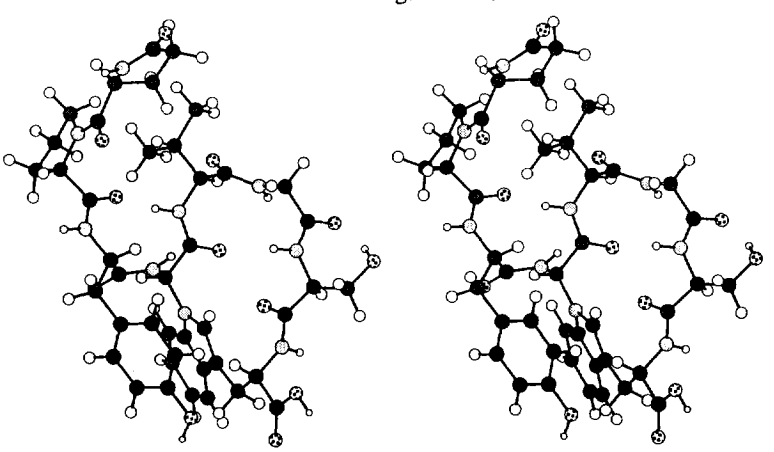

Fig. 3. Stereoscopic view of the global minimum structure of *R* isomer of lyciumin A (**1**)

Conclusion

MC and MD calculation of lyciumin A showed that the *R* isomer of **1**, having a considerably lower energy than the *S* isomer, satisfies the NMR data in pyridine-d5, including the ROE enhancements, dihedral angles derived from $^3J_{NH\text{-}C\alpha}$ coupling constants, and temperature dependences. Further studies on the relations between the solution conformation of lyciumin A and its biological activities are now in progress.

Experimental

Isolation

Cortex (5 kg) of *Lycium chinense* MILL. was exracted with MeOH and from the MeOH extract, lyciumin A was isolated according to the method described previously[4] by Nohara et al. About 1.0 g of pure lyciumin A was obtained as colorless powder, whose spectroscopic data were completely identical with those reported in the literature.[4]

NMR

^1H and ^{13}C-NMR spectra were recorded on JEOL α600, Bruker AM500 and Varian Unity 400 spectrometers. 10 mg of lyciumin A in a 5mm tube (0.5ml pyridine-d5 or DMSO-d6, degassed) was used for the homonuclear and heteronuclear measurements. The spectra were recorded at 300K. A phase sensitive ROESY experiment was made with a mixing time of 200 msec. Temperature effect on NH chemical shifts were measured to assess the solvent accessibilities to the amide protons at 5°C intervals, over the range of 300 - 330 K, by using a linear regression analysis. ^{13}C-NMR assignments in pyridine-d5, PyroGlu[1]: 55.60 (α), 25.06 (β), 30.01 (γ), 178.61 (δ), 171.89 (C=O); Pro[2]: 60.51 (α), 28.71 (β), 25.28 (γ), 46.90 (δ), 173.09 (C=O); Tyr[3]: 56.68 (α), 36.75 (β), 127.12 (γ), 130.72 (δ), 115.94 (ε), 157.55 (ζ), 172.76 (C=O); Gly[4]: 61.96 (α), 168.32 (C=O); Val[5]: 61.58 (α), 29.31 (β), 19.75 (γ), 20.03 (γ), 172.72 (C=O); Gly[6]: 44.11 (α), 170.64 (C=O); Ser[7]: 57.01 (α), 62.77 (β), 170.99 (C=O); Trp[8]: 53.18 (α), 27.39 (β), 125.44 (2), 112.55 (3), 119.23 (4), 119.78 (5), 122.24 (6), 110.06 (7), 137.35 (8), 128.91 (9), 174.82 (C=O).

Computational Methods

Computer modeling experiments were carried out by using the MACROMODEL program (version 4.5) on an IRIS 4D computer (Indy R4600 and Power CHALLENGE M R8000). Molecular mechanics and dynamics calculations were performed with the Amber force field* with a distance-dependent dielectric, $\varepsilon=R_{ij}$. The extended cutoff distances employed were 8 Å for van der Waals, 20 Å for charge/electrostatics and 10 Å for charge/multipole electrostatics. Constrained minimizations and dynamics were calculated with an extra harmonic term of the form $k(\theta-\theta_0)^2$ added to the force field (k=100). The obtained structures were energy minimized by the use of the derivative convergence criteria at a value of 0.001 kJ/Å-mol and GB/SA solvation treatment.

[Pseudo Monte Carlo calculation]

Each MC search was carried out by using the Pseudo Monte Carlo routine in MACROMODEL. The closure bond was chosen at C1-C2 with a closure limit of 1 - 3 Å. A total of 141 starting point structures for *R* isomer and 73 structures for *S* isomer, which were obtained within 25 kJ/mol of the lowest energy conformer, were minimized by the use of molecular mechanics calculation of AMBER* all-atom force field with GB/SA solvation method.

[Molecular Dynamics calculation]

Each MD at 500 K with 1.0-fs time steps for a total of 200 ps were calculated with $\varepsilon=R_{ij}$. Structures were sampled by time at 0.1-ps intervals. The sampled structures derived from the dynamics trajectories were then energy minimized with the AMBER* all-atom force field..

Acknowledgments: The authors thank the Ministry of Education, Science and Culture, Japan, for financial support through Grant-in-Aid for General Scientific Research.

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(*Received in Japan* 18 *October* 1995; *accepted* 12 *December* 1995)