CYCLIC PEPTIDES FROM HIGHER PLANTS. PART 30.[1] THREE NOVEL CYCLIC PEPTIDES, YUNNANINS D, E AND F FROM *STELLARIA YUNNANENSIS*

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Abstract - Three novel cyclic peptides, named yunnanins D [**1**: *cyclo*(-Gly-Ile-Ser-Phe-Arg-Phe-Pro-)], E [**2**: *cyclo*(-Gly-Ser-δ-Hydroxy Ile-Phe-Phe-Ser-)] and F [**3**: *cyclo*(-Gly-Val-Thr-Trp-Tyr-Pro-Ser-Ser-)], have been isolated from *Stellaria yunnanensis* (Caryophyllaceae) and their structures were elucidated by spectroscopic evidence and chemical degradation.

During the course of our investigations in search of new biologically active cyclic peptides from higher plants,[1-3] we have isolated one cyclic hexa- and two heptapeptides, named yunnanins A - C, from the roots of *Stellaria yunnanensis* Franch. (Caryophyllaceae) and characterized their structures and cytotoxic activities.[2] Continued investigation of the roots of *S. yunnanensis* has now resulted in isolation of three new cyclic peptides, named yunnanins D (**1**), E (**2**) and F(**3**), one of which contain a δ-hydroxy isoleucine residue. Here we report the isolation and structural characterization of these cyclic peptides (**1** - **3**).

Repeated fractionation of *n*-BuOH soluble phase of the MeOH extract by Diaion HP-20, silica gel and ODS chromatography led us to isolate three new cyclic peptides, yunnanins D (**1**), E (**2**) and F(**3**).

Yunnanin D (**1**) was obtained as colorless powder: $[\alpha]_D$ -20.0° (c 0.60, MeOH); ir (KBr): 3350 (NH), 1670 and 1630 (amide C=O) cm^{-1}. The FAB ms of **1** showed protonated molecule at *m/z* 805, and the molecular formula has been shown as $C_{40}H_{56}N_{10}O_8$ by HR-FAB ms analysis. The peptide nature of **1** was evident from its ^1H and ^{13}C nmr spectra. The presence of a single stable conformer in DMSO-d_6 on the nmr time scale was displayed by the occurrence of well-resolved sharp ^1H and ^{13}C signals. Extensive 2D nmr analysis, including ^1H-^1H COSY, HOHAHA,[4] HMQC[5] and HMBC,[6] was used to determine the identity of the spin system based on the following seven amino acids, Gly, Ser, Ile, Pro, Phe × 2 and Arg. These amino acids were also confirmed by acid hydrolysis, followed by amino acid analysis. From

the molecular formula as well as the nmr data, it become evident that **1** was a cyclic peptide. As shown in Figure 1, a phase sensitive ROESY[7] analysis suggested the seven amino acid sequence as presented in structure (**1**). A segment, Ile-Ser-Phe-Arg-Phe-Pro-Gly was assigned by the ROE correlations such as Ile^2-NH / Ser^3-NH, Ser^3-Hα / Phe^4-NH, Phe^4-NH / Arg^5-NH, Arg^5-NH / Phe^6-NH, Phe^6-Hα / Pro^7-Hδ, and Pro^7-Hα / Gly^1-NH, and the structure of **1** was determined to be *cyclo*(-Gly-Ile-Ser-Phe-Arg-Phe-Pro-). The ^{13}C chemical shifts (δ 28.88 and 24.40) of β and γ positions in Pro^7 residue suggested that the geometry of the proline amide bond was fixed to be *trans*.[8] The stereochemistry of each amino acid was confirmed to be all L-configuration by Marfey's derivation, followed by hplc analysis.[9]

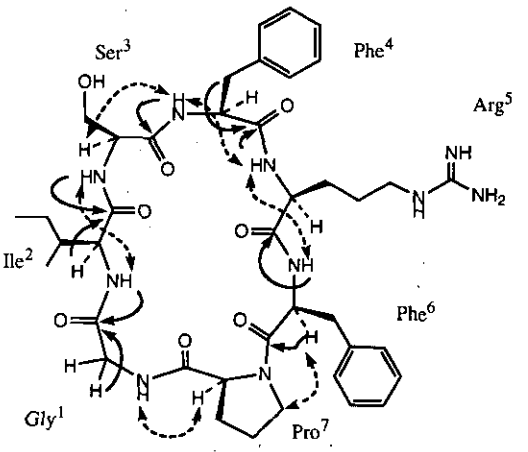

Figure 1 Structure of **1**: Some important ROE and HMBC correlations were shown by dotted arrows and arrows, respectively.

Table 1. ^{1}H and ^{13}C nmr signal assignments of yunnanin D in DMSO-*d*6

assignment	^{1}H nmr δH [int. mult, J(Hz)]	^{13}C nmr δC	assignment	^{1}H nmr δH [int. mult, J(Hz)]	^{13}C nmr δC
Gly^1			Arg^5		
α	4.18 (1H, m)	42.44	α	4.07 (1H, m)	52.92
	3.32 (1H, dd, 4.2, 17.0)		β	1.57 (1H, m)	28.65
NH	8.80 (1H, dd, 4.2, 8.0)			1.37 (1H, m)	
C=O		168.65	γ	1.19 (2H, m)	24.85
			δ	2.98 (2H, m)	40.22
Ile^2			NHε	7.58 (1H, t, 5.2)	
α	4.23 (1H, m)	58.58	ζ		156.73
β	1.76 (1H, m)	37.68	NH	7.70 (1H, d, 9.0)	
γ	1.40 (1H, m)	24.40	C=O		170.38
	1.03 (1H, m)				
Meγ	0.82 (3H, d, 6.7)	15.08			
δ	0.68 (3H, t, 7.3)	10.28	Phe^6		
NH	8.07 (1H, d, 10.5)		α	4.78 (1H, ddd, 6.4,7.3,8.3)	51.72
C=O		169.92	β	2.94 (1H, dd, 7.3, 13.4)	38.19
				2.67 (1H, dd, 6.4, 13.4)	
Ser^3			γ		136.80
α	4.49 (1H, m)	54.13	δ	7.14-7.31 (5H, m)	129.28
β	4.16 (1H, m)	62.72	ε		128.11
	3.71 (1H, m)		ζ		125.27
NH	8.48 (1H, d, 6.4)		NH	7.23 (1H, m)	
C=O		170.38	C=O		168.75
Phe^4			Pro^7		
α	4.21 (1H, m)	56.86	α	4.13 (1H, m)	61.17
β	3.07 (2H, m)	35.54	β	2.06 (1H, m)	28.88
γ		137.44		1.73 (1H, m)	
δ	7.14-7.31 (5H, m)	128.84	γ	1.87 (1H, m)	24.40
ε		128.24		1.75 (1H, m)	
ζ		125.27	δ	3.51 (1H, m)	47.31
NH	8.57 (1H, d, 4.5)			3.05 (1H, m)	
C=O		170.70	C=O		170.91

Yunnanin E (**2**), *colorless powder:* $[\alpha]_D$ -9.6° (*c* 0.25, MeOH), *exhibited a high-resolution* FAB-*ms* spectral quasimolecular ion $(M+Na)^+$ peak at *m/z* 677.2685, corresponding to molecular formula, $C_{32}H_{42}N_6O_9$. Amino acid analysis of the acid hydrolysate showed the presence of two Ser, two Phe and one Gly. The stereochemistry of these amino acids was confirmed to be all L-configuration by Marfey's derivation, followed by hplc analysis.[9] In the nmr spectra, however, ^1H and ^{13}C signals based on six amide protons and six amide carbonyl carbons were observed, showing hexapeptide nature of **2**. The remaining amino acid and the peptide sequence was determined by 2D nmr analysis such as ^1H-^1H COSY, HOHAHA,[4] HMQC,[5] HMBC,[6] and ROESY[7] spectra as follows. Individual ^1H and ^{13}C assignments of the above five usual amino acids were conducted by combination of ^1H-^1H COSY, HOHAHA and HMQC spectra. In the remaining nmr resonances, both of the Hα at δ 4.86 and doublet methyl group at δ 0.92 were coupled with a methine proton at δ 2.60, which was also coupled with two successive methylene protons at δ 1.49, 1.94, 3.79 and 3.85. The later two methylene protons resonated at lower field were considered to be attached to a hydroxy bearing carbon at δ 59.30. These remaining nmr resonances were assigned to a δ-hydroxy isoleucine as in yunnanin B. From these results, all of the constituted six amino acids were determined. The sequencing of them was established to be *cyclo*(-Gly-Ser-δ-hydroxy Ile-Phe-Phe-Ser-) by ROE correlations as shown in Figure 2. A few peptides containing a γ-hydroxy Ile, such as γ-amanitin and so on,[10] have been reported, however, the presence of δ-hydroxy Ile is not known except for yunnanins B and E.[2]

Table 2. ^1H and ^{13}C nmr signal assignments of Yunnanin E in pyridine-d_5

assignment	^1H nmr δ_H [int. mult, J(Hz)]	^{13}C nmr δ_C	assignment	δ_H	δ_C
Gly[1]			Phe[4]		
α	4.01 (1H, dd, 5.6, 12.4)	44.04	α	4.98 (1H, m)	57.17
	4.75 (1H, m)		β	3.58 (1H, m)	37.97
NH	10.05 (1H, t, 5.6)			3.33 (1H, dd, 10.6,14.0)	
C=O		170.30	γ		138.62
			δ	7.33 (2H, d, 7.1)	129.69
			ε	7.27 (2H, m)	128.77
Ser[2]			ζ	7.19 (1H, m)	126.89
α	5.18 (1H, ddd,4.3,5.3,8.0)	56.19	NH	9.14 (1H, d, 7.3)	
β	4.21 (1H, dd, 5.3, 11.5)	62.42	C=O		171.84
	4.49 (1H, dd, 4.3, 11.5)		Phe[5]		
NH	9.39 (1H, d, 8.0)		α	4.78 (1H, m)	55.88
C=O		171.38	β	3.57 (2H, m)	37.97
			γ		138.34
δ-Hydroxy-Ile[3]			δ	7.46 (2H, d, 7.2)	130.22
α	4.86 (1H, m)	59.68	ε	7.25 (2H, m)	128.67
β	2.60 (1H, m)	33.03	ζ	7.19 (1H, m)	126.82
Meγ	0.92 (3H, d, 6.8)	16.62	NH	8.70 (1H, d, 7.7)	
γ	1.49 (1H, m)	35.51	C=O		172.37
	1.94 (1H, m)		Ser[6]		
δ	3.79 (1H, m)	59.30	α	4.91 (1H, m)	58.38
	3.85 (1H, m)		β	4.37 (1H, dd, 6.0, 10.8)	61.88
NH	8.93 (1H, d, 8.0)			4.47 (1H, dd, 6.5, 10.8)	
C=O		172.75	NH	9.25 (1H, d, 4.8)	
			C=O		172.16

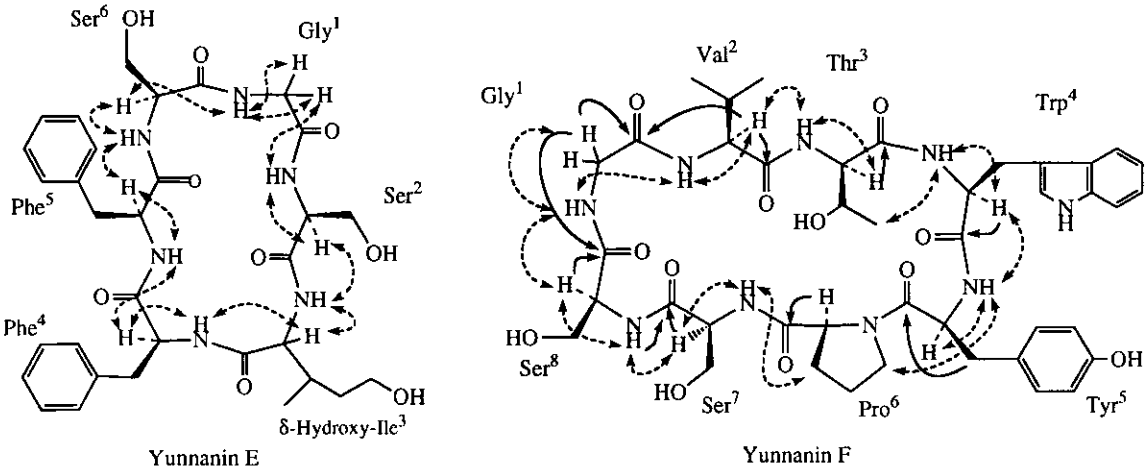

Figure 2 Structures of Yunnanins E (**2**) and F (**3**): Some important HMBC correlations were shown by arrows and ROE ones by dotted arrows.

Table 3. ^{1}H and ^{13}C nmr signal assignments of yunnanin F in pyridine-d_5

assignment		^{1}H nmr δ_H [int. mult, J(Hz)]	^{13}C nmr δ_C	assignment		δ_H	δ_C
Gly^1				Tyr^5			
	α	4.60 (1H, m)	43.90		α	4.91 (1H, m)	56.45
		3.91 (1H, m)			β	3.59 (1H, dd, 9.3, 13.2)	36.42
	NH	8.96 (1H, br s)				3.25 (1H, dd, 2.2, 13.2)	
	C=O		169.39		γ		129.91
					δ	7.88 (2H, d, 8.3)	131.77
Val^2					ε	7.38 (2H, d, 8.3)	116.82
	α	5.34 (1H, dd, 3.2, 9.8)	55.82		ζ		157.85
	β	1.42 (1H, m)	32.84		NH	7.84 (1H, br s)	
	γ	0.49 (3H, d, 6.7)	20.04		C=O		171.66
		0.64 (3H, d, 6.6)	20.04	Pro^6			
	NH	7.37 (1H, m)			α	4.88 (1H, m)	64.65
	C=O		176.10		β	2.28 (1H, m)	30.11
						1.95 (1H, m)	
					γ	1.98 (1H, m)	25.03
Thr^3						1.68 (1H, m)	
	α	4.25 (1H, m)	64.05		δ	3.77 (1H, m)	47.41
	β	4.64 (1H, m)	66.56			2.72 (1H, m)	
	γ	1.41 (3H, d, 6.4)	21.36		C=O		175.29
	NH	9.58 (1H, br s)		Ser^7			
	C=O		172.31		α	4.86 (1H, m)	60.31
Trp^4					β	4.37 (1H, dd, 5.7, 11.2)	61.29
	α	5.23 (1H, br dd, 4.9, 11.9)	55.24			4.24 (1H, dd, 3.3, 11.2)	
	β	3.91 (1H, dd, 4.4, 14.5)	26.69		NH	9.19 (1H, d, 3.2)	
		3.31 (1H, dd, 4.6, 14.5)			C=O		171.58
	1(NH)	12.06 (1H, s)					
	2	7.49 (1H, d, 1.9)	124.96				
	3		109.28	Ser^8			
	4	8.06 (1H, d, 7.7)	118.79		α	5.49 (1H, m)	56.53
	5	7.20 (1H, m)	122.11		β	4.68 (1H, dd, 4.6, 11.1)	63.33
	6	7.25 (1H, t, 7.8)	119.86			4.56 (1H, dd, 7.9, 11.1)	
	7	7.52 (1H, d, 7.8)	112.22		NH	9.04 (1H, d, 9.6)	
	8		137.62		C=O		172.14
	9		128.54				
	NH	7.23 (1H, m)					
	C=O		171.90				

Yunnanin F (**3**), obtained as colorless powder, $[\alpha]_D$ -56.6° (c 0.29, MeOH), had the molecular formula, $C_{42}H_{55}N_9O_{12}$ established by high-resolution FAB-ms spectrometry (*m/z* 878.4062). The ir spectrum (3320 and 1650 cm^{-1}) exhibited bands characteristic of amino and amide carbonyl groups, respectively, and uv absorption bands at 230 and 280 nm indicated the presence of aromatic amino acids. Though the amino acid composition of **3** was revealed to be one Gly, two Ser, one Thr, one Val, one Pro and one Tyr per molecule after complete hydrolysis with 6N HCl, the ^1H and ^{13}C nmr spectra of **3** showed the presence of eight amide NH and eight amide carbonyl groups, indicating the octapeptide nature of **3**. The remaining amino acid was determined to be Trp by a indole ring bearing NH proton singal at δ 12.06 and aromatic carbon signals at δ 109.28, 112.22, 118.79, 119.86, 122.11, 124.96, 128.54 and 137.62. In addition, the lack of terminal amino group protons in the ^1H nmr suggested that **3** might be cyclic octapeptide. A close inspection of the nmr spectra by ^1H-^1H COSY and HMQC experiments led to complete ^1H and ^{13}C assignments of the individual amino acids shown in Table 3. The sequence analysis was conducted by a combination of long range $^2J_{H\text{-}C}$ and $^3J_{H\text{-}C}$ connectivities in HMBC spectrum and ROE correlations in a phase sensitive ROESY spectrum as follows (Figure 2). The structural unit: Ser-Ser-Gly-Val was suggested by $^2J_{H\text{-}C}$ and $^3J_{H\text{-}C}$ correlations. In addition, the ROE correlations between neighboring residues including the above sequence were observed as shown in Figure 2. From these evidences, the structure of **3** was elucidated to be *cyclo*(-Gly-Val-Thr-Trp-Tyr-Pro-Ser-Ser-). The absolute configurations of the component amino acids were confirmed to be all L-configuration by Marfey's method.[9]

Recently, a number of naturally occurring cyclic peptides with unique structures and biological activities have been isolated.[11] Despite their importance, only very few studies on cyclic peptide-containing higher plants have been reported.[12] Yunnanin D, a cyclic heptapeptide, showed cell growth inhibitory activity against P-388 lymphocytic leukemia cells (IC_{50}: 2.1 μg/ml). The investigation of the yunnanins in other biological assays is ongoing.

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EXPERIMENTAL

General Details. - Optical rotations were measured with a JASCO DIP-4 polarimeter and the $[\alpha]_D$ values are given in 10^{-1} deg cm^2 g^{-1}. Mass, uv, and ir spectra were taken with a VG-Autospec spectrometer, a Hitachi 557 spectrophotomer and a JASCO A-302 spectrophotometer, respectively. Hplc was performed with an Inertsil PREP-ODS column (20 mm i.d. × 250 mm, GL Science Inc.) packed with 10 μm ODS. Tlc was conducted on precoated Kieselgel 60 F_{254} (Art. 5715; Merck). ^1H and ^{13}C nmr spectra were recorded on Bruker spectrometers (AM 400 and AM 500) and Varian Unity 400 spectrometer at 303K. Phase sensitive ROESY experiments were made with a mixing time of 0.1 s. The value of the delay to optimize one-bond correlations in the HMQC spectrum and suppress them in the HMBC spectrum was 3.2 Hz and the evolution delay for long-range couplings in the HMBC spectrum was set to 50 msec. The nmr coupling constants (J) are given in Hz.

Materials. - The roots of *S. yunnanensis* were collected in Yunnan, People's Republic of China, in June 1994. 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 Science.

Extraction and isolation of 1 - 3. - The roots of *Stellaria yunnanensis* (7.0 kg) were extracted with hot MeOH (50 l) three times for 1 week to give a MeOH extract which was partitioned between *n*-BuOH and H_2O. The n-BuOH soluble fraction (*ca.* 300 g) was subjected to Diaion HP-20 column chromatography using a H_2O-MeOH gradient system (1:0 - 0:1). The fractions (52 g) eluted with 80% and 100% MeOH, respectively, were further subjected to Si gel column chromatography using a $CHCl_3$-MeOH gradient system (1:0 - 0:1). The fraction (10.4 g) eluted with 10% MeOH was subjected to ODS hplc with a 50% MeOH solvent system to give yunnanin D **1** (160 mg), yunnanin E **2** (470 mg) and yunnanin F **3** (16 mg).

Yunnanin D (1). - Colorless powder, $[\alpha]_D$ -20.0° (c 0.60, MeOH), *m/z* : 805 (Found : $[M+H]^+$, 805.4352 $C_{40}H_{57}N_{10}O_8$; requires : 805.4361), $\nu_{max(KBr)}/cm^{-1}$: 3350, 1670 and 1630, ^1H nmr (DMSO-d_6) : listed in Table 1, ^{13}C nmr (DMSO-d_6) : listed in Table 1.

Yunnanin E (2). - Colorless powder, $[\alpha]_D$ -9.6° (c 0.25, MeOH), *m/z* : 677 (Found : $[M+Na]^+$, 677.2685 $C_{32}H_{42}N_6O_9Na$; requires : 677.2701). Anal. Calcd for $C_{32}H_{42}N_6O_9$: C, 58.72; H, 6.42; N, 12.84. Found: C, 58.49; H, 6.71; N, 12.55. $\nu_{max(KBr)}/cm^{-1}$: 3330, 1650 and 1530, ^1H nmr (pyridine-d_5): listed in Table 2, ^{13}C nmr (pyridine-d_5): listed in Table 2.

Yunnanin F (3). - Colorless powder, $[\alpha]_D$ -56.5° (c 0.29, MeOH), *m/z* : 878 (Found : $[M+H]^+$, 878.4062 $C_{42}H_{56}N_9O_{12}$; requires : 878.4048), $\nu_{max(KBr)}/cm^{-1}$: 3320, 2920, 1650 and 1510, $\lambda_{max/MeOH}$ nm(ε): 276 (2236), 280 (2350), ^1H nmr and ^{13}C nmr (pyridine-d_5): listed in Table 3.

Acid Hydrolysis of 1 - 3. - Solution of **1 - 3** (each containing 1 mg of peptide) in 6N HCl (1 ml) were heated at 110°C for 24 h, respectively. After cooling, each solution was concentrated to dryness. The hydrolysates were dissolved in 0.02N HCl and analyzed by ion-exchange resin chromatography on a Hitachi L-8500 Amino Acid analyzer with ninhydrin detection, respectively.

Absolute Configuration of Amino Acids. - Solution of **1- 3** (each containing 1 mg of peptides) in 6N HCl (1 ml) were heated at 110° for 12 h, respectively. After being cooled, each solution was concentrated to dryness. The residues were dissolved in water and treated with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) and 1M $NaHCO_3$ at 35° for 1 h, respectively. After being cooled, 2M HCl was added to the reactants, which were then concentrated to dryness. These residues were subjected to hplc (Lichrospher 100, RP-18 (10mm), Merck), flow rate 1 ml/min, detection 340nm, solvent : 10 - 50% MeCN / 50mM triethylamine phosphate (TEAP) buffer, respectively.

Cytotoxic activity against P-388 cells. - The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay was performed on a 96-well plate. The blue formazan produced by the mitochondrial dehydrogenase of viable cells was measured spectrophotometrically. An 100 µl aliquot of RPMI-1640 medium supplemented with 5 % fetal calf serum and 100 µg/ml of kanamycin and containing mouse P-388 leukemia cells (3×10^4 cells/ml) was added to each well. After overnight incubation (37 °C, 5 % CO_2), 100, 30, 10, 3, 1, 0.3, and 0.1 µg/ml concentrations of sample solutions were added to the wells and the plates were incubated for 48 h. Then, 20 µl of MTT was added to each well and the plates were incubated for 4 h. The resulting formazan was dissolved in 100 µl of 10% SDS (sodium dodecyl sulfate) containing 0.01 N HCl. Each well was mixed gently with a pipet for 1 or 2 min and the plate was read on a microplate reader (Tosoh MPR-A4i) at 540 nm. The IC_{50} (µg/ml) value was defined as the concentration of sample which achieved 50% reduction of viable cells with respect to the control.

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