Cyclic Peptides from Higher Plants. 24.[1] Yunnanin C, a Novel Cyclic Heptapeptide from *Stellaria yunnanensis*

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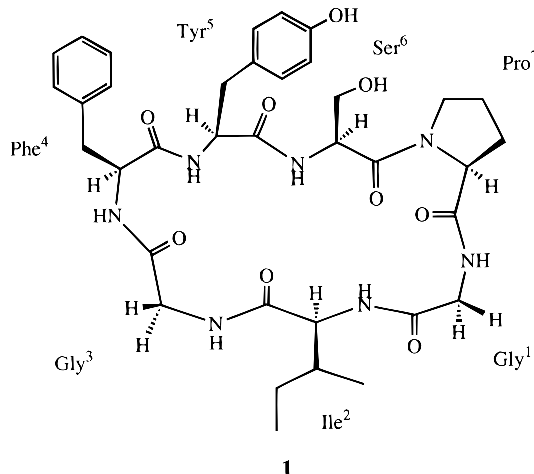
A novel cytotoxic cyclic heptapeptide, yunnanin C (**1**), was isolated from the roots of *Stellaria yunnanensis*. The structure of **1**, *cyclo*(−Gly-Ile-Gly-Phe-Tyr-Ser-Pro−), was elucidated from spectroscopic evidence and by chemical degradation.

As part of our continuing investigation of new biologically active cyclic peptides from higher plants,[2−7] we have previously isolated two cyclic hepta- and hexapeptides, named yunnanins A [*cyclo*(−Gly-Gly-Pro-Phe-Pro-Gly-Tyr−)] and B [*cyclo*(−Gly-Ser-δ-Hydroxy-Ile-Phe-Phe-Ala−)] from the roots of *Stellaria yunnanensis* Franch (Caryophyllaceae), with cytotoxic activity.[8] As a result of further investigation, we have isolated a new cyclic heptapeptide, yunnanin C (**1**), from *S. yunnanensis*. In this paper, we describe the isolation, structure elucidation, and cell growth inhibitory activity of **1**.



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The methanolic extract of the roots of *S. yunnanensis* (7.0 kg) was partitioned between *n*-BuOH and H_2O, and the *n*-BuOH-soluble fraction (300 g) was subjected to Diaion HP-20 column chromatography (H_2O−MeOH gradient system). The 80% and 100% MeOH-eluted fractions were chromatographed on a silica gel column (CH_2Cl_2−MeOH gradient system) followed by HPLC on repeated ODS columns (three solvent systems: 26% CH_3CN/0.05% TFA, 50% MeOH/0.05% TFA, and 55% MeOH) to yield yunnanin C (**1**, 0.0004%).

Yunnanin C (**1**) was obtained as colorless needles (from MeOH), mp 255 °C, $[\alpha]_D$ −48.1° (c = 0.21, MeOH). The IR absorptions at 3300 and 1630 cm^{-1} were attributed to amino and amide carbonyl groups, respectively, and the UV absorption at 276 nm (ϵ 2300) indicated the presence of aromatic functions. The FAB MS spectrum of **1** showed a pseudomolecular ion at m/z 722, and the molecular formula has been shown to be $C_{36}H_{47}N_7O_9$ by high-resolution FABMS analysis [found 722.3523 $(M + H)^+$, calcd 722.3514]. The amino acid composition of **1** was determined to be 1 mol each of serine (Ser), isoleucine (Ile), phenylalanine (Phe), tyrosine (Tyr), and proline (Pro) residues and 2 mol of glycine (Gly) residue, and their absolute configuration was determined to be L by using Marfey's method.[9] In spite of the presence of a proline residue, 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 ^{1}H and ^{13}C signals. In the ^{1}H-NMR spectrum of **1**, six NH protons corresponding to all amino acids present except for one proline residue were observed at 7.53−8.78 ppm. Detailed analysis of the ^{1}H−^{1}H COSY, HOHAHA,[10] and HMQC[11] NMR spectra led to the complete assignment of the ^{1}H- and ^{13}C-NMR chemical shifts of each amino acid residue of **1** (Table 1). The amino acid sequence of **1** was determined by extensive 2D NMR analysis including the HMBC[12] and ROESY[13] spectra measured in DMSO-d_6 (Figure 1). The sequence of the two structural units, Gly-Phe-Tyr-Ser and Pro-Gly-Ile, was assigned by two-bond ^{1}H−^{13}C correlations in the HMBC spectrum. The whole sequence of **1** was established by ROE enhancements such as Ile^2-Hα/Gly^3-NH and Ser^6-Hα/Pro^7-Hδ. Accordingly, the structure of yunnanin C (**1**) was determined as *cyclo*(−Gly-Ile-Gly-Phe-Tyr-Ser-Pro−). The chemical shifts of the β- and γ-carbons of the Pro residue were δ 28.88 and 24.59, respectively, which gave evidence for the presence of a *trans*-proline amide bond.[14] The strong ROE enhancement between Ser-Hα and Pro-Hδ gave further evidence of the *trans*-geometry.

Recently, a number of naturally occurring cyclic peptides with unique structures and biological activities have been isolated.[15] Despite their importance, only very few studies on cyclic peptide-containing higher plants have been reported.[16−19] Yunnanins A and C, of which both are cyclic heptapeptides, showed cell growth inhibitory activity against P-388 lymphocytic leukemia cells (IC_{50}: yunnanin A 2.1 μg/mL; yunnanin C 2.2 μg/mL). The investigation of the yunnanins in other biological assays is ongoing.

Experimental Section

General Experimental Procedures. The melting point was obtained with a Yanagimoto MP-3 micromelting point apparatus and is uncorrected. The optical rotation was measured on a JASCO DIP-4 polarimeter. The IR spectrum (KBr) was obtained on a Perkin-Elmer 1710 spectrophotometer. Mass spectra were recorded

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Table 1. ^1H and ^{13}C NMR Assignments for Yunnanin C (**1**) in DMSO-d_6

position		$^{\delta}H$ (int, mult, J (Hz))	$^{\delta}C$
Gly[1]			
	α	3.99 (1H, dd, 7.6, 16.7)	42.60
		3.37 (1H, dd, 4.9, 16.9)	
	NH	8.78 (1H, dd, 5.2, 7.2)	
	C=O		168.44
Ile[2]			
	α	4.15 (1H, m)	58.30
	β	1.75 (2H, m)	36.83
	γ	1.38 (1H, m)	24.28
		0.90 (1H, m)	
	γ-CH_3	0.81 (3H, d, 6.8)	15.26
	δ	0.77 (3H, t, 7.3)	10.37
	NH	7.73 (1H, d, 10.2)	
	C=O		170.65
Gly[3]			
	α	3.95 (1H, dd, 4.4, 17.1)	42.60
		3.59 (1H, dd, 3.4, 17.0)	
	NH	7.98 (1H, t, 3.9)	
	C=O		169.11
Phe[4]			
	α	3.88 (1H, br m)	56.67
	β	2.86 (1H, m)	35.76
		2.71 (1H, dd, 5.2, 14.0)	
	γ		137.51
	δ	7.03 (2H, d, 7.2)	128.81
	ε	7.24 (2H, m)	128.19
	ζ	7.20 (1H, m)	126.31
	NH	8.58 (1H, d, 5.7)	
	C=O		170.88
Tyr[5]			
	α	4.18 (1H, m)	55.74
	β	3.13 (1H, dd, 4.1, 13.8)	35.58
		2.84 (1H, m)	
	γ		128.45
	δ	6.99 (2H, d, 8.5)	129.93
	ε	6.67 (2H, d, 8.5)	114.95
	ζ		155.79
	NH	8.23 (1H, d, 8.6)	
	C=O		170.65
Ser[6]			
	α	4.78 (1H, m)	52.42
	β	3.52 (2H, m)	61.27
	NH	7.53 (1H, d, 8.6)	
	C=O		169.04
Pro[7]			
	α	4.12 (1H, t, 7.4)	60.78
	β	2.08 (1H, m)	28.88
		1.79 (1H, m)	
	γ	2.05 (1H, m)	24.59
		1.85 (1H, m)	
	δ	3.81 (1H, m)	47.57
		3.69 (1H, m)	
	C=O		171.62

on a VG Autospec instrument. HPLC was performed on an Inertsil PREP-ODS packed with 10 μm ODS. TLC was conducted on precoated Kieselgel 60 F_{254} (Art. 5715; Merck), and the spots were detected by spraying with Dragendorff's reagent. ^1H-NMR and ^{13}C-NMR spectra were run in DMSO-d_6 using a Bruker AM-500 instrument, with chemical shifts (δ) reported in ppm. An 8 mg sample of yunnanin C in a 5-mm tube (0.5 mL of DMSO-d_6, degassed) was used for the homonuclear and heteronuclear measurements. The spectra were recorded at 303 K. A phase-sensitive ROESY NMR experiment was acquired with mixing times of 100 ms. 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 ms.

Plant material. 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.

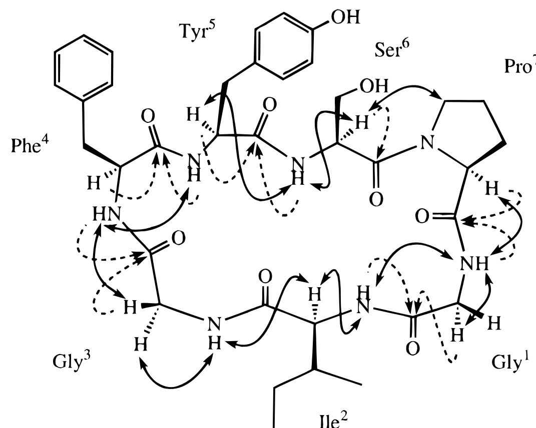

Figure 1. HMBC correlations (dashed arrows) and NOE correlations (arrows) for yunnanin C (**1**) in DMSO-d_6.

Extraction and Isolation. The roots of *S. yunnanensis* (7.0 kg) were extracted with 90% hot MeOH three times 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 eluted with 80% and 100% MeOH, respectively, were further subjected to Si gel column chromatography using a CH_2Cl_2−MeOH gradient system (1:0−0:1). The fraction eluted with 10% MeOH was subjected to ODS HPLC with a 26% CH_3CN/0.05% TFA, 50% MeOH/0.05% TFA, and 55% MeOH solvent system to give yunnanin C (**1**) (30.0 mg) as colorless needles: mp 255 °C; $[\alpha]_D$ −48.1° (c = 0.21, MeOH); IR (KBr) ν_{max} 3300 and 1630 cm^{-1}; ^1H-NMR and ^{13}C-NMR data, see Table 1; FABMS m/z $[M + H]^+$ 722 (base peak); HRFABMS m/z found 722.3523, calcd for $C_{36}H_{47}N_7O_9$ 722.3514.

Absolute Configuration of Amino Acids. A solution of **1** (1 mg) in 6 N HCl was heated at 110 °C for 12 h. The solution was concentrated to dryness. The residue was dissolved in H_2O 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, 2 M HCl was added and then concentrated to dryness. This residue was subjected to HPLC [Lichrospher 100, RP-18 (10 μm), Merck], flow rate 1 mL/min, detection 340 nm, solvent 10−50% CH_3CN/50 mM triethylamine phosphate (TEAP) buffer. The t_R values (min) were L-Ser 20.28, L-Pro 27.42, L-Tyr 30.21, and L-Phe and L-Ile 44.81, 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. A 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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