Cycloleonuripeptides E and F, Cyclic Nonapeptides from *Leonurus heterophyllus*

Hiroshi Morita,*,† Toru Iizuka,† Akira Gonda,‡ Hideji Itokawa,‡ and Koichi Takeya‡

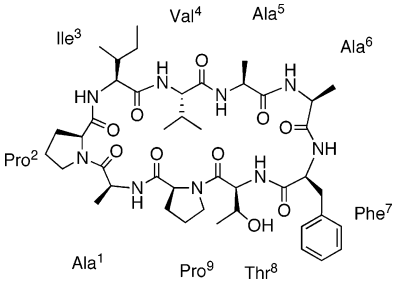
Faculty of Pharmaceutical Sciences, Hoshi University, Ebara 2-4-41 Shinagawa-ku, Tokyo 142-8501, Japan, and School of Pharmacy, Tokyo University of Pharmacy & Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan

Received December 24, 2005

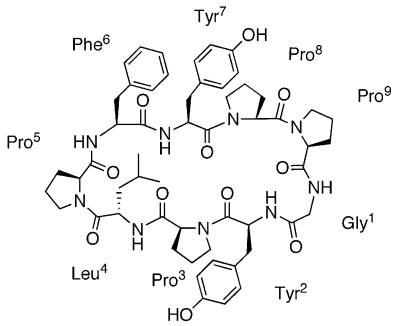
Two new cyclic nonapeptides, cycloleonuripeptide E, *cyclo* (-Ala-Pro-Ile-Val-Ala-Ala-Phe-Thr-Pro-), and cycloleonuripeptide F, *cyclo* (-Gly-Tyr-Pro-Leu-Pro-Phe-Tyr-Pro-Pro-), have been isolated from the fruits of *Leonurus heterophyllus*, and their structures were elucidated by 2D NMR analysis and chemical degradation. Cycloleonuripeptides E (**1**) and F (**2**) showed moderate vasorelaxant effects on rat aorta.

Cyclic peptides exhibit a range of biological activities.[1,2] Their cyclic nature often provides more lipophilicity and membrane permeability, because of reduced zwitterionic character. Furthermore, restricted bond rotation results in rigid backbone conformation with more affinity and selectivity for binding proteins.

In our search for structurally unique and biologically interesting peptides from higher plants, we previously isolated four cyclic peptides, cycloleonuripeptides A−D,[3−5] from the fruits of *Leonurus heterophyllus* (Labiatae), which have been used as a Chinese drug for invigorating blood circulation, for regulating menstrual disturbance, and to dispel edema,[6] By further fractionation of the fruit extract, we obtained two new cyclic nonapeptides, cycloleonuripeptides E (**1**) and F (**2**), which showed vasorelaxant effects on rat aorta. In this paper, we describe the isolation and structure elucidation of **1** and **2**.



1



2

Cycloleonuripeptide E (**1**), colorless solid, $[\alpha]^{20}_{D}$ −177 (*c* 0.4, MeOH), showed the molecular formula $C_{43}H_{65}N_9O_{10}$, which was determined by HRFABMS, indicating 16 degrees of unsaturation in the molecule. The IR absorption bands were characteristic of amino (3322 cm^{-1}) and amide carbonyl (1647 cm^{-1}) groups. Amino acid analysis of **1** showed it to consist of Ala × 3, Pro × 2, Ile, Val, Phe, and Thr, all of which were proved to be L-amino acids by Marfey's derivatization, followed by HPLC analysis.[7] In the NMR spectra, seven amide proton signals and nine amide carbonyl signals corresponding to the above nine amino acids were observed. Because the two-proline-containing nonapeptide structure with one phenylalanine residue satisfies the 15 degrees of unsaturation, the remaining unsaturation is explained by a cyclic structure. Complete assignments for the ^1H and ^{13}C NMR signals in pyridine-d_5 were accomplished using a combination of 2D NMR experiments, such as $^1H-^1H$ COSY, HOHAHA, HMQC, and HMBC spectra (Table 1).

The sequencing of **1** was analyzed by correlations observed in the NOESY spectrum. The NOESY correlations Ala[1]-NH/Pro[2]-Hδ, Pro[2]-Hα/Ile[3]-NH, Ile[3]-Hα/Val[4]-NH, Val[4]-Hα/Ala[5]-NH, Ala[5]-Hα/Ala[6]-NH, Ala[6]-Hα/Phe[7]-NH, Phe[7]-Hβ/Thr[8]-NH, Thr[8]-Hα/Pro[9]-Hδ, and Pro[9]-Hα/Ala[1]-NH elucidated the nine amino acid sequence in **1** to be -Ala-Pro-Ile-Val-Ala-Ala-Phe-Thr-Pro-, and the structure was determined to be *cyclo* (-Ala-Pro-Ile-Val-Ala-Ala-Phe-Thr-Pro-).

Cycloleonuripeptide F (**2**), colorless solid, $[\alpha]^{20}_{D}$ −129 (*c* 0.6, MeOH), showed the molecular formula $C_{55}H_{69}N_9O_{11}$ by HR-FABMS, indicating 26 degrees of unsaturation in the molecule. IR absorptions at 3310 and 1650 cm^{-1} were attributed to amino and amide carbonyl groups, respectively. Amino acid analysis of **2** showed that it consisted of Tyr × 2, Pro × 4, Gly, Leu, and Phe, all of which were proved to be L-amino acids by Marfey's derivatization, followed by HPLC analysis.[7]

In the NMR spectra (pyridine-d_5), two sets of signals ascribable to two conformational isomers were observed. This phenomena could result from rotation of the proline amide bond with a rotation rate slow enough to give separate signals in the NMR. The sequence analysis of **2** was carried out using DMSO-d_6, because a single stable conformer was observed in this solvent. The assignments of ^1H and ^{13}C NMR signals of **2** were made using a combination of $^1H-^1H$ COSY, HMQC, and HMBC spectra (Table 2).

Two partial segments, Pro-Pro-Gly-Tyr and Pro-Leu-Pro-Phe, were assigned by HMBC correlations as follows: Hβ (Pro[8])/CO (Pro[8]), Hα (Pro[9])/CO (Pro[8]), Hα (Pro[9])/CO (Pro[9]), NH (Gly[1])/CO (Pro[9]), Hα (Gly[1])/CO (Gly[1]), NH (Tyr[2])/CO (Gly[1]), Hα (Pro[3])/CO (Pro[3]), NH (Leu[4])/CO (Pro[3]), Hα (Leu[4])/CO (Leu[4]), Hα (Pro[5])/CO (Leu[4]), Hα (Pro[5])/CO (Pro[5]), NH (Phe[6])/CO (Pro[5]), Hα (Phe[6])/CO (Phe[6]), and NH (Tyr[7])/CO (Phe[6]). Two structural units (-Pro-Leu-Pro-Phe-Tyr- and -Pro-Pro-Gly-Tyr-) analyzed by the HMBC correlations could be combined by a NOESY correlation between Tyr[2]-Hα and Pro[3]-Hα in the NOESY spectrum. Another NOESY correlation between Pro[8]-Hα and Pro[9]-Hα was indicative of a *cis* peptide bond between Pro[8] and Pro[9]. Thus, the structure of **2** was determined to be *cyclo* (-Gly-Tyr-Pro-Leu-Pro-Phe-Tyr-Pro-Pro-).

* To whom correspondence should be addressed. Tel: (03)5498-5778. Fax: (03)5498-5778. E-mail: moritah@hoshi.ac.jp.
† Hoshi University.
‡ Tokyo University of Pharmacy & Life Sciences.

10.1021/np050544k CCC: $33.50
Published on Web 04/27/2006

Table 1. ^{1}H and ^{13}C NMR Data of Cycloleonuripeptide E (**1**) in Pyridine-d_5[a]

assignment		δ_H (int. mult, J(Hz))	δ_C			δ_H	δ_C
Ala[1]				Ala[6]			
	α	5.13 (1H, m)	46.5		α	4.67 (1H, dd, 3.6, 7.2)	51.3
	β	1.45 (3H, d, 6.7)	17.6		*β*	1.38 (3H, d, 7.2)	17.1
	NH	8.12 (1H, m)			NH	10.07 (1H, br s)	
	C═O		172.5		C═O		173.5
Pro[2]				Phe[7]			
	α	5.17 (1H, m)	62.4		α	4.57 (1H, m)	57.6
	β	2.11 (1H, m)	29.6		*β*	3.93 (2H, m)	34.7
		2.40 (1H, m)			*γ*		140.2
	γ	1.89 (1H, m)	25.5		*δ*	7.28 (2H, d, 7.6)	128.7
		1.94 (1H, m)			*ϵ*	7.33 (2H, m)	129.9
	δ	3.70 (1H, m)	48.5		*ζ*	7.22 (1H, m)	126.6
		4.06 (1H, m)			NH	9.71 (1H, br d, 6.5)	
	C═O		172.7		C═O		170.7
Ile[3]				Thr[8]			
	α	4.60 (1H, t, 6.8)	60.3				
	β	1.97 (1H, m)	36.0		α	5.41 (1H, dd, 3.0, 9.1)	58.1
	γ	1.26 and 1.68 (1H, m)	26.5		*β*	4.56 (1H, m)	68.5
	γ-Me	1.01 (3H, d, 6.8)	16.1		*γ*	1.56 (3H, d, 6.4)	19.4
	δ-Me	0.69 (3H, t, 7.4)	11.2		OH	5.98 (1H, br d)	
	NH	7.98 (1H, m)			NH	7.96 (1H, m)	
	C═O		171.7		C═O		170.9
Val[4]				Pro[9]			
	α	4.81 (1H, m)	60.8				
	β	2.54 (1H, m)	31.0		α	4.84 (1H, m)	61.3
	γ	1.12 (3H, d, 6.7)	19.9		*β*	2.03 (1H, m)	29.5
		1.14 (3H, d, 6.7)	19.9			2.35 (1H, m)	
	NH	8.15 (1H, m)			*γ*	1.10 (1H, m)	25.6
	C═O		171.7			1.76 (1H, m)	
Ala[5]					*δ*	3.53 (1H, m)	48.6
	α	5.06 (1H, m)	49.6			3.64 (1H, m)	
	β	1.59 (3H, d, 6.6)	19.4		C═O		170.4
	NH	8.25 (1H, d, 6.6)					
	C═O		173.9				

[a] δ in ppm.

Vasorelaxant effects of cycloleonuripeptides E (**1**) and F (**2**) were examined by observing contractions of isolated rat thoracic aorta. Relaxant effects (**1**, 32%; **2**, 40%) on the aorta precontracted with 3×10^{-7} M norepinephrine (NE) were moderately induced by both cycloleonuripeptides E (**1**, 10^{-4} M) and F (**2**, 10^{-4} M). The same relaxant effects were shown on aorta rings without endothelium.

Experimental Section

General Experimental Procedures. IR and UV spectra were recorded on a JASCO A-302 spectrometer and Hitachi 557 spectrophotometers, respectively. Optical rotation was measured with a JASCO DIP-4 spectrometer. FAB and high-resolution mass spectra were taken with a VG Autospec spectrometer. High-pressure liquid chromatography (HPLC) was performed with an Inertsil PREP-ODS column (20 mm i.d × 250 mm and 30 mm i.d. × 250 mm, GL Science Inc.) packed with 10 μm ODS. ^{1}H and 2D NMR spectra in pyridine-d_5 and DMSO-d_6 were recorded on a 500 MHz spectrometer at 300 K, while ^{13}C NMR spectra were measured on a 125 MHz spectrometer. Chemical shifts were reported using residual pyridine (δ_H 7.21 and δ_C 135.5) or DMSO (δ_H 2.50 and δ_C 39.5) as internal standards. Standard pulse sequences were employed for the 2D NMR experiments. ^{1}H−^{1}H COSY, HOHAHA, and NOESY spectra were measured with spectral widths of both dimensions of 4800 Hz, and 32 scans with two dummy scans were accumulated into 1K data points for each of 256 t_1 increments. NOESY and HOHAHA spectra in the phase-sensitive mode were measured with a mixing time of 800 and 30 ms, respectively. For HMQC spectra in the phase-sensitive mode and HMBC spectra, a total of 256 increments of 1K data points were collected. For HMBC spectra with the *Z*-axis PFG, a 50 ms delay time was used for long-range C−H coupling. Zero-filling to 1K for F_1 and multiplication with squared cosine-bell windows shifted in both dimensions were performed prior to 2D Fourier transformation. FAB and high-resolution mass spectra were recorded on a VG Autospec instrument by using a glycerol matrix. Amino acid analysis was carried out using a Hitachi L-8500 amino acid analyzer. TLC was conducted on precoated Kieselgel 60 F_{254} (Art. 5715; Merck), and the spots were detected by spraying with Dragendorff's reagent.

Plant Material. The fruits of *Leonurus heterophyllus* were purchased from the Shanghai Corporation of Chinese Traditional Drugs, People's Republic of China, in August 1995. The botanical identification was made by Dr. Zhi-Sheng Qiao, Department of Pharmacognosy, College of Pharmacy, Second Military Medical University, Shanghai, China. A voucher specimen has been deposited in the herbarium of Tokyo University of Pharmacy & Life Sciences.

Extraction and Isolation. After the fruits of *L. heterophyllus* (10 kg) were defatted with *n*-hexane two times, they were extracted with hot 80% MeOH two times to give a MeOH extract (1.6 kg), which was treated with *n*-BuOH and H_2O. The *n*-BuOH-soluble fraction (1.0 kg) was subjected to Diaion HP-20 column chromatography using a H_2O−MeOH gradient system (1:0 → 0:1). The fractions (100 g) eluted by 80% MeOH were further subjected to silica gel column chromatography using a CH_2Cl_2−MeOH gradient system (1:0 → 0:1). The fraction (10 g) eluted by 10% MeOH was subjected to ODS MPLC with 68% MeOH solvent system and ODS HPLC (Inertsil PREP-ODS, 10 μm ODS, 20 × 250 mm; flow rate 8.0 mL/min; UV detection at 210 nm; eluent, 59% MeOH and 32% CH_3CN) to give cycloleonuripeptide E (**1**, 13 mg, 0.00013%) and cycloleonuripeptide F (**2**, 71 mg, 0.00071%) as colorless solids.

Cycloleonuripeptide E (1): colorless solid; $[\alpha]^{20}_D$ −177 (*c* 0.4, MeOH); UV (MeOH) λ_{max} 258 (ϵ 1500); IR (KBr) ν_{max} 3322 (NH) and 1647 (amide C═O) cm^{-1}; ^{1}H and ^{13}C NMR (Table 1); FABMS *m*/*z* 868 $(M + H)^+$; HRFABMS *m*/*z* 868.4910 $(M + H)^+$, calcd for $C_{43}H_{66}N_9O_{10}$ 868.4933.

Cycloleonuripeptide F (2): colorless solid; $[\alpha]^{20}_D$ −129 (*c* 0.6, MeOH); UV (MeOH) λ_{max} 280 nm (ϵ 3500); IR (KBr) ν_{max} 3310 (NH) and 1650 (amide C═O) cm^{-1}; ^{1}H and ^{13}C NMR (Table 2); FABMS *m*/*z* 1032 $(M + H)^+$; HRFABMS *m*/*z* 1032.5216 $(M + H)^+$, calcd for $C_{55}H_{70}N_9O_{11}$ 1032.5195.

Acid Hydrolysis of 1 and 2. Cycloleonuripeptides E and F (each 1 mg) were hydrolyzed in 1 mL of 6 N HCl in a sealed vial at 110 °C for 24 h. After cooling, each solution was concentrated to dryness under reduced pressure, and the residue was dissolved in 0.02 N HCl. Amino

Table 2. ^{1}H and ^{13}C NMR Data of Cycloleonuripeptide F (**2**) in DMSO-d_6[a]

assignment		δ_H (int. mult, J(Hz))	δ_C			δ_H	δ_C
Gly[1]				Phe[6]			
	α	3.39 (1H, m)	40.9		α	4.83 (1H, m)	51.0
	β	4.52 (1H, m)			*β*	2.68 (1H, dd, 5.0, 13.7)	38.9
	NH	8.06 (1H, dd, 5.1, 7.7)				2.98 (1H, dd, 5.4, 13.7)	
	C=O		168.4		*γ*		136.0
Tyr[2]					*δ*	7.13 (2H, d, 7.0)	130.1
	α	4.09 (1H, br dd, 4.0, 9.7)	54.2		*ϵ*	7.23 (2H, t, 7.0)	127.6
	β	2.80 (2H, m)	36.7		*ζ*	7.17 (1H, t, 7.0)	126.4
	γ		125.6[b]		NH	6.27 (1H, d, 8.2)	
	δ	6.89 (2H, d, 8.5)	130.2		C=O		170.1
	ϵ	6.68 (2H, d, 8.5)	115.4	Tyr[7]			
	ζ		156.6		α	4.49 (1H, m)	51.1
	NH	8.24 (1H, s)			*β*	2.55 (1H, dd, 3.7, 12.3)	39.8
	C=O		170.0			2.77 (1H, m)	
Pro[3]					*γ*		125.6[b]
	α	2.96 (1H, d, 8.1)	59.8		*δ*	6.82 (2H, d, 8.3)	129.8
	β	1.23 (1H, m)	30.7		*ϵ*	6.65 (2H, d, 8.3)	115.0
		1.87 (1H, m)			*ζ*		156.3
	γ	1.30 (1H, m)	21.9		NH	9.07 (1H, d, 9.5)	
		1.65 (1H, m)			C=O		168.9
	δ	3.10−3.50 (2H, m)	46.4[c]				
	C=O		171.0	Pro[8]			
Leu[4]					α	3.48 (1H, m)	58.6
	α	4.24 (1H, m)	49.7		*β*	1.66 (2H, m)	29.8
	β	0.80 (1H, m)	35.9		*γ*	1.63 (1H, m)	23.9
		1.71 (1H, m)				1.85 (1H, m)	
	γ	1.57 (1H, m)	25.8		*δ*	3.10−3.50 (2H, m)	46.2[c]
	δ	0.81 (3H, d, 6.7)	21.2		C=O		169.6
		0.84 (3H, d, 6.7)	22.1				
	NH	8.57 (1H, d, 8.2)		Pro[9]			
	C=O		172.1		α	4.13 (1H, d, 7.6)	60.3
Pro[5]					*β*	2.09 (2H, m)	31.7
	α	4.29 (1H, dd, 1.6, 8.3)	59.9		*γ*	1.82 (1H, m)	21.4
	β	1.79 (1H, m)	28.6			1.97 (1H, m)	
		2.00 (1H, m)			*δ*	3.10−3.50 (2H, m)	45.9[c]
	γ	1.49 (1H, m)	21.9		C=O		170.7
		1.65 (1H, m)					
	δ	3.10−3.50 (2H, m)	46.2[c]				
	C=O		169.0				

[a] δ in ppm. [b,c] Assignment may be interchanged.

acids were determined by ion-exchange resin chromatography on a Hitachi L-8500 amino acid analyzer with ninhydrin detection. Absolute configuration of amino acids was analyzed by Marfey's method.[7]

Vasodilator Assay.[8] A male Wistar rat weighting 230 g was sacrificed by bleeding from carotid arteries under anesthetization. A section of the thoracic aorta between the aortic arch and the diaphragm was removed and placed in oxygenated, modified Krebs-Henseleit solution (KHS: 118.0 mM NaCl, 4.7 mM KCl, 25.0 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM $MgSO_4$, and 11.0 mM glucose). The aorta was cleaned of loosely adhering fat and connective tissue and cut into ring preparations 3 mm in length. The tissue was placed in a well-oxygenated (95% O_2, 5% CO_2) bath of 10 mL of KHS solution at 37 °C with one end connected to a tissue holder and the other to a force-displacement transducer (Nihon Kohden, TB-611T). The tissue was equilibrated for 60 min under a resting tension of 1.0 g. During this time the KHS in the tissue bath was replaced every 20 min.

After equilibration, each aortic ring was contracted by treatment with 3×10^{-7} M norepinephrine (NE). The presence of functional endothelial cells was confirmed by demonstrating relaxation to 10^{-5} M acetylcholine (Ach), and aortic rings, in which 80% relaxation occurred, were used as tissues with endothelium. When the NE-induced contraction reached a plateau, test compounds were added.

Animal experimental studies were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, and under the supervision of the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Science, Sports Culture, and Technology of Japan.

Acknowledgment. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and a grant from Tokyo Crude Drugs Association.

References and Notes

(1) Itokawa, H.; Takeya, K.; Hitotsuyanagi, Y.; Morita, H. Macrocyclic Peptide Alkaloids from Plants. *The Alkaloids* **1997**, *49*, 301−387.
(2) Fusetani, N.; Matsunaga, S. *Chem. Rev.* **1993**, *93*, 1793−1806, and references therein.
(3) Morita, H.; Gonda, A.; Takeya, K.; Itokawa, H. *BioMed. Chem. Lett.* **1996**, *6*, 767−770.
(4) Morita, H.; Gonda, A.; Takeya, K.; Itokawa, H.; Iitaka, Y. *Tetrahedron* **1997**, *53*, 1617−1626.
(5) Morita, H.; Gonda, A.; Takeya, K.; Itokawa, H.; Shirota, O. *Chem. Pharm. Bull.* **1997**, *45*, 161−164.
(6) *The Pharmacology of Chinese Herbs*; Huang, K. C., Ed.; CRC Press, Inc.: Boca Raton, 1993; p 249.
(7) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591−596.
(8) Nagai, M.; Noguchi, M.; Iizuka, T.; Otani, K.; Kamata, K. *Biol. Pharm. Bull.* **1996**, *19*, 228−232.

NP050544K