Pergamon

PII: S0031-9422(97)00056-3

Phytochemistry, Vol. 45, No. 4, pp. 841–845, 1997

0031-9422/97 $17.00+0.00

CYCLIC OCTAPEPTIDES FROM *STELLARIA DICHOTOMA* VAR. *LANCEOLATA*

HIROSHI MORITA, KOICHI TAKEYA and HIDEJI ITOKAWA

Department of Pharmacognosy, School of Pharmacy, Tokyo University of Pharmacy and Life Science, Horinouchi 1432-1, Hachioji, Tokyo 192-03, Japan

(*Received* 3 *October* 1996)

Key Word Index—*Stellaria dichotoma* var. *lanceolata*; Caryophyllaceae; roots; cyclic octapeptide; dichotomin H; dichotomin I.

Abstract—Two new cyclic octapeptides, dichotomin H, *cyclo*(-Ala-Pro-Thr-Phe-Tyr-Pro-Leu-Ile-), and dichotomin I, *cyclo*(-Val-Pro-Thr-Phe-Tyr-Pro-Leu-Ile-) have been isolated from the roots of *Stellaria dichotoma* L. var. *lanceolata* Bge., and their structures were elucidated by extensive two-dimensional NMR methods and chemical degradation.

INTRODUCTION

Cyclic peptides are interesting natural products exhibiting a wide range of biological activities. As part of our continuing study of cyclic peptides from higher plants [1, 2], we have investigated several cyclic peptides, named dichotomins. These have cytotoxic or cyclooxygenase inhibitory activities from *Stellaria dichotoma* var. *lanceolata* and were used as folk medicines for the treatment of fever [3, 4]. Separation of the methanolic extract led to two peptidic compounds, named dichotomins H and I (**1** and **2**). Here, we report a full account of the structure elucidation of **1** and **2** by two-dimensional NMR and chemical degradation.

RESULTS AND DISCUSSION

The concentrated methanolic extracts of the roots of *Stellaria dichotoma* var. *lanceolata* was extracted with 1-butanol. The 1-butanol phase was concentrated, successively treated with HP-20 with gradient system (water–methanol), silica gel column chromatography with gradient system (methylene chloride–methanol), and was finally subjected to HPLC on ODS column chromatography with 38% CH_3CN and 67% methanol to furnish two new cyclic octapeptides, dichotomins H (**1**, 0.0001% yield) and I (**2**, 0.0005% yield).

The FAB-mass spectrum of **1** gave a $(M+1)^+$ ion at 903 and the molecular formula, $C_{47}H_{66}N_8O_{10}$, derived from the HR-FAB mass spectrum, indicated 19 degrees of unsaturation. The IR absorption bands were (3310 and 1635 cm^{-1}), characteristic of amino and amide carbonyl groups. The ^{13}C NMR spectrum contained eight signals due to amide carbonyl carbons, which were involved in amide linkage. Standard amino acid analysis demonstrated 1 mol each of Ala, Thr, Leu, Ile, Phe, Tyr, and 2 mol of Pro. The ^1H NMR spectrum in pyridine-d_5 indicated six amide NH signals between δ 7.91 and 10.50, which support the result of amino acid analysis, showing two Pro residues. The relatively high intensity of the molecular ion peak in the FAB mass spectrum suggested that the presumed octapeptide might be in a cyclic form. Complete assignments for the ^1H and ^{13}C NMR resonances in pyridine-d_5 (Table 1), were accomplished using combination of 2D-NMR experiments such as ^1H-^1H COSY, HOHAHA, HMQC [5] and HMBC [6] spectra.

The HMBC analyses suggested the seven amino acid sequence shown in structure **1**. Two partial segments, Pro-Thr-Phe and Pro-Leu-Ile were assigned by two bond ^1H-^{13}C correlations as follows; NH (Thr^3)/CO(Pro^2), Hα(Pro^2)/CO(Pro^2), NH(Phe^4)/CO (Thr^3), Hα(Thr^3)/CO(Thr^3), NH(Leu^7)/CO(Pro^6), Hα(Pro^6)/CO(Pro^6), NH(Ile^8)/CO(Leu^7), and Hα(Leu^7)/CO(Leu^7) (Fig. 1). Two structural units analysed by the HMBC correlations and the two remaining amino acids, Ala and Tyr could be combined by ROE enhancements; Phe^4-Hα/Tyr^5-NH, Tyr^5-Hα/Pro^6-Hα, Ile^8-Hα/Ala^1-NH, and Ala^1-Hα/Pro^2-Hα in a phase sensitive ROESY spectrum [7]. The structure of **1** was determined to be *cyclo*(-Ala-Pro-Thr-Phe-Tyr-Pro-Leu-Ile).

Dichotomin I (**2**) was also a cyclic octapeptide with very similar amino acids and sequencing as follows. Dichotomin I (**2**), colourless powder, $[\alpha]_D$ −99.6° (*c* 0.54, MeOH), showed a high-resolution FAB-mass spectral quasimolecular ion peak at *m/z* 931.5304 (MH^+, Δ + 1.1 mmu), corresponding to molecular for-

Table 1. ^1H and ^{13}C NMR signal assignments of dichotomin H (**1**) in pyridine-d_5

Assignment	^1H NMR δ_H (int, mult, J(Hz))	^{13}C NMR δ_C		δ_H	δ_C
Ala[1]			Tyr[5]		
α	4.84 (1H, *m*)	47.61	α	4.69 (1H, *m*)	55.91
β	1.38 (3H, *d*, 7.1)	17.00	β	3.18 (2H, *m*)	37.21
NH	10.50 (1H, *br s*)		γ		128.91
C=O		173.04	δ	7.42 (2H, *d*, 7.1)	131.00
Pro[2]			ε	7.23 (2H, *d*, 7.1)	116.70
α	4.97 (1H, *d*, 7.8)	62.07	ζ		157.99
β	2.19 (1H, *m*)	32.20	NH	10.04 (1H, *d*, 3.9)	
	2.55 (1H, *m*)		C=O		171.23
γ	1.78 (1H, *m*)	22.29	Pro[6]		
	2.05 (1H, *m*)		α	4.26 (1H, *d*, 7.6)	61.66
δ	3.77 (2H, *m*)	46.94	β	1.44 (1H, *m*)	31.19
C=O		173.46		2.42 (1H, *m*)	
Thr[3]			γ	1.58 (2H, *m*)	22.29
α	4.72 (1H, *dd*, 4.4, 6.8)	62.54	δ	3.55 (1H, *m*)	46.80
β	4.54 (1H, *m*)	67.02		3.71 (1H, *m*)	
γ-Me	1.28 (3H, *d*, 6.3)	21.88	C=O		171.04
NH	7.91 (1H, *br d*, 6.8)		Leu[7]		
C=O		171.04	α	5.07 (1H, *m*)	55.61
Phe[4]			β	2.05 (1H, *m*)	42.10
α	5.54 (1H, *br dd*, 7.5, 14.9)	53.98		2.12 (1H, *m*)	
β	3.25 (1H, *m*)	37.54	γ	1.89 (1H, *m*)	25.56
	3.70 (1H, *m*)		δ-Me	0.91 (3H, *d*, 6.6)	21.56
γ		139.17		0.96 (3H, *d*, 6.6)	22.98
δ		129.63	NH	8.65 (1H, *d*, 8.4)	
ε	7.11–7.24 (5H, *m*)	128.53	C=O		172.87
ζ		126.45	Ile[8]		
NH	9.07 (1H, *d*, 8.7)		α	5.09 (1H, *m*)	57.51
C=O		174.17	β	2.33 (1H, *m*)	38.91
			γ	1.35 (1H, *m*)	25.34
				1.80 (1H, *m*)	
			γ-Me	1.15 (3H, *d*, 6.7)	15.91
			δ-Me	0.80 (3H, *t*, 7.4)	11.32
			NH	8.28 (1H, *d*, 8.9)	
			C=O		172.81

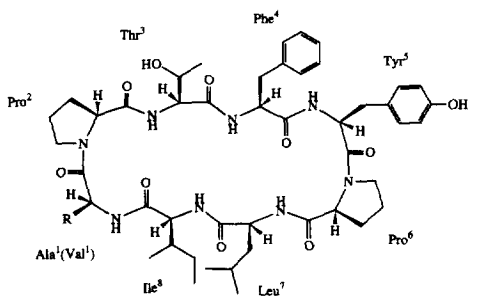

Fig. 1. Structures of dichotomins H(**1**) and I(**2**). **1**, R = CH_3; **2**, R = isopropyl.

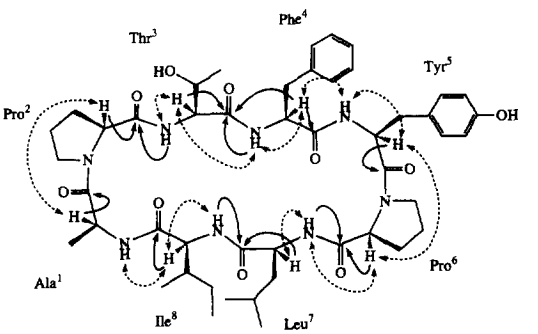

Fig. 2. Some important HMBC and ROE correlations of dichotomin H (**1**); Arrows show HMBC and dashed arrows ROE correlations.

mula, $C_{49}H_{70}N_8O_{10}$. The IR absorptions at 3309 and 1649 cm^{-1} were attributed to amino and amide carbonyl groups, respectively. The octapeptide nature of **2** was evident from its ^1H and ^{13}C NMR spectra, showing six amide NH and eight amide carbonyl groups (Table 2). Further, the relatively high intensity of the molecular ion and the lack of terminal amino group protons in the ^1H NMR suggested that **2** might be a cyclic peptide. In order to elucidate the amino acid composition, **2** was subjected to complete hydrolysis with 6 M HCl by heating at 110° for 24 hr in a sealed tube. The hydrolysate was then analysed by HPLC and was shown to contain 1 mol each of

Table 2. ^{1}H and ^{13}C NMR signal assignments of dichotomin I (**2**) in pyridine-d_5

Assignment	^{1}H NMR δ_H (int. mult, J(Hz))	^{13}C NMR δ_C		δ_H	δ_C
Val[1]			Tyr[5]		
α	4.76 (1H, *br t*, 8.5)	57.17	α	4.61 (1H, *m*)	55.80
β	2.31 (1H, *m*)	31.13	β	3.21 (2H, *m*)	37.62
γ-Me	0.97 (6H, *d*, 6.4)	19.0 × 2	γ		128.80
NH	10.52 (1H, *br s*)		δ	7.45 (2H, *d*, 7.1)	131.11
C=O		172.79	ε	7.22 (2H, *d*, 7.1)	116.76
Pro[2]			ζ		158.23
α	5.15 (1H, *d*, 7.5)	62.61	NH	10.16 (1H, *br s*)	
β	2.04 (1H, *m*)	32.11	C=O		171.40
	2.55 (1H, *m*)		Pro[6]		
γ	1.54 (1H, *m*)	22.23	α	4.09 (1H, *d*, 7.5)	61.73
	1.84 (1H, *m*)		β	2.31 (2H, *m*)	31.33
δ	3.78 (1H, *m*)	46.59	γ	1.54 (1H, *m*)	22.23
	3.89 (1H, *m*)			1.84 (1H, *m*)	
C=O		173.10	δ	3.51 (1H, *m*)	46.75
Thr[3]				3.67 (1H, *m*)	
α	4.56 (1H, *m*)	62.82	C=O		171.02
β	4.56 (1H, *m*)	66.96	Leu[7]		
γ-Me	1.32 (3H, *d*, 5.2)	22.03	α	4.92 (1H, *m*)	55.70
NH	7.61 (1H, *br s*)		β	2.04 (2H, *m*)	42.19
C=O		171.08			
Phe[4]			γ	1.85 (1H, *m*)	25.65
α	5.45 (1H, *m*)	54.54	δ-Me	0.89 (3H, *d*, 6.6)	21.68
β	3.19 (1H, *m*)	37.96		1.02 (3H, *d*, 6.6)	23.02
	3.61 (1H, *m*)		NH	8.42 (1H, *br s*)	
γ		139.17	C=O		172.95
δ		129.70	Ile[8]		
ε	7.11-7.23 (5H, *m*)	128.61	α	5.05 (1H, *br t*, 9.9)	57.85
ζ		126.69	β	2.28 (1H, *m*)	38.35
NH	9.20 (1H, *d*, 7.5)		γ	1.25 (1H, *m*)	25.23
C=O		173.46		1.76 (1H, *m*)	
			γ-Me	1.02 (3H, *d*, 6.2)	16.04
			δ-Me	0.76 (3H, *t*, 7.3)	10.71
			NH	8.32 (1H, *br s*)	
			C=O		173.90

Val, Thr, Leu, Ile, Phe, Tyr, and 2 mol of Pro. These eight amino acids accounted for the observed mass molecular weight and 19 degrees of unsaturation.

Structure elucidation of each NMR spin system was conducted by extensive analysis of ^{1}H–^{1}H COSY, HOHAHA and HMQC spectra (Table 2). The sequencing of the amino acids for **2** was elucidated by connecting the individual amino acids on the basis of HMBC correlations (Fig. 3). From the HMBC correlations among each Hα, NH and the intermediate carbonyl carbon, the sequence was identified as *cyclo* (-Val-Pro-Thr-Phe-Tyr-Pro-Leu-Ile-). The deduced structure of dichotomin I was also in good agreement with the result of the ROE correlations in a phase sensitive ROESY spectrum.

The absolute stereochemistry of the component amino acids in **1** and **2** was determined to be L-configuration by derivatization of the acid hydrolysate with Marfey's reagent, followed by HPLC analysis [8].

Space interactions between Ala[1]-Hα and Pro[2]-Hα,

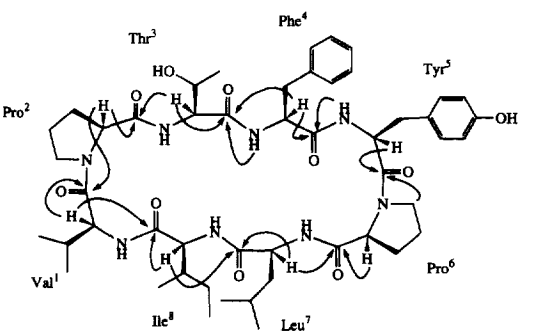

Fig. 3. HMBC correlations of dichotomin I (**2**).

and between Tyr[5]-Hα and Pro[6]-Hα in **1**, and between Val[1]-Hα and Pro[2]-Hα, and between Tyr[5]-Hα and Pro[6]-Hα in **2** are diagnostic of *cis* peptide bonds of each proline amide bonds in **1** and **2**, which is also supported by the chemical shift difference of Cβ and Cγ carbons of each proline residue in **1** and **2** (**1** Δδ 9.9 and 8.9; **2** Δδ 9.9 and 9.1) [9], and the occurrence of a

doublet signal of Hα in the proline residues in **1** and **2** has also been correlated with the *cis* peptide bond [10].

Dichotomins H and I showed a moderate cell growth inhibitory activity against P-388 cells (IC_{50} **1** 3.0 μg ml^{-1}, **2** 2.3 μg ml^{-1}).

EXPERIMENTAL

General. IR and UV spectra were recorded on JASCO A-302 spectrometer and Hitachi 557 spectrophotometer, respectively. Optical rotation was measured with a JASCO DIP-4 spectrometer and $[\alpha]_D$ values are given in 10^{-1} deg cm^2 g^{-1}. FAB and high resolution mass spectra were taken with a VG Autospec spectrometer. HPLC was performed with an Inertsil PREP-ODS column (20 mm i.d. × 250 mm and 30 mm i.d. × 250 mm, GL Science Inc.) packed with 10 μm ODS. Peptide-containing frs were traced by TLC on precoated TLC plated 60 F_{254} (Merk) using a solvent system consisting of $CHCl_3$–MeOH = 4:1 in a satd chamber. All NMR spectroscopy were carried out on Bruker AM400, AM500, and Varian Unity 400 spectrometer. The spectra were recorded at 300 K for dichotomin H and 315 K for dichotomin I and each concn was 10 mg ml^{-1} in pyridine-d_5. The phase sensitive ROESY experiments were acquired with mixing times of 300 msec. The values of the delay to optimize one-bond correlations in the HMQC spectrum and suppress then in the HMBC spectrum was 150 msec and the evolution delay for long-range couplings in the HMBC spectrum was set to 90 msec.

Plant material. The roots of *Stellaria dichotoma* L. var. *lanceolata* Bge. were purchased in Shanghai, 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 the Tokyo University of Pharmacy and Life Science.

Extraction and isolation. The roots (10 kg) were extracted with hot MeOH × 3 to give a MeOH extract which was treated with 1-BuOH and H_2O. The 1-BuOH soluble (230 g), was subjected to HP-20 column chromatography using MeOH–H_2O gradient system (0:1–1:0), followed by silica gel column chromatography of the fraction eluted by 80% MeOH using a CH_2Cl_2–MeOH gradient system (1:0–0:1). The fr. eluted by 20% MeOH was finally subjected to ODS HPLC with an 38% CH_3CN and 67% MeOH solvent system to give dichtomins H (0.001%) and I(0.0005%).

Dichotomin H **1**. Colourless powder, $[\alpha]_D$ −77.5° (*c* 0.93, MeOH); *m/z* 903 (Found: M^++H, 903,4990. $C_{47}H_{67}N_8O_{10}$ requires, 903.4980); ν_{max} (KBr)/cm^{-1} 3310 (NH) and 1635 (amid C = 0); λ_{max} (MeOH)/nm 257 (ε 2400).

Dichotomin I **2**. Colourless powder, $[\alpha]_D$ −99.6° (*c* 0.54, MeOH); *m/z* 931 (Found: M^++H, 931.5304. $C_{49}H_{71}N_8O_{10}$ requires, 931.5293); ν_{max} (KBr)/cm^{-1} 3309 (NH) and 1649 (amid C = O); λ_{max} (MeOH)/nm 260 (ε 2500).

Amino acid analysis. Each of dichotomins H and I (1 mg) was hydrolysed in 1 ml 6 M HCl in a sealed vial at 110° for 24 hr. HCl was removed under red. pres., and residue was dissolved in 0.02 M HCl. Amino acids were determined by ion-exchange resin chromatography on Hitachi L-8500 Amino Acid analyser with ninhydrin detection.

Absolute configuration of amino acid. Each soln of dichotomins H and I in 6 M HCl was heated at 110° for 12 hr. After being cooled, each soln was concd to dryness. The residue was soluble in H_2O and treated with 1-fluoro-2,4-dinitrophenyl-5-L-alanin amide (Marfey's reagent) and 1 M $NaHCO_3$ at 35° for 1 hr. After being cooled, 2 M HCl was added and then concd to dryness. This residue was subjected to HPLC (Lichrospher 100, RP-18 (10 μm), Merck), flow rate 1 ml min^{-1}, detection 340 nm, solvent: 10–50% CH_3CN–50 mM triethylamine phosphate (TEAP) buffer.

Cytotoxic activity on P388 cells. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) colorimetric assay was performed in a 96-well plate. The blue formazan produced by the mitochondrial dehydrogenase of viable cells was measured spectrophotometrically. 100 μl of RPMI-1640 medium supplemented with 5% fetal calf serum and 100 μg ml^{-1} of kanamycin and containing mouse P388 leukemia cells (3×10^4 cells ml^{-1}) was added to each well. After overnight incubation (37°, 5% CO_2), 100, 30, 10, 3, 1, 0.3, and 0.1 μg ml^{-1} of sample solns 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 sulphate) containing 0.01 M 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^{-1}) value was defined as the concn of sample which achieved 50% reduction of viable cells with respect to the control.

Acknowledgements—The authors thank the Ministry of Education, Science and Culture, Japan, for financial support through Grant-in-Aid for General Scientific Research.

REFERENCES

1. Cyclic peptide from higher plant. Part. 40.
2. Morita, H., Kayashita, T., Takeya, K. and Itokawa, H., *Chemical and Pharmaceutical Bulletin*, 1996, **44**, 2177 and refs cited therein.
3. Morita, H., Kayashita, T., Shishido, A., Takeya, K., Itokawa, H. and Shiro, M., *Tetrahedron*, 1996, **52**, 1165.
4. Morita, H., Shishido, A., Kayashita, T., Takeya,

K. and Itokawa, H., *Journal of Natural Products*, (in press).
5. Bax, A. and Subramanian, S., *Journal of Magnetic Resonance*, 1986, **67**, 565.
6. Hurd, R. E. and John, B. K., *Journal of Magnetic Resonance*, 1991, **91**, 648.
7. Bother-By, A. A., Stephens, R. L., Lee, J., Warren, C. D. and Jeanloz, R. W., *Journal of the American Chemical Society*, 1984, **106**, 811.
8. Marfey, P., *Carlsberg Research Communications*, 1984, **49**, 591.
9. Siemion, I. Z., Wieland, T. and Pook, K.-H., *Angewandte Chemie, International Edition*, 1974, **14**, 702.
10. Kopple, K. D., Schumper, T. J. and Go, A., *Journal of the American Chemical Society*, 1974, **96**, 2597.