Dichotomins J and K, Vasodilator Cyclic Peptides from *Stellaria dichotoma*

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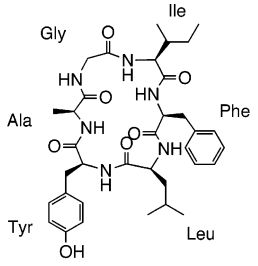
Two new cyclic peptides, dichotomins J (**1**) and K (**2**), have been isolated from the roots of *Stellaria dichotoma*, and their structures were elucidated by chemical degradation and extensive 2D NMR methods. Dichotomins J (**1**) and K (**2**) showed a moderate vasorelaxant effect on rat aorta.

Higher plants, microorganisms, and marine organisms are rich sources of cyclic peptides.[1,2] Recently, much attention has been devoted to cyclic peptides because of their variety of essential biological functions.[1,2]

As part of our continuing investigation of new biologically active cyclic peptides from higher plants, we have focused our attention on the isolation of cyclic peptides from the Caryophyllaceae plants.[2] We have previously isolated new cyclic peptides, dichotomins A−I, from the *n*-BuOH-soluble fraction of the roots of *Stellaria dichotoma* L. var. *lanceolata* Bge., which has been used as an antifebrile.[3−5] Further purification of the *n*-BuOH-soluble fraction resulted in the isolation of two new cyclic peptides, dichotomins J (**1**) and K (**2**), which inhibited vasocontraction induced by norepinephrine (NE) on rat aorta. In this paper, the isolation, structure elucidation by chemical degradation and extensive 2D NMR methods, and vasorelaxant effect on rat aorta of **1** and **2** are described.



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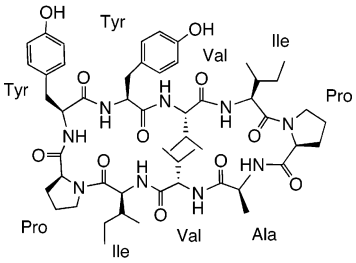

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The MeOH extract of the roots of *S. dichotoma* L. var. *lanceolata* Bge. was partitioned between *n*-BuOH and H_2O. The *n*-BuOH-soluble materials were subjected to a Diaion HP-20 column (H_2O → MeOH), and 80% MeOH eluted fractions were chromatographed on a silica gel column. Final purification by C_{18} HPLC afforded dichotomins J (**1**, 0.0001%) and K (**2**, 0.0002%).

Dichotomin J (**1**) was isolated as colorless solid, $[\alpha]^{20}_D$ −85° (*c* 0.2, MeOH), and gave an $(M + H)^+$ ion in the HRFABMS at *m/z* 665.3662, corresponding to the molecular formula $C_{35}H_{48}N_6O_7$, with 15 degrees of unsaturation. IR absorption bands at 3320 and 1648 cm^{-1} attributed to amino and amide carbonyl groups, respectively, and the ^1H and ^{13}C NMR spectra of **1** (see Table 1) contained resonances that were characteristic of peptides. Amino acid analysis of **1** with 6 N HCl at 110 °C for 24 h in a sealed tube showed the presence of 1 mol each of Gly, Phe, Tyr, Ala, Leu, and Ile, which were confirmed to be all L-configured by Marfey's derivatization, followed by HPLC analysis.[6] The molecular formula of **1** corresponded to the above amino acid composition if **1** was a cyclic peptide. Detailed analysis of the $^1H-^1H$ COSY and HOHAHA spectra recorded in pyridine-d_5 allowed the coupling sequence of each amino acid resonance, and the corresponding carbon resonances were elucidated on the basis of the HMQC spectrum as shown in Table 1. The gross structure including the sequence of the amino acids was assembled by connecting the individual amino acids on the basis of connectivity observed in the HMBC experiment (Figure 1). The HMBC correlations between each amide carbonyl carbon and neighboring amide NH and Hα protons indicated the partial sequence of −Gly-Ile-Phe-Leu-Tyr-Ala−. In addition, NOESY correlations of Gly-Hα/Ile-NH, Phe-Hα/Leu-NH, Leu-Hα/Tyr-NH, Tyr-Hα/Ala-NH, and Ala-Hα/Gly-NH indicated the structure of **1** to be *cyclo*(−Gly-Ile-Phe-Leu-Tyr-Ala−).

Dichotomin K (**2**) showed an HRFABMS quasimolecular ion peak at *m/z* 1038.5664 ($[M + Na]^+$, Δ −2.4 mmu), corresponding to the molecular formula $C_{53}H_{77}N_9O_{11}$.

Amino acid analysis of **2** with 6 N HCl at 110 °C for 24 h in a sealed tube showed the presence of 1 mol Ala and 2 mol each of Val, Ile, Pro, and Tyr, which were confirmed to be all L-configured by Marfey's derivatization, followed by HPLC analysis.[6] The ^1H NMR spectrum in conventional NMR solvents such as pyridine-d_5 and DMSO-d_6 gave broad signals resulting from the slow rate of interconversion not only at room temperature but also at high temperature (80 °C). Therefore, the identification of the individual amino acids and sequence analysis by use of NMR were abandoned in favor of employing the Edman sequencing method to determine the primary structure.[7]

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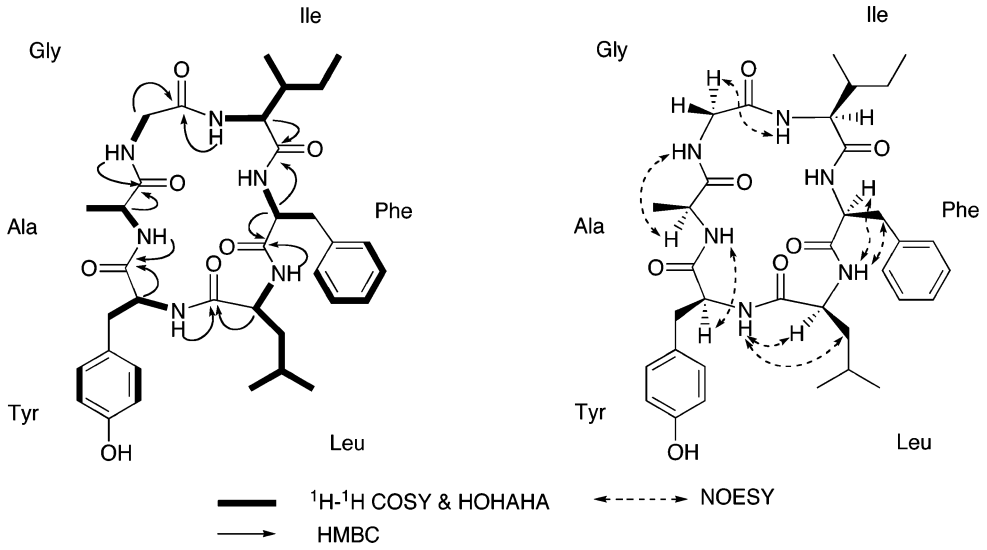

Figure 1. Selected two-dimensional NMR correlations for dichotomin J (**1**).

Table 1. ^{1}H and ^{13}C NMR Data of Dichotomin J (**1**) in Pyridine-d_5[a]

	position	^{1}H	^{13}C
Gly	α	3.98 (1H, dd, 6.0, 15.0)	44.6
		4.58 (1H, dd, 5.1, 15.0)	
	NH	9.59 (1H, dd, 5.1, 6.0)	
	C=O		170.7
Ile	α	4.73 (1H, m)	59.3
	β	2.24 (1H, m)	36.1
	γ	1.19 (1H, m)	25.1
		1.49 (1H, m)	
	Me*γ*	0.92 (3H, d, 6.8)	15.9
	Me*δ*	0.73 (3H, t, 7.4)	11.4
	NH	8.92 (1H, d, 8.1)	
	C=O		172.3
Phe	α	4.94 (1H, m)	56.5
	β	3.66 (2H, m)	37.4
	γ		138.9
	δ	7.39 (2H, d, 6.9)	129.7
	ϵ	7.26 (2H, t, 6.9)	128.7
	ζ	7.22 (1H, m)	126.7
	NH	8.92 (1H, d, 8.1)	
	C=O		172.6
Leu	α	4.45 (1H, m)	54.8
	β	1.91 (2H, m)	40.5
	γ	1.66 (1H, m)	24.8
	Me*δ*	0.75 (3H, d, 6.5)	21.8
		0.76 (3H, d, 6.6)	23.0
	NH	9.15 (1H, d, 6.2)	
	C=O		173.4
Tyr	α	4.76 (1H, m)	57.3
	β	3.46 (1H, dd, 8.6, 13.9)	36.7
		3.58 (1H, dd, 6.4, 13.9)	
	γ		128.6
	δ	7.32 (2H, d, 8.4)	131.0
	ϵ	7.13 (2H, d, 8.4)	116.2
	ζ		157.8
	NH	9.19 (1H, d, 6.9)	
	C=O		172.3
Ala	α	4.60 (1H, m)	50.5
	β	1.73 (3H, d, 6.8)	17.2
	NH	8.98 (1H, d, 6.7)	
	C=O		173.7

[a] *δ* in ppm.

The acyclic peptide fragment generated by digestion with α-chymotrypsin produced an *m/z* 870 $(M + H)^+$ ion corresponding to the molecule without one tyrosine. This fragment was then analyzed by a peptide sequencer to be Val-Ile-Pro-Ala-Val-Ile-Pro-Tyr. Therefore, the structure of **2** was unequivocally established to be a new cyclic nonapeptide, representing *cyclo*(−Val-Ile-Pro-Ala-Val-Ile-Pro-Tyr-Tyr−).

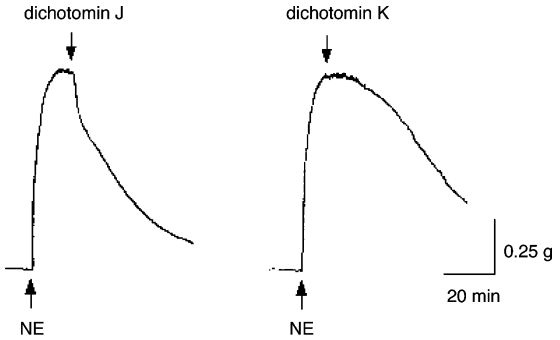

Figure 2. Relaxation responses induced by dichotomins J and K on aortic rings precontracted with 3×10^{-7} M norepinephrine (NE). NE: 3×10^{-7} M; dichotomin J: 3×10^{-5} M; dichotomin K: 10^{-4} M.

Vasorelaxant effects of dichotomins J (**1**) and K (**2**) were examined in contractions of isolated rat thoracic aorta. Figure 2 indicated the relaxant effects of **1** and **2** on the aorta precontracted with 3×10^{-7} M norepinephrine (NE). The vasodilator effect of dichotomin J (**1**) was shown to be more potent than that of dichotomin K (**2**).

Experimental Section

General Experimental Procedures. Optical rotations were measured on a JASCO DIP-4 polarimeter. UV spectra were recorded on a Shimadzu UV1600PC spectrophotometer and IR spectra on a Perkin-Elmer 1710 spectrophotometer. ^{1}H and 2D NMR spectra in pyridine-d_5 were recorded on a 500 MHz spectrometer at 300 K, while ^{13}C NMR spectra were measured on a 125 MHz spectrometer. Chemical shifts were reported using residual pyridine (δ_H 7.21 and δ_C 135.5) as internal standard. Standard pulse sequences were employed for the 2D NMR experiments. ^{1}H−^{1}H COSY, HOHAHA, and NOESY spectra were measured with spectral widths of both dimensions of 4800 Hz, and 32 scans with two dummy scans were accumulated into 1 K data points for each of 256 t_1 increments. NOESY and HOHAHA spectra in the phase-sensitive mode were measured with a mixing time of 800 and 30 ms, respectively. For HMQC spectra in the phase-sensitive mode and HMBC spectra, a total of 256 increments of 1 K data points were collected. For HMBC spectra with the *Z*-axis PFG, a 50 ms delay time was used for long-range C−H coupling. Zero-filling to 1 K for F_1 and multiplication with squared cosine-bell windows shifted in both dimensions were performed

prior to 2D Fourier transformation. FAB and high-resolution mass spectra were recorded on a VG Autospec instrument by using a glycerol matrix. Amino acid analysis was carried out using a 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.

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 Sciences.

Extraction and Isolation. The roots of *S. dichotoma* L. var. *lanceolata* Bge. (8.5 kg) were extracted with MeOH (30 L) three times to give a MeOH extract (2.4 kg), which 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% MeOH were further subjected to silica gel column using a CH_2Cl_2−MeOH gradient system (1:0 → 0:1). The fraction eluted with 10% MeOH was subjected to C_{18} HPLC (YMC-Pack ODS AM, 5 μm, 20 × 250 mm; flow rate 8.0 mL/min; UV detection at 210 nm; eluent, 25% CH_3CN/25% MeOH/50% H_2O) to afford dichotomin J (**1**, 8.5 mg, 0.0001%) and dichotomin K (**2**, 17 mg, 0.0002%). Compounds **1** and **2** give a positive reaction for Dragendorff's reagent on TLC but negative for ninhydrin reagent.

Dichotomin J (1): colorless solid; $[\alpha]^{20}_D$ −85° (*c* 0.2, MeOH); UV (MeOH) λ_{max} 278 nm (ϵ 3340); IR (KBr) ν_{max} 3320 (NH) and 1648 (amide C=O) cm^{-1}; ^1H and ^{13}C NMR (Table 1); FABMS *m/z* 665 $(M + H)^+$; HRFABMS *m/z* 665.3662 $(M + H)^+$, calcd for $C_{35}H_{49}N_6O_7$ 665.3662.

Dichotomin K (2): colorless solid; $[\alpha]^{20}_D$ −101° (*c* 0.8, MeOH); UV (MeOH) λ_{max} 275 nm (ϵ 4540); IR (KBr) ν_{max} 3310 (NH) and 1650 (amide C=O) cm^{-1}; FABMS *m/z* 1038 $(M + H)^+$; HRFABMS *m/z* 1038.5664 $(M + Na)^+$, calcd for $C_{53}H_{77}N_9O_{11}Na$ 1038.5640.

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 a 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 acetone (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). Retention times (min) of the amino acid derivatives were as follows: L-Ala (26.1), L-Pro (28.0), L-Tyr (32.0), L-Val (33.9), L-Phe (40.0), L-Leu (40.4), and L-Ile (41.0), which were identical with those derived from authentic amino acids.

Enzymatic Hydrolysis of 2. α-Chymotrypsin (500 μg dissolved in 50 μL of 0.001% HCl, Wako Pure Chemical Industries) was added to **2** (0.5 mg) in NH_4HCO_3 solution (1%, 0.9 mL), and the digestion was performed at 37 °C with the pH maintained at 8.0 by the manual addition of 0.1 N HCl. After 16 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 22% CH_3CN/0.05%TFA, flow rate 9 mL/min) to give an acyclic peptide (0.3 mg, t_R 92.1 min) as amorphous powder: FABMS *m/z* 870 $(M + H)^+$.

Vasodilator Assay.[8] A male Wistar rat weighting 230 g was sacrificed by bleeding from carotid arteries under anesthetization. A section of the thoracic aorta between the aortic arch and the diaphragm was removed and placed in oxygenated, modified Krebs-Henseleit solution (KHS: 118.0 mM NaCl, 4.7 mM KCl, 25.0 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM $MgSO_4$, and 11.0 mM glucose). The aorta was cleaned of loosely adhering fat and connective tissue and cut into ring preparations 3 mm in length. The tissue was placed in a well-oxygenated (95% O_2, 5% CO_2) bath of 10 mL of KHS solution at 37 °C with one end connected to a tissue holder and the other to a force-displacement transducer (Nihon Kohden, TB-611T). The tissue was equilibrated for 60 min under a resting tension of 1.0 g. During this time the KHS in the tissue bath was replaced every 20 min.

After equilibration, each aortic ring was contracted by treatment with 3×10^{-7} M norepinephrine (NE). The presence of functional endothelial cells was confirmed by demonstrating relaxation to 10^{-5} M acetylcholine (Ach), and aortic rings in which 80% relaxation occurred were regarded as tissues with endothelium. When the NE-induced contraction reached plateau, each sample was added.

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