New Cyclic Peptides from *Citrus medica* var. *sarcodactylis* SWINGLE

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Two new cyclic peptides were isolated from the fruit peels of *Citrus medica* var. *sarcodactylis* SWINGLE. Their structures were elucidated as cyclo(–Gly–Asp–Leu–Thr–Val–Tyr–Phe–) and cyclo(–Gly–Leu–Pro–Trp–Leu–Ile–Ala–Ala–) by intensive two-dimensional (2D) NMR analysis and chemical evidence.

Key words *Citrus medica* var. *sarcodactylis*; Rutaceae; cyclic peptide; fruit peel; cyclopeptide

Many cyclic peptides with unique structures such as kapakahines[1] microsclerodermins[2] have been isolated from marine organisms, and cyclomarins[3] and methylsulfomycin[4] from microorganisms. These natural products exhibit a wide range of biological activity such as antibiotic, antiinflammatory and cytotoxic activity. As part of our continuing study of cyclic peptides from higher plants,[5—14] we examined the isolation of fruit peels of *Citrus medica* var. *sarcodactylis* (Rutaceae). Cyclic peptides from the Rutaceous plants evolidine[15] and citrusins[16] have been reported. In the present study, we isolated two new cyclic peptides from *C. medica* var. *sarcodactylis*, and their structures were determined to be cyclo(–Gly–Asp–Leu–Thr–Val–Tyr–Phe–) and cyclo(–Gly–Leu–Pro–Trp–Leu–Ile–Ala–Ala–).

Silica gel column chromatography of the $CHCl_3$ soluble fraction of a 50% aqueous MeOH extract prepared from the fruit peels of *C. medica* var. *sarcodactylis*, followed by HPLC on octadecyl silica (ODS), yielded peptidic compounds **1** and **2**.

Compound **1**, a colorless powder, had the molecular formula $C_{39}H_{54}N_7O_{11}$ as revealed by the high resolution (HR)-FAB-MS quasimolecular ion peak at *m/z* 796.3892 $[M+H]^+$. The IR absorptions at 3434 and 1641 cm^{-1} were assigned to an amine and amide carbonyl, respectively. In the ^{1}H-NMR spectrum in pyridine-d_5, most of the signals were quite broad, whereas **1** gave well resolved sharp signals when the NMR data were recorded in CD_3OD. Determination of the component amino acids and the complete amino acid sequence for **1** were performed by two-dimensional (2D) NMR spectroscopy. Assignment of the ^{1}H-NMR chemical shifts in the individual amino acid residues was obtained by a 2D homonuclear correlation spectroscopy (COSY) experiment to show the complete spin systems of one asparagine (Asp), one glycine (Gly), one leucine (Leu), one phenylalanine (Phe), one threonine (Thr), one tyrosine (Tyr) and one valine (Val) (Table 1). The corresponding carbon resonances were determined on the basis of ^{1}H-detected heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond correlation (HMBC) experiments. HMBC correlation analysis revealed two partial sequences, –Phe–Gly–Asp–Leu– and –Val–Thr–Tyr–, (Fig. 1); however, no additional information was available from the nuclear Overhauser effect

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

	^{1}H-NMR Assignment δ_H [int, mult, *J* (Hz)]	^{13}C-NMR δ_C		^{1}H-NMR δ_H [int, mult, *J* (Hz)]	^{13}C-NMR δ_C
Gly[1]			Thr[5]		
α	3.53 (1H, m)	44.17	α	4.42 (1H, m)	60.47
	3.65 (1H, m)		β	4.41 (1H, m)	69.14
CO		171.06	γ_{CH3}	1.20 (3H, d, 6.3)	20.91
Asp[2]			CO		173.18
α	4.73 (1H, t, 4.3)	50.50	Tyr[6]		
β	2.78 (1H, dd, 4.4, 16.3)	37.84	α	4.49 (1H, dd, 4.1, 7.4)	57.20
	3.11 (1H, dd, 4.0, 16.4)		β	3.01 (1H, m)	36.10
γ_{CO}		175.89		3.28 (1H, m)	
CO		173.74	γ		129.33
Leu[3]			δ	6.94 (2H, d, 8.4)	131.48
α	3.89 (1H, dd, 4.1, 11.1)	56.29	ε	6.56 (2H, d, 8.5)	116.42
β	1.71 (1H, ddd, 4.2, 9.8, 13.9)	39.70	ζ		157.42
	2.03 (1H, m)		CO		173.65
γ	1.62 (1H, m)	26.07	Phe[7]		
δ	0.88 (3H, d, 6.5)	21.45	α	4.39 (1H, m)	57.66
	0.92 (3H, d, 6.5)	23.59	β	2.97 (1H, dd, 7.3, 13.9)	37.20
CO		174.23		3.13 (1H, dd, 5.8, 14.1)	
Val[4]			γ		138.01
α	4.18 (1H, d, 9.1)	62.74	δ	7.24 (2H, m)	130.47
β	2.00 (1H, m)	32.25	ε	7.33 (2H, m)	129.59
γ	0.97 (6H, d, 6.5)	19.39	ζ	7.28 (1H, m)	128.08
		19.94	CO		173.74
CO		174.17			

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spectroscopy (NOESY) experiment. So, we carried out a detailed examination of mass fragments to determine the amino acid sequence. In the FAB-MS spectrum, compound **1** showed fragment ions due to the elimination of Asp–Leu–Val–Thr– and –Thr–Tyr–Phe–Gly– from *m/z* 795.83 at *m/z* 368.36 and *m/z* 327.32, respectively (Fig. 2). The above facts are evidence that compound **1** has a cyclic nature, as shown in Fig. 1. Thus, the structure of compound **1** was determined to be cyclo(–Gly–Asp–Leu–Thr–Val–Tyr–Phe–). The absolute stereochemistry of all of the component amino acids was determined to be of L-configuration by HPLC analysis.[17)]

Compound **2** showed an HR-FAB-MS spectral quasimolecular ion peak at *m/z* 822.4856 $[M+H]^+$, corresponding to the molecular formula $C_{42}H_{64}N_9O_8$. The IR absorption bands at 3434 and 1640 cm^{-1} were assigned to amino and amide carbonyl groups, respectively. The octapeptide nature of **2** was evident from its ^{13}C-NMR spectrum in CD_3OD, in which eight amide carbonyl groups were identified (Table 2). The ^1H- and ^{13}C-NMR signals for the individual amino acids were readily assigned by the extensive analysis of ^1H–^1H COSY and HMQC spectra. The gross structure, including the amino acid sequence, was determined by linking the individual amino acids identified by the phase sensitive NOESY and HMBC experiments. The HMBC correlation analysis revealed two partial sequences, –Ala^7–Ala^8–Gly^1– and –Pro^3–Trp^4–Leu^5–Ile^6–. The NOESY correlation analysis revealed a partial sequence, –Leu^2–Pro^3–, whereas its NOESY spectrum in pyridine-d_5 provided good information regarding cross signals between Hβ of Ile^6 and NH of Ala^7, between NH of Ala^7 and NH of Ala^8, between NH of Ala^8 and NH of Gly^1, and between NH of Gly^1 and NH of Leu^2 (Fig. 3). Thus, the structure of compound **2** was identified as cyclo (–Gly–Leu–Pro–Trp–Leu–Ile–Ala–Ala–). The absolute stereochemistry of each amino acid in **2** was determined to be of an L-configuration by HPLC analysis of the derivatives of the acid hydrolysate by treatment with Marfey's reagent.

The amide bond between Leu^2 and Pro^3 residues was determined to be *trans* on the basis of the strong NOE correlation between Hα in Leu^2 and Hδ in Pro^3, as well as the chemical shifts of the β and δ carbons of the Pro^3 residue at δ 30.13 and 26.34.[18)]

The immunosuppressive activity of **1** and **2** was examined on the mouse lymphocyte proliferation induced by Concanavalin A; they did not show any activity (IC_{50}: >100 μg/ml, respectively).

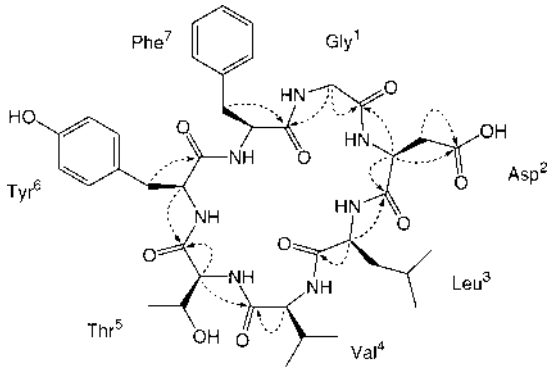

Fig. 1. HMBC Correlations (Dashed Arrows) for Compound **1** in CD_3OD

Experimental

General Experimental Procedures Optical rotation was measured on a JASCO DIP-4 spectrometer, and the $[\alpha]_D$ values are given in 10^{-1} deg cm^2 g^{-1}. We also conducted FAB-MS using a VG Autospec spectrometer. IR and UV spectra were taken on JASCO FT/IR 620 and Hitachi 557 spectrometers, respectively, and ^1H- and ^{13}C-NMR spectra on a Bruker DRX-500 spectrometer with chemical shifts (δ) reported in ppm recorded at 300 K.

Plant Material The fruit peels of *Citrus medica* var. *sarcodactylis* for this study were collected in the Republic of China, in 2000. The plant material was confirmed by Dr. Takeya, one of the authors. A voucher specimen has been deposited at the Department of Pharmacognosy, Tokyo University of Pharmacy & Life Science.

Extraction and Isolation The fruit peels (500 g) were extracted with 50% MeOH. The MeOH extract was concentrated and the residue was partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ layer gave 13.6 g, which was chromatographed on a silica gel column using a $CHCl_3$–AcOEt–MeOH gradient system. The fraction was eluted with MeOH and was then subjected to ODS MPLC with 75% MeOH/H_2O to give two peptide fractions, each of which was purified by ODS HPLC with MeOH/H_2O (1/1) and MeOH/H_2O (11/9), respectively, to give compounds **1** (21.2 mg, 0.0042%) and **2** (10.6 mg, 0.0021%).

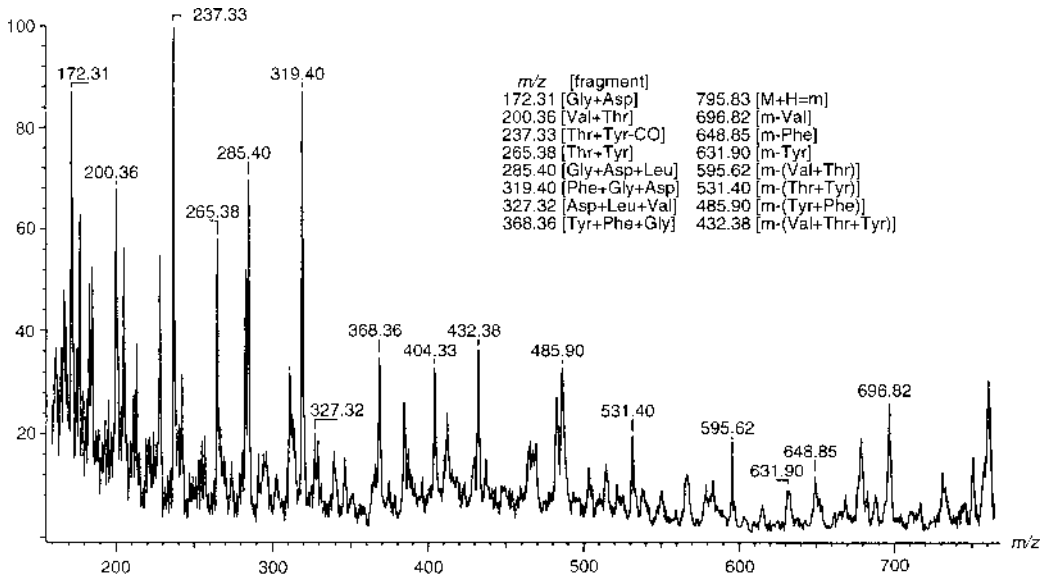

Fig. 2. FAB Mass Spectrum Obtained for Compound **1**

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

	^{1}H-NMR Assignment δ_H [int, mult, J (Hz)]	pyr[a)]	^{13}C-NMR δ_C		^{1}H-NMR δ_H [int, mult, J (Hz)]	pyr[a)]	^{13}C-NMR δ_C
Gly[1]				Leu[5]			
α	3.55 (1H, d, 17.0)	3.87	43.97	α	4.48 (1H, dd, 3.9, 9.4)	4.76	53.57
	4.01 (1H, d, 16.9)	4.53		β	1.42 (1H, m)	1.86	40.65
CO			171.31		1.64 (1H, m)	1.95	
NH		8.79		γ	1.58 (1H, m)	1.88	26.79
Leu[2]				δ	0.91 (3H, d, 6.5)	1.02	23.59
α	4.66 (1H, m)	5.13	48.49—49.51		1.04 (3H, d, 6.4)	1.12	22.50
β	0.15 (1H, m)	0.54	41.38	CO			175.55
	0.87—0.95 (1H, m)	1.45		NH		10.03	
γ	1.44 (1H, m)	1.95—2.05	24.85	Ile[6]			
δ	0.76 (3H, d, 6.5)	0.94	20.93	α	4.02 (1H, d, 7.0)	4.73	63.48
	0.80 (3H, d, 6.8)	1.00	23.66	β	1.81 (1H, m)	2.16	36.66
CO			174.76	γ	1.30 (1H, m)	1.47	27.31
NH		7.46			1.53 (1H, m)	1.83	
Pro[3]				γ_{CH3}	0.94 (3H, d, 6.9)	1.17	15.92
α	4.06 (1H, m)	4.37	63.93	δ	0.89 (3H, t, 7.4)	0.81	11.36
β	1.93 (1H, m)	1.95—2.05	30.19	CO			176.99
	2.30 (1H, m)	2.12		NH		7.93	
γ	1.96 (1H, m)	1.63	26.34	Ala[7]			
	2.11 (1H, m)	1.92		α	4.10 (1H, m)	4.69	52.86
δ	2.99 (1H, m)	3.10	48.06	β	1.37 (3H, d, 8.7)	1.50	16.77
	3.62 (1H, m)	3.54		CO			175.21
CO			173.80	NH		9.05	
Trp[4]				Ala[8]			
α	4.70 (1H, m)	4.94	55.10	α	4.53 (1H, q, 7.3)	5.15	48.49—49.51
β	3.23 (1H, dd, 5.0, 14.6)	3.37	26.79	β	1.38 (3H, d, 8.7)	1.76	18.36
	3.56 (1H, dd, 4.9, 14.5)	3.91		CO			174.92
2	7.03 (1H, s)	7.38	125.12	NH		8.32	
3			109.29				
4	7.59 (1H, d, 7.8)	7.76	119.11				
5	7.10 (1H, t, 7.1)	7.21	120.35				
6	7.17 (1H, t, 7.2)	7.31	122.91				
7	7.59 (1H, d, 8.2)	7.61	113.40				
8			137.92				
9			129.00				
CO			172.95				
1NH		12.17					

a) pyr: This column shows the proton chemical shifts in pyridine-d_5.

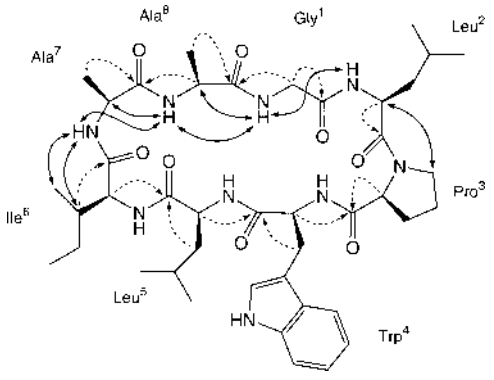

Fig. 3. HMBC Correlations (Dashed Arrows) for Compound **2** in CD_3OD and NOE Correlations (Arrows) for Compound **2** in Pyridine-d_5

Compound **1**: Colorless powder, $[\alpha]_D^{24}$ −22.3° (c=0.25, MeOH); HR-FAB-MS m/z 796.3892 $[M+H]^+$ (Calcd for $C_{39}H_{54}N_7O_{11}$, 796.3881); IR (film) ν_{max} 3434 and 1641 cm^{-1}; UV (MeOH) λ_{max} nm: 212 (ε 14200) and 278 (ε 1800). ^{1}H- and ^{13}C-NMR see Table 1.

Compound **2**: Colorless powder, $[\alpha]_D^{24}$ −81.1° (c=0.15, MeOH); HR-FAB-MS m/z 822.4856 $[M+H]^+$ (Calcd for $C_{42}H_{64}N_9O_8$; 822.4878); IR (film) ν_{max} 3434 and 1640 cm^{-1}; UV (MeOH) λ_{max} nm: 224 (ε 13300) and 282 (ε 4400). ^{1}H- and ^{13}C-NMR see Table 2.

Absolute Configuration of Amino Acids A solution of peptide (1 mg) in 6 N HCl was heated at 110 °C for 24 h in a sealed tube. After removal of HCl by evaporation *in vacuo*, the hydrolyzate was dissolved in water and treated with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) and 1 M $NaHCO_3$ at 35 °C for 1 h. After cooling, it was treated with 2 M HCl and then concentrated to dryness. This residue was subjected to HPLC, at a flow rate 1 ml/min, detection at 340 nm, solvent: 10—80% MeOH (80 min gradient added on 5 min to be eluted with 100% MeOH)/50 mM triethylamine phosphate (TEAP) buffer (pH 3.2). Retention times (min) of authentic amino acids were as follows: L-Ala (45.4), D-Ala (54.2), L-Asp (39.9), D-Asp (45.9), L-Phe (61.1), D-Phe (70.7), L-Ile (63.1), D-Ile (73.5), L-Leu (64.2), D-Leu (74.3), L-Pro (47.0), D-Pro (52.1), L-Thr (38.3), D-Thr (49.1), L-Val (56.8), D-Val (67.9), L-Trp (58.3), D-Trp (65.5), L-Tyr (72.9), D-Tyr (83.2).

Immunosuppressive Activity The bio-assay method using mouse lymphocyte was as follows. After dislocation of the 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 the test compound was added to a final concentration of 10—100000 ng/ml. The plate was incubated for 3 d in 5% CO_2/95% air at 37 °C. After termination of the cell culture, 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 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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