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NEW CYCLIC PEPTIDES FROM *CITRUS AURANTIUM*

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Abstract — Two new cyclic peptides were isolated from the fruit peels of *Citrus aurantium*. Their structures were elucidated as *cyclo* (-Gly-Leu-Val-Leu-Pro-Ser-) and *cyclo* (-Gly-Gly-Leu-Leu-Leu-Pro-Pro-Phe-) by intensive 2D NMR analysis and chemical evidence.

Cyclic peptides with unique structures, such as kapakahines,[1] microsclerodermins,[2] have been isolated from marine organisms and cyclomarins,[3] methylsulfomycin[4] from micro organisms, and hibispeptin A[5] and mahafacyclin A[6] from higher plants. These natural products exhibit a wide range of biological activities such as antibiotic, anti-inflammatory and cytotoxicity. As part of our continuing study of cyclic peptides from higher plants,[7-16] we examined fruit peels of *Citrus aurantium* (Rutaceae) which are listed in Japanese Pharmacopoeia XIV and used for aromatic stomachic. From rutaceous plants, the cyclic peptides evolidine[17] and citrusins[18] have been isolated. In the present study, we isolated two new cyclic peptides and determined their structures as *cyclo* (-Gly-Leu-Val-Leu-Pro-Ser-) and *cyclo* (-Gly-Gly-Leu-Leu-Leu-Pro-Pro-Phe-).

Results and Discussion

Silica gel column chromatography of the $CHCl_3$ soluble fraction of a 50% MeOH extract of the fruit peels of *C. aurantium*, followed by HPLC on ODS, yielded peptidic compounds (**1**) and (**2**).

Compound (**1**), colorless powder, had a molecular formula $C_{27}H_{47}N_6O_7$ as revealed by the HRFABMS quasimolecular ion peak at *m/z* 567.3483 $[M+H]^+$. The IR spectral absorptions at 3308 and 1652 cm^{-1} were assigned to an amine and amide carbonyl groups, respectively. In the ^1H NMR spectrum in pyridine-d_5, most of the signals were quite broad suggesting the presence of two conformers, whereas in CD_3OD, **1** gave well resolved sharp signals indicating the presence of a minor conformer, when the NMR spectral data were recorded in CD_3OD. The conformer ratio of major to minor in CD_3OD was 77: 23 from proton signal integration. Determination of component amino acids and the complete amino acid sequence for **1** was performed by 2D NMR spectroscopy. Assignment of ^1H NMR chemical shifts to specific protons in individual residues was obtained by a 2D homonuclear COSY experiment to show the

Table 1. ^{1}H and ^{13}C NMR Signal Assignments for Conformers of Compound (**1**) in CD_3OD at 300 K

assignment		Conformer I δ_H [int, mult, J(Hz)]	Conformer II δ_H [int, mult, J(Hz)]	Conformer I δ_C	Conformer II δ_C
Gly[1]	α	3.74 (1H, d, 15.4)	3.72-3.76 (1H, m)	44.94	43.96
		3.94 (1H, d, 15.2)	4.29 (1H, d, 16.5)		
	CO			172.20[*1]	171.65
Leu[2]	α	4.18 (1H, m)	4.25-4.31 (1H, m)	56.00	56.00
	β	1.61 (2H, m)	1.62-1.66 (1H, m)	42.03	41.78
			1.70-1.75 (1H, m)		
	γ	1.72 (1H, m)	1.67-1.77 (1H, m)	26.58	26.50
	δ	0.91 (3H, d, 6.5)	0.93-0.96 (3H, m)	22.35	22.57
		0.97 (3H, m)	0.97-0.99 (3H, m)	23.73	23.55
	CO			175.54	175.46
Val[3]	α	4.19 (1H, m)	4.14 (1H, d, 7.6)	59.17	62.32
	β	2.02 (1H, m)	2.32 (1H, m)	32.84	32.09
	γ	0.89 (3H, d, 6.7)	0.92-0.93 (3H, m)	19.20	19.16
		0.99 (3H, m)	0.95-0.96 (3H, m)	20.11	20.39
	CO			174.06	173.77
Leu[4]	α	4.24 (1H, m)	4.64 (1H, t, 6.6)	53.63	51.80
	β	1.36 (1H, ddd, 3.0, 10.0, 13.6)	1.57-1.62 (2H, m)	40.52	41.50
		1.68 (1H, m)			
	γ	1.77 (1H, m)	1.67-1.77 (1H, m)	26.36	26.82
	δ	0.95 (3H, d, 6.5)	1.00-1.01 (3H, m)	22.18	21.80
		0.96 (3H, m)	0.92-0.94 (3H, m)	24.36	24.25
	CO			174.41[*2]	173.38
Pro[5]	α	4.44 (1H, d, 7.9)	4.23-4.25 (1H, m)	63.07	64.01
	β	2.20 (1H, m)	1.85-1.90 (1H, m)	33.42	30.78
		2.38 (1H, m)	2.30 (1H, m)		
	γ	1.82 (1H, m)	1.99-2.03 (1H, m)	23.37	27.07
		1.93 (1H, m)	2.11 (1H, m)		
	δ	3.51 (1H, m)	3.57-3.60 (1H, m)	48.50	49.00
		3.59 (1H, m)	3.79 (1H, m)		
	CO			174.34[*2]	175.19
Ser[6]	α	4.66 (1H, t, 5.1)	4.03 (1H, dd, 3.5, 5.4)	57.67	60.08
	β	3.85 (1H, dd, 4.8, 11.3)	3.90 (1H, dd, 3.4, 11.5)	63.78	62.25
		3.94 (1H, m)	4.17-4.20 (1H, m)		
	CO			172.26[*1]	172.20

* : Assignments may be interchanged.

Table 2. ^{1}H and ^{13}C NMR Signal Assignments of Compound (**2**) in CD_3OD at 300 K

assignment		^{1}H NMR δ_H [int, mult, J(Hz)]	^{13}C NMR δ_C
Gly[1]			
	α	3.63 (1H, d, 16.3)	44.57
		4.41 (1H, d, 16.4)	
	CO		173.68
Gly[2]			
	α	3.53 (1H, d, 15.8)	44.14
		4.09 (1H, d, 15.9)	
	CO		172.61
Leu[3]			
	α	4.02 (1H, t, 7.5)	55.66
	β	1.59 (2H, m)	41.79[*1]
	γ	1.59-1.75 (1H, m)	26.39[*2]
	δ	0.89 (3H, d, 6.3)	22.12
		0.94-0.97 (3H, m)	23.86
	CO		176.02
Leu[4]			
	α	4.31 (1H, dd, 4.1, 11.2)	54.35
	β	1.65 (1H, m)	41.60[*1]
		1.82 (1H, m)	
	γ	1.59-1.75 (1H, m)	26.67
	δ	0.89 (3H, d, 6.3)	21.84
		0.94-0.97 (3H, m)	23.98
	CO		175.06
Leu[5]			
	α	4.57 (1H, t, 7.0)	51.72
	β	1.44 (1H, m)	41.87[*1]
		1.72 (1H, m)	
	γ	1.59-1.75 (1H, m)	26.43[*2]
	δ	0.94-0.97 (3H, m)	23.49
		0.94-0.97 (3H, m)	23.58
	CO		171.54
Pro[6]			
	α	4.44 (1H, m)	61.60
	β	1.75 (1H, m)	29.94
		2.23 (1H, m)	
	γ	2.00 (2H, m)	26.19
	δ	3.57 (2H, m)	48.91
	CO		174.13
Pro[7]			
	α	4.37 (1H, m)	62.83
	β	1.68 (1H, m)	33.04
		2.00 (1H, m)	
	γ	0.86 (1H, m)	22.86
		1.53 (1H, m)	
	δ	3.34 (2H, m)	48.51
	CO		175.65
Phe[8]			
	α	4.73 (1H, dd, 3.7, 12.4)	58.19
	β	3.03 (1H, m)	38.43
		3.46 (1H, m)	
	γ		139.21
	δ	7.29 (2H, m)	130.42
	ε	7.31 (2H, m)	130.29
	ζ	7.24 (1H, m)	128.61
	CO		174.40

* : Assignments may be interchanged.

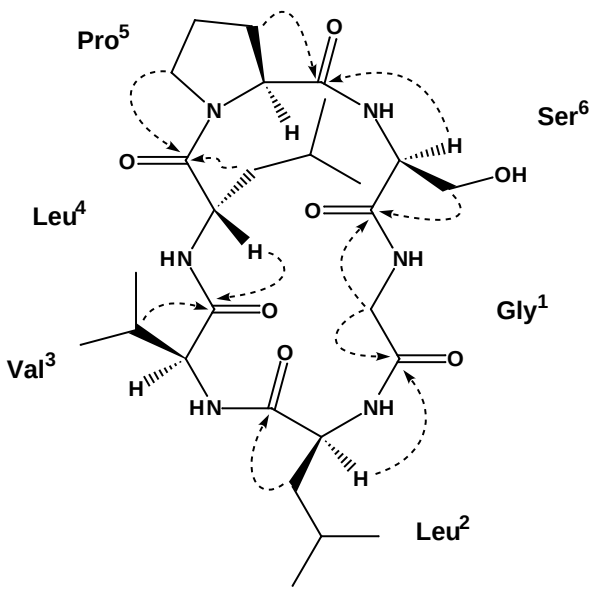
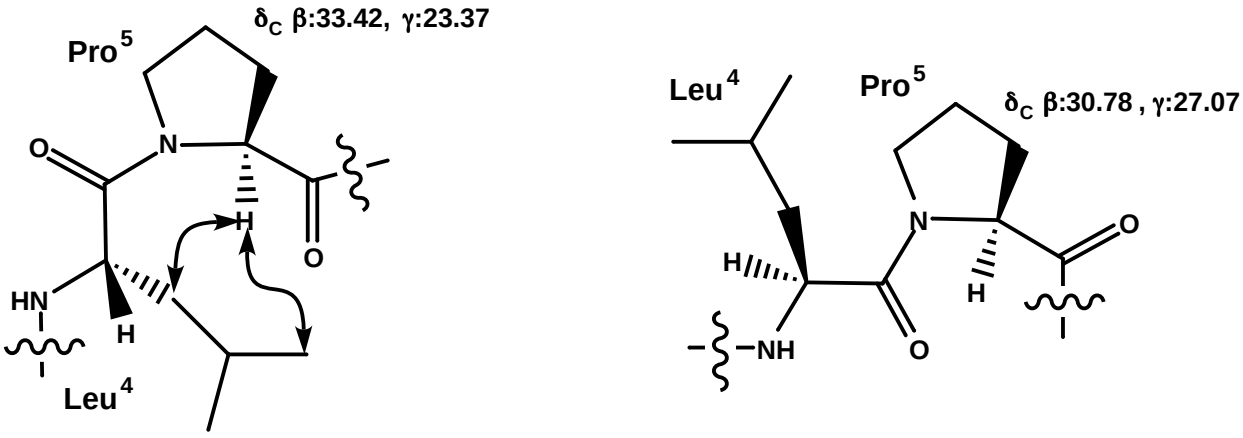


Figure 1. HMBC correlations (dashed arrows) for compound (**1**) in CD_3OD.

complete spin systems of two Leu, one Pro, one Ser, one Gly and one Val (Table 1). The corresponding carbon resonance was determined on the basis of HMQC and HMBC experiments. The HMBC correlation between each amide carbonyl carbon and Hα proton except a correlation between carbonyl carbon of Leu^2 and Hα proton of Val^3 were expected to be due to linear nature of **1** (Figure 1). But the observed MS spectral molecular weight suggested the lack of terminal NH_2 and COOH groups of corresponding linear peptide, that is, the molecular weight was less 18 amu than the expected linear one. The chemical shifts of the two conformers showed shift



Major conformer (77%)

Minor conformer (23%)

Figure 2. NOE correlations (arrows) for partial structures of compound (**1**) in CD_3OD

differences of ± 1.0 ppm in the ^{13}C NMR spectrum with the exception of the proline β and γ carbon signals. The β and γ carbons of the Pro residue in the major conformer were at δ 33.42 (β) and 23.37 (γ), whereas the corresponding signals of the minor conformer were at δ 30.78 (β) and 27.07 (γ). NOE correlations between Hβ and Hδ of Leu^4 and Hα of Pro^5, were clear in the major conformer but absent in the minor conformer (Figure 2). The above results indicate that the major conformer contains a *cis* amide bond[19] between Leu^4 and Pro^5. Thus the structure of compound (**1**) was determined as *cyclo* (-Gly-Leu-Val-Leu-Pro-Ser-). The absolute stereochemistry of all of the component amino acids was determined to be of L-configuration by HPLC analysis.[20]

Compound (**2**) showed a HRFABMS quasimolecular ion peak at *m/z* 795.4751 $[M+H]^+$, corresponding to

the molecular formula, $C_{41}H_{63}N_8O_8$. The IR spectral absorption bands at 3431 and 1647 cm^{-1} were assigned to amino and amide carbonyl groups, respectively. The octapeptide nature of **2** was evident from its ^{13}C NMR spectrum which suggested eight carbonyl groups (Table 2). The ^{1}H and ^{13}C NMR spectral signals for individual amino acids were readily assigned by extensive analysis of ^{1}H-^{1}H COSY and HMQC spectra. The gross structure including the amino acid sequence was determined by linking the individual amino acids according to the phase sensitive NOESY and HMBC experiments. The HMBC correlation analysis revealed two partial sequences, -Leu-Leu-Leu- and -Pro-Pro-Phe-Gly-Gly-. The NOE correlation between Hα of Leu^5 and Hδ of Pro^6 was indicated that two partial sequences from HMBC experiment were connected (Figure 3). Furthermore, the observed MS spectral molecular weight was less 18 amu than expected linear peptide. Thus the structure of compound (**2**) was identified as *cyclo* (-Gly-Gly-Leu-Leu-Leu-Pro-Pro-Phe-). The absolute stereochemistry of each amino acid in **2** was determined to be of L-configuration by HPLC analysis of the derivatives of the acid hydrolyzate by treating with Marfey's reagent. The amide bonds between Leu^5- Pro^6 and Pro^4- Pro^7 residues were determined to be *trans* and *cis*, respectively, on the basis of the strong NOE correlations between Hα in Leu^5 and Hδ in Pro^6, and between Hα in Pro^6 and Hα in Pro^7, as well as the chemical shifts of the β and γ carbons of the Pro^7 residue at δ 33.04 and 22.86.

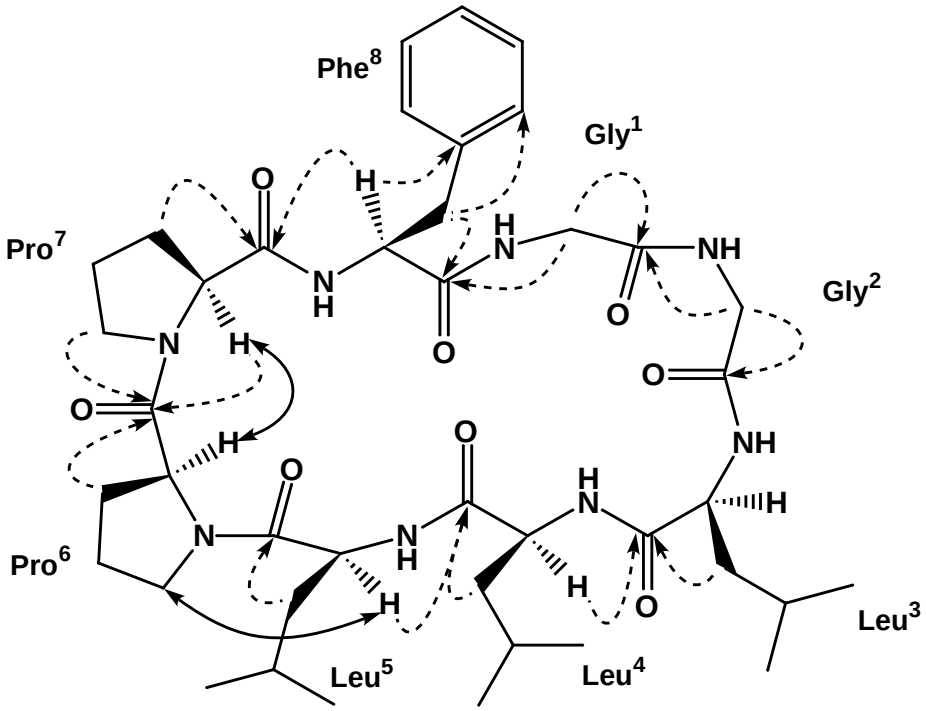


Figure 3. HMBC correlations (dashed arrows) and NOE correlations (arrows) for structure of compound (**2**) in CD_3OD

The cytotoxic and immunosuppressive activities against murine splenocytes of these compounds were examined, but did not indicate positive activity. The IC_{50} values of compounds (**1**) and (**2**) were >50 μg/mL in immunosuppressive activities.

EXPERIMENTAL

General Experimental Procedures. Optical rotations were measured on a JASCO DIP-4 spectrometer and the $[\alpha]_D$ values are given in 10^{-1}deg cm^2 g^{-1}. FABMS measurements were done on a VG Autospec spectrometer. IR and UV spectra were taken on JASCO FT/IR 620 and Hitachi 557 spectrometers, respectively. ^{1}H and ^{13}C NMR spectra were recorded on Bruker DRX-500 spectrometer at 300 K. Chemical shifts (δ) were using residual CD_3OD peaks (δ_H 3.30 and δ_C 49.00) as internal references. Preparative HPLC was carried out on a JASCO HPLC system using an Inertsil PREP-ODS (20 × 250 mm) packed with 10 μm ODS.

Plant Material. The fruit peels of *Citrus aurantium* for this study were collected in the Republic of Haiti. A voucher specimen (No. Tochi-240898) has been deposited in the School of Pharmacy, Tokyo University of Pharmacy & Life Science.

Separation of cyclic peptides. The fruit peels (1 kg) were extracted with 50% aqueous MeOH (3 L x 3) at 50 ºC for 12 h. The MeOH extract was concentrated and the residue (410 g) was partitioned between $CHCl_3$ and H_2O. The $CHCl_3$-soluble fraction gave 13.6 g, which was chromatographed on a silica gel column (ϕ50 × 200 mm) using a $CHCl_3$—AcOEt—MeOH gradient system. The fraction (5.18 g) which eluted with MeOH was subjected to ODS MPLC with 35% aqueous MeOH to give two peptide fractions, each of which was purified by ODS HPLC with MeOH / H_2O (1 / 1) and MeOH / H_2O (13 / 7), respectively, to give compounds (**1**) (110.4 mg, 1.1×10^{-2}%) and (**2**) (13.8 mg, 1.4×10^{-3}%).

Compound (1). Colorless powder, $[\alpha]_D^{23}$ -123.6º (*c* 0.23, MeOH); HRFABMS *m/z* 567.3483 $[M+H]^+$ (calcd for $C_{27}H_{47}N_6O_7$, 567.3506); IR (film) ν_{max} 3308 and 1652 cm^{-1}; UV (MeOH) λ_{max} 226 nm (ε 6100). ^{1}H and ^{13}C NMR data were listed in Table 1.

Compound (2). Colorless powder, $[\alpha]_D^{23}$ -109.6º (*c* 0.30, MeOH); HRFABMS *m/z* 795.4751 $[M+H]^+$ (calcd for $C_{41}H_{63}N_8O_8$); 795.4769; IR (film) ν_{max} 3431 and 1647 cm^{-1}; UV (MeOH) λ_{max} 280 nm (ε 1700) and 228 (ε 7500). ^{1}H and ^{13}C NMR data were listed in Table 2.

Absolute Configuration of Amino Acids. A solution of peptide (1 mg) in 6N HCl (100 μL) was heated at 110 ºC for 24 h in a sealed tube. After removal of HCl by evaporation *in vacuo*, the hydrolysate was dissolved in water and treated with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) (1 mg) and 1M $NaHCO_3$ (17 μL) at 35 ºC for 1 h. After cooling, it was treated with 2M HCl and then concentrated to dryness. This residue was subjected to HPLC, flow rate 1 mL/min, detection at 340 nm, solvent: 10 - 80% MeOH (80 min gradient) / 50 mM triethylamine phosphate (TEAP) buffer (pH 3.2). From the hydrolysate of compound (**1**), when analyzed under the same conditions, peaks with retention times of 36.8, 47.3, 56.7 and 64.5 min were found. In similar way, the hydrolyzate of compound (**2**) was showed three peaks with retention times of 46.9, 61.5 and 64.5 min. Retention times (min) of authentic amino acids were as follows: L-Phe (61.1), D-Phe (70.7), L-Leu (64.2), D-Leu (74.3), L-Pro (47.0), D-Pro (52.1), L-Ser (36.6), D-Ser (39.8), L-Val (56.8), D-Val (67.9).

Biological assay. The bio-assay method using mouse lymphocyte was as follows. After dislocation of cervical vertebrae of ICR mice aged 5 to 8 weeks, the spleen was removed and mashed. Then, the cells were suspended in RPMI 1640 medium and passed through a stainless steel mesh. The single cell suspension was washed twice with the medium and finally suspended in RPMI 1640 medium containing 10% fetal calf serum, and 10 mg/mL Kanamycin to give 3.5×10^6 cells/mL. 200 μL of this suspension was placed in each well of a microtiter plate with 96 flatbottom wells. Concanavalin A was added to each well to a final concentration of 2.5 μg/mL. Subsequently, 4 μL of serially diluted EtOH solution of test compound was added to a final concentration of 10 - 100000 ng/mL. The plate was incubated for 3 days in 5% CO_2 / 95% air at 37 °C. After termination of cell culture, cytotoxic activity was checked from 10 μL of each dose by observing under microscope after dyed with 10 μL trypanblue solution (0.3%, in PBS), and 20 μL of 5 mM 1-methoxy PMS and 0.2 mM WST-1 (DOJINDO Laboratories) in phosphate buffered saline was added to every well and the plate was incubated again at 37 °C in 5% CO_2 / air for a 4 h. The plate was read on a microplate reader (Corona MT P-32, Corona Co., Japan) at 415 nm. A dose response curve was plotted for each drug, and the concentration which gave 50% inhibition of cell growth (IC_{50}) was recorded.

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