Cyclic Peptides from Higher Plants. 39. Dichotomins F and G, Cyclic Peptides from *Stellaria dichotoma* var. *lanceolata*†

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Two new cyclic peptides, dichotomins F (**1**) and G (**2**), have been isolated from the roots of *Stellaria dichotoma* L. var. *lanceolata* Bge., and the structures were elucidated by chemical degradation, ESIMS−MS, and extensive 2D NMR methods.

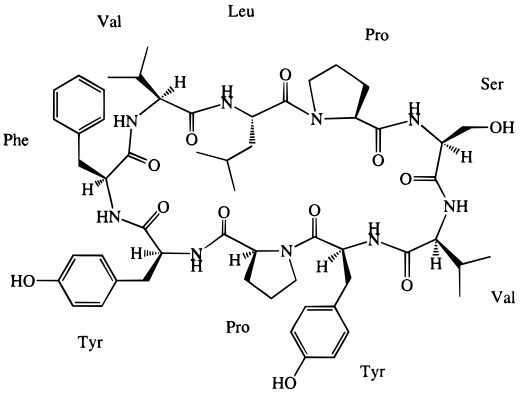
Recently, much attention has been devoted to cyclic peptides because these interesting natural products exhibit a wide variety of essential biological functions. Many cyclic peptides with unique structures and interesting pharmacological activities have been isolated from marine and micro organisms,[2] but only a few examples have been reported from higher plants.[1]

As part of our continuing investigation of new biologically active cyclic peptides from higher plants, we have focused our attention on the isolation of new cyclic peptides from the Caryophyllaceae plants.[3] Among them, we have previously isolated new cyclic peptides dichotomins A−E from *n*-BuOH-soluble fraction of the roots of *Stellaria dichotoma* L. var. *lanceolata* Bge., which was used in folk medicine as an antifebrile.[4] Some dichotomins showed cytotoxic activity and/or cyclooxygenase inhibitory activity. By the guidance of this assay, further purification of the *n*-BuOH-soluble fraction resulted in the isolation of two new cyclic peptides, named dichotomins F (**1**) and G (**2**). Here, we report a full account of the structure elucidation of dichotomins F and G by chemical degradation, ESIMS−MS and extensive 2D NMR methods; their cyclooxygenase inhibitor activities are also described.

The MeOH extract of the roots of *S. dichotoma* L. var. *lanceolata* Bge. was partitioned between *n*-BuOH and H_2O. The *n*-BuOH-soluble material was subjected to Diaion HP-20 column (H_2O−MeOH), 80% and 100% MeOH eluted fractions were chromatographed on a Si gel column, followed by HPLC on ODS to yield two peptides, which we named dichotomins F (**1**: 0.0003%) and G (**2**: 0.0006%).

Dichotomin F (**1**) showed a molecular formula, $C_{56}H_{75}N_9O_{12}$ (HRFABMS), with 24 degrees of unsaturation. Amino acid analysis of **1** showed the presence of Pro × 2, Phe, Tyr × 2, Val × 2, Leu, Ser, which were all confirmed to be of L-configuration by Marfey's derivatization, followed by HPLC analysis.[5]

In the ^{1}H-NMR spectrum, most of the signals were much broadened in both pyridine-d_5 and DMSO-d_6 because of a slow rate of interconversion not only at room temperature but also at high temperature (80 °C). In addition, four conformers in pyridine-d_5 and three conformers in DMSO-d_6 were observed, respectively. Therefore, the identification of the individual amino acids and sequence analysis by use of NMR was abandoned in favor of employing the electrospray ionization (ESI)[6] tandem mass spectrometry (MS−MS) method to determine the primary structure. The combination of ESI and tandem mass spectrum can provide useful structural information from fragment ions that may be lacking in the ESI spectrum. We have already reported that the possibility of using ESIMS−MS techniques as a tool for sequence determination of the peptides.[7] The ESIMS spectrum of **1** in 50% MeOH solution produced only stable ions such as $[M + Na]^+$ and $[M + K]^+$. Two peptide fragments (**3** and **4**) generated by digestion with α-chymotrypsin, however, produced the corresponding $[M + H]^+$ ion, which is then analyzed in a second mass spectrometer. As can be seen from fragmented ions of **3** and **4** (Figure 1), the sequences of **3** and **4** were determined to be Pro-Tyr-Phe and Val-Leu-Pro-Ser-Val-Tyr, respectively. These findings were also confirmed by Edman sequencing.



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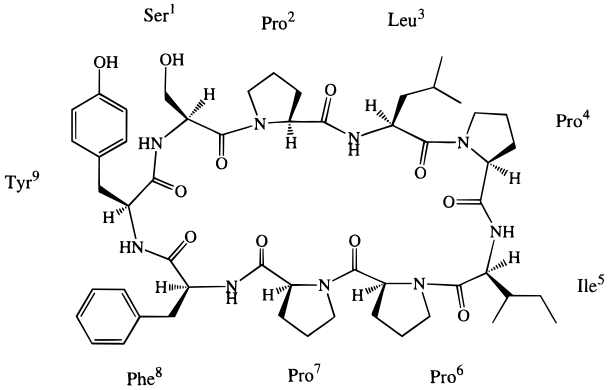

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† For part 38 of this series, see Morita *et al.*[1]

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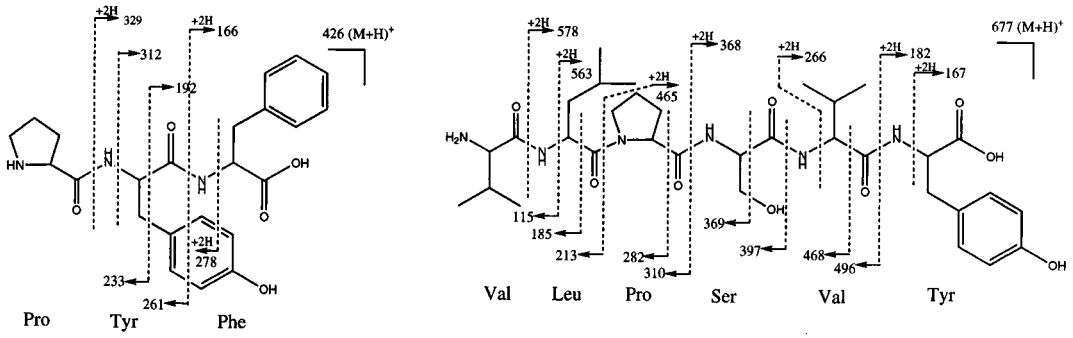

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Figure 1. MS−MS fragmentations of **3** and **4**.

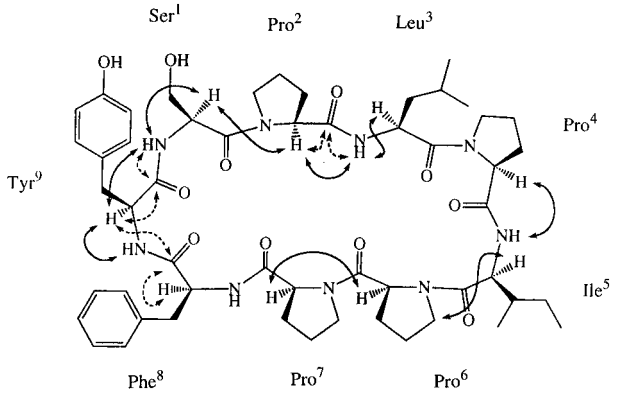

Figure 2. NOE correlations (arrows) and HMBC correlations (dashed arrows) for dichotomin G (**2**) in pyridine-d_5.

Therefore, the structure of **1** was unequivocally established to be a novel cyclic nonapeptide, representing *cyclo* (−Pro-Tyr-Phe-Val-Leu-Pro-Ser-Val-Tyr−).

Dichotomin G (**2**) showed a HRFABMS spectral quasimolecular ion peak at *m/z* 1012.5523 ([M + H]$^+$, Δ +1.5 mmu), corresponding to the molecular formula, $C_{53}H_{73}O_{11}N_9$. The IR absorption bands at 3315 and 1675 cm^{-1} were attributed to amino and amide carbonyl groups, respectively. The nonapeptide nature of **2** was evident from its ^{13}C-NMR spectrum, showing nine amide carbonyl groups, as shown in Table 1. Amino acid analysis of the acid hydrolysate of **2** revealed the presence of Ser, Leu, Ile, Phe, Tyr, and Pro × 4. The absolute stereochemistry of each amino acid in **2** was determined to be L-configuration by derivation of the acid hydrolysate with Marfey's reagent, followed by HPLC analysis.[5] The ^{1}H- and ^{13}C-NMR signals for individual amino acid were readily assigned by extensive analysis of ^{1}H−^{1}H COSY and HMQC spectra. The gross structure including the two sequences, −Phe-Tyr-Ser-Pro-Leu− and −Pro-Ile-Pro-Pro− was deduced on the basis of connectivity observed in the ROESY and HMBC experimental results (Figure 2). Finally, this sequence was confirmed by tandem MS fragmentation analysis of an acyclic peptide (**5**) generated by digestion with α-chymotrypsin (Figure 3). From the foregoing evidence, the whole structure was identified as *cyclo* (−Ser-Pro-Leu-Pro-Ile-Pro-Pro-Phe-Tyr−).

Dichotomins F and G showed moderate cyclooxygenase inhibitory activities (B: 72.6% inhibition at 100 μM; C: 62.6% inhibition at 100 μM). The investigation of dichotomins F and G in other biological assays is ongoing.

Experimental Section

General Experimental Procedures. The optical rotation was measured on a JASCO DIP-4 polarimeter. The IR spectrum (KBr) was obtained on a Perkin-Elmer 1710 spectrophotometer. FABMS and HRMS were recorded on a VG Autospec instrument and ESIMS−MS spectra on a TSQ-700 spectrometer. Amino acid analysis was carried out using Hitachi L-8500 amino acid analyzer. 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. ^{1}H-NMR and ^{13}C-NMR spectra were run in pyridine-d_5 and DMSO-d_6 using a Bruker AM-500 instruments, with chemical shifts (δ) reported in

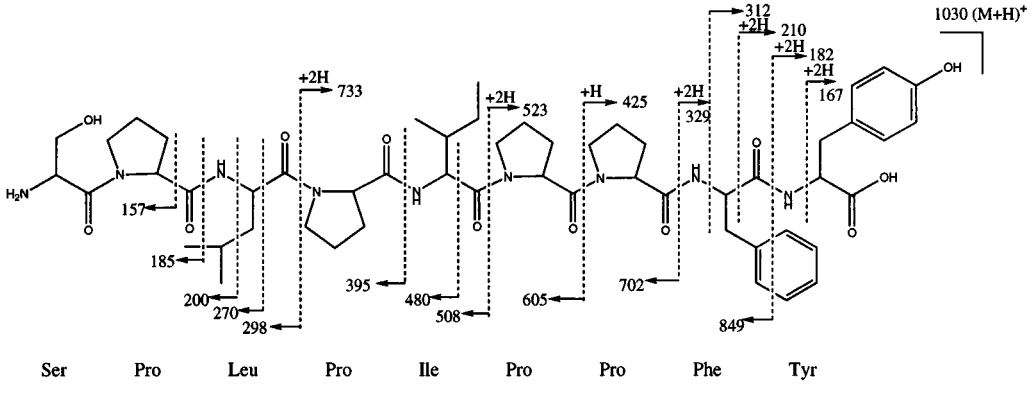

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Figure 3. MS−MS fragmentation of **5**.

Table 1. ^{1}H- and ^{13}C-NMR Assignments for Dichotomin G (**2**) in Pyridine-d_5

	position	$^\delta$H [int, mult, *J* (Hz)]	$^\delta$C
Ser[1]			
	α	5.20 (1H, ddd, 4.7, 6.4, 10.0)	54.70
	β	4.19 (1H, t, 10.0)	64.17
		4.60 (1H, dd, 4.7, 10.0)	
	NH	7.97 (1H, d, 6.4)	
	C=O		171.72
Pro[2]			
	α	5.57 (1H, dd, 2.3, 8.8)	61.77
	β	2.22 (1H, m)	32.15
		2.32 (1H, m)	
	γ	1.70 (1H, m)	23.07
		1.90 (1H, m)	
	δ	3.48 (1H, m)	47.62
		3.84 (1H, m)	
	C=O		172.78
Leu[3]			
	α	5.32 (1H, m)	49.07
	β	1.34 (1H, m)	41.01
		1.64 (1H, m)	
	γ	1.76 (1H, m)	25.21
	δ	0.85 (3H, d, 6.7)	20.96
		1.06 (3H, d, 6.5)	23.34
	NH	8.52 (1H, d, 9.5)	
	C=O		174.00
Pro[4]			
	α	5.35 (1H, m)	60.34
	β	1.77 (2H, m)	25.60
	γ	2.05 (1H, m)	25.55
		2.80 (1H, m)	
	δ	3.52 (1H, m)	47.20
		3.60 (1H, m)	
	C=O		170.62
Ile[5]			
	α	4.95 (1H, dd, 7.3, 9.6)	55.15
	β	1.91 (1H, m)	38.14
	γ	0.89 (1H, m)	24.23
		1.50 (1H, m)	
	*γ*Me	1.09 (3H, d, 6.7)	15.53
	δ	0.60 (3H, t, 7.4)	11.10
	NH	8.67 (1H, d, 9.6)	
	C=O		170.73
Pro[6]			
	α	4.07 (1H, m)	59.27
	β	1.74 (2H, m)	28.40
		2.40 (1H, m)	
	γ	1.91 (2H, m)	24.86
	δ	3.68 (1H, m)	48.05
		3.89 (1H, m)	
	C=O		170.80
Pro[7]			
	α	4.43 (1H, d, 8.2)	61.28
	β	1.80 (1H, m)	31.66
		2.01 (1H, m)	
	γ	0.79 (1H, m)	21.63
		1.34 (1H, m)	
	δ	3.43 (1H, m)	47.13
		3.49 (1H, m)	
	C=O		171.53
Phe[8]			
	α	5.34 (1H, m)	56.97
	β	4.05 (2H, m)	39.31
	γ		138.37
	δ	7.24 (2H, m)	129.26
	ϵ	7.16 (2H, m)	128.78
	ζ	7.15 (1H, m)	126.95
	NH	7.90 (1H, br s)	
	C=O		173.21
Tyr[9]			
	α	4.82 (1H, m)	59.27
	β	3.70 (2H, m)	35.01
	γ		130.16
	δ	7.26 (2H, d, 8.2)	131.08
	ϵ	7.07 (2H, d, 8.2)	115.88
	ζ		156.86
	NH	9.01 (1H, br d, 5.3)	
	C=O		170.45

ppm. The spectra were recorded at 303 K. A phase-sensitive ROESY experiment was acquired with mixing times of 100 msec. 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. dichotoma* L. var. *lanceolata* Bge. were purchased from the Shanghai Corporation of Chinese Traditional Drugs, People's Republic of China, in August 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. The roots of *S. dichotoma* L. var. *lanceolata* Bge. (8.5 kg) were extracted with hot MeOH (30 L) three times to give a MeOH extract (2.4 kg) that was treated with *n*-BuOH−H_2O (1:1). The *n*-BuOH-soluble fraction (294 g) was subjected to Diaion HP-20 column chromatography using a H_2O−MeOH gradient system (1:0−0:1). The two fractions eluted with 80% and 100% MeOH was 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 60% MeOH and 30% CH_3CN solvent system to give dichotomin F (**1**, 0.0003%) and dichotomin G (**2**, 0.0006%). Compounds **1** and **2** give a positive reaction for Dragendorff's reagent on TLC but negative for ninhydrin reagent.

Dichotomin F (1): colorless needles; mp 150−151 °C; $[\alpha]_D$ −85.1° (*c* 0.23, MeOH); FABMS *m/z* $[M + H]^+$ 1066 (found $[M + H]^+$ 1066.5595, $C_{56}H_{76}N_9O_{12}$ requires 1066.5613); IR (KBr) ν_{max} 3318 (NH) and 1649 (amide C=O) cm^{-1}; UV (MeOH) λ_{max} 277 nm (ϵ 4350).

Dichotomin G (2): colorless powder; $[\alpha]_D$ −100.5° (*c* 0.76, MeOH); IR (KBr) ν_{max} 3315 (NH) and 1675 (amide C=O) cm^{-1}; ^{1}H-NMR and ^{13}C-NMR data, see Table 1; FABMS *m/z* $[M + H]^+$ 1012 (found $[M + H]^+$ 1012.5523, $C_{53}H_{74}N_9O_{11}$ requires 1012.5508).

Acid Hydrolysis of 1 and 2. Solutions of **1** and **2** (each containing 1 mg of peptide) in 6 N HCl were heated at 110 °C for 24 h in a sealed tube. After cooling, each solution was concentrated to dryness. The hydrolysates were dissolved in 0.02 N HCl and amino acids were determined by ion-exchange resin chromatography on Hitachi L-8500 amino acid analyzer with ninhydrin detection.

Absolute Configuration of Amino Acids. Each solution of **1** and **2** (1 mg) in 6 N HCl (1 mL) was heated at 110 °C for 12 h. The solution was concentrated to dryness. The residue was dissolved in H_2O (100 μL) and treated with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent, 1 mg) in Me_2CO (100 μL) and 1 M $NaHCO_3$ (20 μL) at 35 °C for 1 h. After cooling, 2 N HCl (15 μL) 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 (pH 3.2). The t_R values (min) were L-Ser 19.69, L-Pro 28.66, L-Tyr 32.09, L-Val 33.85, L-Phe 40.02, L-Leu 40.42, and L-Ile 40.63, respectively.

Enzymatic Hydrolysis of 1 and 2. α-Chymotrypsin (250 μg dissolved in 25 μL of 0.001% HCl, Wako Pure Chemical Industries) was added to **1** and **2** (each 0.6 mg) in NH_4HCO_3 solution (1%, 0.45 mL), and the digestion was performed at 35 °C with the pH maintained at 8.0 by the manual addition of 0.1 N HCl. After 24 h the reaction was stopped by adjusting the solution to pH 2.2 with 1 N HCl. The digestion mixture was lyophilized to dryness, and hydrolysates were subjected to HPLC (Inertsil PREP-ODS column, 20 mm i.d. × 250 mm, GL Science Inc., packed with 10 μm ODS, eluted with 20% CH_3CN−0.05%TFA, flow rate 9 mL/min) to give compounds **3** (0.2 mg, t_R 28.17 min) and **4** (0.1 mg, t_R 32.38 min) and eluted with 30% CH_3CN−0.05%TFA (flow rate 9 mL/min) to give **5** (0.8 mg, t_R 30.33 min) as amorphous powder.

Assay for Cyclooxygenase Inhibitors. This assay was performed by the use of cyclooxygenase + PGH_2/PGE_2 isomerase kit (Eldan Tec. Co. Ltd., Israel); that is, 2 mL of samples in various concentrations and indometacin (1×10^{-4} M) solutions, and 10 mL of cofactors' solution (includes epinephrine, tryptophan, hydroquinone, and GSH) were added to 100 mL of sheep vesicular gland microsome solutions (0.2 mg/mL) that were dissolved in 50 mM Tris−HCl buffer solution. Their mixture solutions were preincubated with shaking for 3 min at 25 °C. After preincubation, 2 mL of arachidonic acid solution (1 mg/mL) was added to the above enzymatic solutions and incubated continually for further 3 min. At the end of the reaction was added 10 mL of $FeCl_3$ solution (25 mM) to the reaction mixtures. After completion of the reactions, the mixtures were centrifuged at 3000 × *g* at 4 °C for 10 min. The contents of PGE_2 in the supernatant solutions were determined by using the prostaglandin E_2 enzyme immunoassay kit (Cayman Chemical Co.).

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